A factor analysis approach to examining relationships among ovarian steroid concentrations, gonadotrophin concentrations and menstrual cycle length characteristics in healthy, cycling women

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Running title: Integration of menstrual function within cycles

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Abstract

Title: A factor analysis approach to examining relationships among ovarian steroid concentrations, gonadotropin concentrations, and menstrual cycle length characteristics in healthy, cycling women.

Study question: How are ovarian steroid concentrations, gonadotropins, and menstrual cycle characteristics inter-related within normal menstrual cycles?

Summary answer: Within cycles, measures of estradiol production are highly related to one another, as are measures of progesterone production, however the two hormones also show some independence from one another, and measures of cycle length and gonadotropin concentrations show even greater independence, indicating minimal integration within cycles.

What is known already: The menstrual cycle is typically conceptualized as a cohesive unit, with hormone levels, follicular development, and ovulation all closely inter-related within a single cycle. Empirical support for this idea is limited, however, and to our knowledge, no analysis has examined the relationships among all of these components simultaneously.

Study design, size, duration: 206 healthy, cycling Norwegian women participated in a prospective cohort study (EBBA-I) over the duration of a single menstrual cycle. Of these, 192 contributed hormonal and cycle data used in the current analysis.

Participants/materials, setting, methods: Subjects provided daily saliva samples throughout the menstrual cycle from which estradiol and progesterone concentrations were measured. FSH and LH concentrations were measured in serum samples from three points in the same menstrual cycle and cycle length characteristics were calculated based on hormonal data and menstrual records. A factor analysis was conducted to examine the underlying relationships among 22 variables derived from the hormonal data and menstrual cycle characteristics.

Main results and the role of chance: Six rotated factors emerged, explaining 80% of the variance in the data. Of these, factors representing estradiol and progesterone concentrations accounted for 37% and 13% of the variance, respectively. There was some association between measures of

estradiol and progesterone production within cycles, however cycle length characteristics and gonadotropin concentrations showed little association with any measure of ovarian hormone concentrations.

Limitations, reasons for caution: Our summary measures of ovarian hormones may be imprecise in women with extremely long or short cycles, which could affect the patterns emerging in the factor analysis. Given that we only had data from one cycle on each woman, furthermore, we cannot how address cycle characteristics may covary within individual women across multiple cycles.

Wider implications of the findings: Our findings are generalizable to other healthy populations with typical cycles, however may not be applicable to cycles that are anovulatory, extreme in length, or otherwise atypical. The results support previous findings that measures of estradiol production are highly correlated across the cycle, as are measures of progesterone production. Estradiol and progesterone concentrations are associated with one another, furthermore. However factor analysis also revealed more complex underlying patterns in the menstrual cycle, highlighting the fact that gonadotropin concentrations and cycle length characteristics are virtually independent of ovarian hormones. These results suggest that despite integration of follicular and luteal ovarian steroid production across the cycle, cycle quality is a multi-faceted construct, rather than a single dimension.

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Keywords: menstrual cycle length, gonadotropin, estradiol, progesterone, ovarian function;

Introduction

The menstrual cycle is typically characterized as a single, cohesive unit in which hormone levels, follicular development, and ovulation are all closely inter-related. From this perspective, high quality cycles are not only ovulatory, but have high estradiol and progesterone concentrations, characteristic mid-cycle LH and FSH peaks, and are approximately 28 days in length. At the other end of the spectrum, low quality cycles are not only anovulatory, but may have low estradiol and progesterone concentrations, lack discernible LH and FSH peaks, and be atypical in length. In other words, the classical view of the menstrual cycle implies that the quality of a given cycle is consistent across multiple measures, including follicular development, ovarian steroid and gonadotropin concentrations, endometrial development, and cycle length characteristics. This concept of consistent quality across the cycle is often implicitly accepted, however few studies have directly examined this question. Certainly at the extreme end of the spectrum of impaired ovarian function, multiple aspects of hormone production and cycle characteristics can all be compromised, with possible cessation of menses and hormone cycling (Ellison, 1990). Within the range of typical, healthy cycling, however, the degree of cohesiveness or integration of cycle quality remains unclear. Empirically, if the quality of a cycle really is a single dimension, the various measureable components should show a high degree of covariance within cycles. The goal of the current analysis is to examine patterns of association among component parts of the menstrual cycle (estradiol concentrations, progesterone concentrations, gonadotropin concentrations, and cycle length variables) in order to better understand the normal menstrual cycle.

Theoretical support for close correlation of estradiol and progesterone production comes from the fact that both ovarian steroids derive from the same underlying structures. That is, the very cells

that produce estradiol in the follicular phase- the theca and granulosa cells of the preovulatory follicle- are those that go on to comprise the progesterone-producing cells of the corpus luteum after ovulation (Strauss and Williams, 2004). Based purely on the underlying cellular physiology, therefore, we might predict consistency of ovarian steroid production across the menstrual cycle (e.g. robust follicular estradiol production associated with robust luteal estradiol and progesterone production). On the other hand, estrogen and progesterone play different physiological roles in reproduction, and epidemiological evidence indicates that they can vary independently of one another (Lipson and Ellison, 1996, Nunez-De La Mora, et al., 2008, Nunez-de la Mora, et al., 2007, Venners, et al., 2006). For instance, one study measuring daily ovarian hormone profiles in premenopausal women found very low correlations between urinary estrogen and progesterone metabolite concentrations within a cycle (r=-0.003 to 0.13) (Windham, et al., 2002). Thus, although a high degree of consistency between estradiol and progesterone indices might be expected, empirical support for that prediction is mixed.

Much as we might expect consistency of ovarian steroid hormone production within a cycle, so might we predict that ovarian steroid production is closely associated with production of pituitary gonadotropins. Ovarian steroid hormone production and release comes from coordination of ovarian theca and granulosa cell activity and is dependent upon gonadotropin input from the pituitary gland, with luteinizing hormone (LH) stimulating theca cell function, while follicle stimulating hormone (FSH) influences estradiol production by the granulosa cells (Strauss and Williams, 2004). Although some follicular development can proceed in the absence of FSH stimulation suggesting there is limited ovarian hormone activity independent of pituitary input (Oktay, et al., 1998), gonadotropin stimulation is essential for advancing further to the steroid-producing antral phase (Irving-Rodgers, et al., 2001). The complex feedback interactions between gonadotropins and ovarian steroids continue throughout mid-cycle, when rising follicular estradiol levels drive preovulatory surges in FSH and LH (Richards, et al., 2002). The apparent

interdependence of gonadotropin and ovarian steroid activity characteristic of normal ovarian function suggests that the two may be closely associated throughout the cycle, at least during certain periods. For instance, in one study of cycling women, there were weak correlations between estradiol and FSH early in the follicular phase, but higher (inverse) correlations in the mid-follicular phase (Robertson, et al., 2009) and other studies have found that estradiol has inhibitory effects on FSH secretion in the luteal phase (de Ziegler, et al., 1992, Lahlou, et al., 1999, Lasley, et al., 1975). In the luteal phase, moreover, both LH and FSH show weak to moderate negative correlations with progesterone concentrations, while LH and estradiol concentrations show a weak positive correlation (Robertson, et al., 2009). Thus there is evidence to suggest some coordination of pituitary gonadotropin and ovarian steroid production within the cycle, although the strength and direction of the relationship may vary at different points in the cycle.

That cycle length variables should be linked to hormone concentrations and follicular development is less obvious, but this prediction follows from the physiology nonetheless. The length of the follicular phase reflects the speed at which the antral follicle is recruited and develops, and thus by extension, follicular phase length should be related to gonadotropin and ovarian steroid concentrations as well (Cabral and de Medeiros, 2007, Harlow, et al., 2000). Experimental evidence in primates suggests that if the antral follicle is destroyed, the characteristic preovulatory gonadotropin surge is delayed, extending both the length of follicular phase and that of the total cycle (Goodman, et al., 1977). In humans, a limited body of work suggests associations between ovarian steroid concentrations and cycle length parameters, including total cycle length, follicular phase length, and luteal phase length, arguing further for consistency of cycle quality across multiple domains (Harlow, et al., 2000, Landgren, et al., 1980, Windham, et al., 2002). In particular, short follicular phases and short cycles may be associated with relatively high estrogen and progesterone concentrations, whereas longer follicular phases

may be characterized by lower average estrogen concentrations (Harlow, et al., 2000, Landgren, et al., 1980). Other studies have also observed positive correlations between progesterone levels and luteal phase length (Landgren, et al., 1980, Windham, et al., 2002).

To date, it has been difficult to study associations among different measures of ovarian function across the menstrual cycle because of the difficulty of obtaining repeated measures of hormonal variables across the entire cycle. Only a handful of studies have measured complete, daily estradiol and progesterone profiles over the course of one or more cycles (De Souza, et al., 1998, Liu, et al., 2004, Matthews, et al., 2006, Santoro, et al., 2004, Windham, et al., 2002). The convenience and non-invasiveness of saliva collection compared to blood or urine makes it an ideal medium for measuring daily ovarian steroid profiles. However the extremely low concentrations of estradiol in saliva made these analyses prohibitively difficult until relatively recently (O'Rourke and Ellison, 1993). In this analysis using data from healthy, cycling women participating in the Norwegian Energy Balance and Breast Cancer Aspects-I (EBBA-I) study, we adopt a factor analysis approach to examine whether the menstrual cycle truly is a cohesive unit. In particular, we focus on the relationships among four aspects of the menstrual cycle (estradiol concentrations, progesterone concentrations, gonadotropin concentrations, and measures of cycle length), looking at the strength of the associations between these components within cycles.

Methods

Subject population, participants, and study design

Women were recruited for the EBBA-I study, based in Tromsø, Norway, between 2000 and 2002. The study's goal was to examine the role of energetics and other lifestyle variables on known breast cancer risk factors in healthy, premenopausal women. To participate, women had to be age 25-35 with regular menstrual cycles and could not have been pregnant, lactated, or used hormonal

contraception in the previous six months. Women with known histories of infertility, gynecological disorders, and chronic illnesses (such as type II diabetes) were excluded as well. In total, 206 women participated in EBBA-I, and the subject population, recruitment methods, and study design have been described elsewhere in detail (Furberg, et al., 2005). Subjects received 1000 Norwegian kroner (approximately $160 US dollars at the time) to cover transportation and other expenses related to their participation.

Ethical approval

Participating women signed informed consent and the study was approved by the Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate as well as the human subjects review boards at all participating institutions.

Salivary steroid assay

As part of the study, subjects collected daily waking saliva samples over an entire menstrual cycle according to protocols developed by the Reproductive Ecology Laboratory at Harvard University (Lipson and Ellison, 1989). Free estradiol concentrations were assayed in samples from 20 cycle days (reverse cycle days -5 to -24), and progesterone concentrations were assayed for the last 14 days of each cycle (reverse cycle days -1 to -14). Levels of both hormones were measured using I-125-based radioimmunoassay (RIA) kits (Diagnostics Systems Laboratory, Webster, TX, USA) using methods reported elsewhere (Furberg, et al., 2005). The sensitivity of the estradiol assay was 4 pmol/L (1.1 pg/mL), the average intra-assay variability was 9%, and the interassay variability ranged from 23% to 13% for the low and high pools, respectively. The sensitivity of the progesterone assay was 13 pmol/L (4.1 pg/mL). Based on our assayed samples, the average intra-assay variability was 10%, and the inter-assay variability ranged from 19% to 12% for the low and high pools, respectively.

Once estradiol assays had been completed, the daily concentrations across each cycle were examined in order to identify the day of the greatest mid-cycle drop in estradiol using methods described elsewhere (Lipson and Ellison, 1996). For each cycle, a mid-cycle estradiol "drop day" was first determined. The drop day was defined as the second of the two consecutive days in a mid-cycle window during which the greatest decrease in estradiol occurred. The mid-cycle window for identifying peak estradiol was days -18 to -12, thus the drop day was constrained to fall between days -17 to -11. This estradiol drop provides a good marker for the timing of ovulation, and the drop day was subsequently designated as day '0'. Thus days in the follicular phase have negative prefixes (e.g. day -1, day -2), whereas days in the luteal phase have positive prefixes (e.g. day +1, day +2). A drop day could not be assigned for 14 subjects. Eight of the 14 had missing hormone data for at least one day during the interval between reverse cycle days -18 to -12. The remaining six subjects had no discernable rise or drop in estradiol during the critical time window and their mid-cycle LH levels were low as well, suggesting that the cycles were anovulatory. Because determination of drop day is needed to calculate hormonal indices and separate cycles into follicular and luteal phases, these 14 women were excluded and only the 192 women with aligned cycles were included in analyses.

Creation of ovarian hormone indices

From the daily estradiol and progesterone concentrations we were able to calculate a number of different indices of hormone levels, representing different components or periods of ovarian function. Each index was calculated as the mean hormone concentration across samples collected during a particular period of the cycle relative to ovulation (day 0). Seven estradiol indices were calculated for each cycle: *total estradiol* (mean estradiol for all cycle days measured) reflects average estradiol exposure across the cycle; *follicular estradiol* (mean estradiol, days -10 to -1) reflects average estradiol prior to ovulation; *mid-follicular estradiol* (mean estradiol, days -10 to -6) reflects estradiol production around the time of the emergence of the dominant follicle; *late*

follicular estradiol (mean estradiol, days -5 to -1) reflects the secretory activity of the dominant follicle prior to ovulation; *maximum follicular estradiol* (highest estradiol concentration measured between days -10 to -1), *maximum estradiol* (highest estradiol at any point in the cycle), and *magnitude of the midcycle estradiol drop* (maximum estradiol minus estradiol on day 0) reflect midcycle estradiol secretion as well as the decrease in circulating estradiol accompanying ovulation of the dominant follicle. If follicular phase length was shorter than 10 days, follicular estradiol and mid-follicular estradiol calculations were adjusted accordingly (e.g. if the follicular phase was only 9 days, was calculated as the mean of days -9 through -1, rather than -10 through -1). Similarly, if data were missing (for instance, due to estradiol concentrations below the sensitivity limit at the beginning of the cycle), indices were calculated as the mean across the days with measurable concentrations in that interval.

Six indices of progesterone concentrations were calculated as well. *Total progesterone* (mean progesterone, days 0 to +14) reflects average progesterone exposure during the luteal phase; *early-mid luteal progesterone* (mean progesterone, days 0 to +9) represents the average circulating progesterone concentrations during the beginning and middle of the luteal phase; *mid-luteal progesterone* (mean progesterone, days +5 to +9) reflects the level of progesterone secretion at the peak of the luteal phase; *very early luteal progesterone* (mean progesterone, days 0 to +2) and *early luteal progesterone* (mean progesterone, days +3 to +5) together reflect the early luteal progesterone rise, before any possible effects of human chorionic gonadotropin (hCG) from a potential conceptus; and *late luteal progesterone* (mean progesterone, days +10 to +14) reflects post-peak secretion of progesterone during the regression of the corpus luteum prior to menstruation. If luteal phase length was shorter than 14 days, total progesterone and late luteal progesterone calculations were adjusted accordingly (e.g. if the luteal phase was 12 days, total progesterone was calculated as the mean of days 0 through +12, rather than 0 through +14). When progesterone values were missing for individual days, the hormone indices were calculated

as the means of those days with data. Summary statistics for the hormonal variables are provided in Table 2.

Calculation of menstrual cycle phase lengths

Three cycle length variables were measured using the hormone data and self-reported dates of menses. *Overall cycle length* was the number of days from menstrual onset to menstrual onset, as determined by self-reported menses. *Follicular phase length* was the number of days from menstrual onset to the mid-cycle estradiol drop day. Finally, *luteal phase length* was the number of days from the day after the mid-cycle estradiol drop day to onset of subsequent self-reported menses.

Serum sample collection and gonadotropin assay

At three points in the cycle, fasting serum samples were taken by trained nurses at the University Hospital of Northern Norway, Tromsø. These collections were done between days 1-2, 7-12, and 21, reflecting the early follicular, pre-ovulatory, and luteal phases of the cycle. Luteinizing hormone (LH) and follicular stimulating hormone (FSH) were measured in serum samples from all three time points using Techicon Immuno1 immunometric assays (Bayer Corp, Tarrytown, NY). Both assays were standardized against the WHO 2nd International Standard (for FSH: IRP 78/549 and for LH: IRP 68/40). The sensitivity of the FSH assay was 0.1 IU/L and the coefficient of variation was less than 7 percent. For LH, the assay sensitivity was 0.3 IU/L and the coefficient of variation was 5-10 percent.

Statistical analyses

All statistical analyses were carried out in SAS Enterprise 4.3 (SAS Corporation, Cary, NC). Because hormone values typically follow non-normal distributions, all hormone indices were first log transformed to normalize variances. We first examined bivariate correlations between the

ovarian hormone indices in our analysis. We then conducted a factor analysis by principal components extraction, with and without orthogonal varimax rotation of axes on the correlation matrix of the study variables. The goal of factor analysis is to condense a large number of correlated variables into a smaller number of factors and in doing so, reveal underlying relationships among the variables. Orthogonal varimax rotation then rotates these factors so that they are uncorrelated with one another, creating factors for which one or more variables have high loadings, while loadings for the other variables are close to zero (Manly, 2005). Each factor has an eigenvalue, which indicates the amount of variance in all of the variables that is accounted for by that factor, and following the conventionally used Kaiser criterion, only factors with eigenvalues greater than 1 (i.e. explaining more than 1 percent of the total variance) are retained in the analysis (Kaiser, 1958). Thus factors with large eigenvalues explain a large amount of variance in the overall data, whereas factors with small eigenvalues explain little of the variance. A Scree plot (which helps to visually discriminate between those factors explaining a large fraction of the variance and those which are relatively unimportant) was made to confirm the number of factors that should be included in the analysis. For each factor with an eigenvalue greater than 1, we examined the loading of each menstrual cycle variable, which is similar to a standardized regression coefficient when the factor is regressed on the variables (DeCoster, 1998). Loadings of ≥ 0.7 were considered strong loadings, while those <0.7, but ≥ 0.35 were considered moderate loadings. Loadings <0.35 were considered weak to negligible.

One of the useful aspects of principal component extraction is the collapsing of highly correlated variables into a smaller number of axes representing linear functions of those correlated variables. Here, for instance, although high correlations might be expected among the different indices of each steroid (particularly those that overlap), it is not necessarily the case that seemingly related indices would all cluster on the same rotated axes resulting from factor analysis. Luteal and follicular estradiol secretion, for example, might be governed by different patterns of

gonadotropin secretion and hence manifest significant independence. Similarly mid-follicular estradiol might reflect the combined secretory activity of a recruited cohort of follicles under FSH stimulation, whereas late follicular and maximum follicular estradiol presumably reflect secretion by the dominant follicle alone. The degree to which these aspects of estradiol production are independent will affect the degree to which they individually correlate with average estradiol levels over the entire follicular phase or the entire cycle as well. Thus the current analyses allow us to examine the relationships among the specified variables without making any *a priori* assumptions about the independence (or multicollinearity) of different indices of ovarian steroid levels and other cycle characteristics. Instead, factor analysis allows us to identify those clusters of variables that are highly redundant and thus reduce the number of indices studied.

Results

General characteristics of the study subjects are provided in Table 1. The study population was predominantly Caucasian and highly educated with a mean age of 30 years. 61% of subjects were married and half had at least one child. The average cycle length was 28 days (range: 20-47), of which 15 were spent in the follicular phase and 13 in the luteal phase. Bivariate analyses indicate moderate to strong positive correlations between the estradiol and progesterone indices and are presented in Table 2. Most of the estradiol indices have correlation coefficients with each other in the range 0.7 to 0.95. The exception is the magnitude of the midcycle estradiol drop, which correlates very weakly, and typically negatively, with the other estradiol variables. Similarly, the progesterone indices have correlation coefficients with each other in the range of 0.57 to 0.97. Except for magnitude of the estradiol drop, the correlations between the estradiol and progesterone indices are moderate, ranging from 0.38 to 0.60.

The unrotated factor matrix (not shown) generated six factors with eigenvalues greater than 1. Typically, the first unrotated factor represents the single vector that captures the greatest amount of the multivariate variance and in this case, all of the estradiol and progesterone measures (aside from the magnitude of the estradiol drop) had loadings of 0.70 or greater on Factor 1. Of the remaining variables, seven had loadings between 0.05 and 0.16, with the remaining three having loadings less than 0.05. Factor 1 of the unrotated matrix accounted for only 38% of the total multivariate variance in the sample, however, indicating that the majority of the multivariate variance could not be captured by a single axis.

Subsequent orthogonal varimax rotation of the axes obtained from the factor analysis maximized the separation of the factor loadings of the original variables onto different axes and generated six rotated factors with eigenvalues greater than 1, which together explained 80 percent of the variation in the data. Factor loadings for the six rotated factors are presented in Table 3. The varimax rotation largely succeeded in separating the original variables onto different axes, each of which was orthogonal to, or independent of, the others. All variables loaded on at least one factor, but no variable had a strong loading (≥ 0.7) on more than one factor. Two variables, mid-cycle LH and mid-cycle FSH, showed split moderate loadings on more than one factor. Mid-cycle LH loaded strongly on Factor 5 and moderately (and negatively) on Factor 3, while mid-cycle FSH showed a moderate, negative loading on Factor 3, and moderate, positive loadings on Factors 4 and 5. Only two original variables, mid-cycle FSH and magnitude of the mid-cycle estradiol drop, did not have a strong loading on any of the six rotated factors. Mid-cycle FSH instead had moderate loadings on three factors, while the magnitude of the mid-cycle estradiol drop had a moderate loading on one factor.

The first rotated factor explained 37% of the variance and included all of the measures of estradiol except for the magnitude of the mid-cycle estradiol drop. The second rotated factor

included the six progesterone indices and explained 13% of the variance. The third rotated factor accounted for 11% of the variance and included cycle length and follicular phase length with minor loadings on mid-cycle gonadotropin concentrations. The fourth rotated factor explained 9% of the variance and included luteal gonadotropin concentrations, with a minor loading on mid-cycle FSH concentrations and the magnitude of the estradiol drop. The fifth rotated factor, explaining 5% of the variance, had major loadings on early follicular and mid-cycle LH concentrations and minor loadings on early follicular and mid-cycle FSH loadings. Finally, only luteal phase length was included in the sixth rotated factor, which accounted for 5% of the variance in the data set. Sensitivity analyses (not shown) using only subjects with complete daily hormone data did not change the basic relationships among variables and factors.

Discussion

The purpose of this analysis is to examine the extent to which the menstrual cycle is a cohesive unit in healthy, reproductive-age women, as measured by the strength of the relationships among hormonal measurements and cycle characteristics. Or, phrased as a question, to what extent is any one measure of menstrual function predictive of or independent of others within the same cycle? Of particular interest is the extent to which there is coordination of ovarian steroid production across the follicular and luteal phases of the cycle. In our study population, the relationship between follicular phase estradiol and luteal phase progesterone is significantly positive, as reflected in the bivariate correlations. These correlations are much higher than reported in at least one other study in cycling women, in which correlations between urinary estradiol and progesterone metabolite concentrations were 0.13 or lower (Windham, et al., 2002). Because urinary assays measure conjugated metabolite concentrations and are thus one or more steps removed from circulating free hormone concentrations, such assays may introduce additional noise related to inter-individual metabolic variation (Gann, et al., 2001). For that reason, the

stronger correlations found in the current study based on free (bioactive) salivary steroid concentrations may be a more accurate reflection of the true relationship between estradiol and progesterone concentrations in healthy, cycling women.

In the current study, the positive association between follicular and luteal steroid profiles is further illustrated by sorting the study subjects into quartiles on the basis of the indices of one steroid and comparing the full daily profiles of the other. Sorting the subjects into quartiles by mean follicular estradiol concentrations shows that women with high mean follicular estradiol concentrations also tend to have high progesterone concentrations throughout the luteal phase (Figure 1). Similarly, when subjects are sorted by mean luteal progesterone concentrations, those with the highest quartile of luteal progesterone concentrations tend to have high follicular estradiol concentrations as well (Figure 2). In both cases, the quartiles are clearly distinct from one another. Thus crude analyses suggest that, across women, within a cycle, levels of one of these hormones are indicative of levels of the other.

The subsequent factor analysis allowed simultaneous examination of the relationships between the ovarian steroid concentrations and other measures of cycle quality to identify more complex underlying patterns. The factors obtained after varimax rotation represent the "sorting" of variables into groups that are highly correlated among the group while being orthogonal, or independent, of the groups represented by other factors. Factor 1 has very strong loadings (0.85 or greater) for all the estradiol indices except the magnitude of the estradiol drop. It reflects the high consistency of estradiol production across the ovarian cycle and supports previous work finding high correlations between estradiol measures at multiple points across the cycle (Windham, et al., 2002). The progesterone indices load weakly on this factor (0.20 to 0.30), with loadings being highest for early luteal progesterone measures and lower for indices capturing the later part of the luteal phase. This suggests that estradiol and progesterone production cannot be fully

disentangled, particularly in the early luteal phase. Factor 2 has very high loadings (0.73 or greater) for all the progesterone indices, suggesting that progesterone production is highly consistent across the luteal phase. In Factor 2 there are weak loadings (0.2 to 0.34) for most of the estradiol indices, again indicating that there is some aspect of the relationship between progesterone and estradiol production that cannot be disarticulated, as suggested in the crude analyses.

Nevertheless, the degree to which indices of the two ovarian steroids separate onto different axes in the factor analysis reflects the degree to which they are actually independent of each other. It is noteworthy, that no other variables have loadings on the first two rotated factors, which we therefore regard as the estradiol and progesterone factors, respectively. In particular, the loadings for both the gonadotropin variables and the cycle length measures are extremely low. This suggests that a woman's circulating estradiol and progesterone concentrations are not a clear function of her circulating gonadotropin concentrations, nor are they closely related to her cycle length. Rather, other factors including gonadotropin receptor densities or sub-types, co-gonadotropins such as insulin and IGF-1, or other unknown genetic, developmental, or constitutional components may explain inter-individual variance in ovarian steroid concentrations. It remains possible that differences in gonadotropin concentrations may account for more of the documented within-individual variance in ovarian steroid concentrations (between multiple cycles in the same woman, for instance) (Lipson and Ellison, 1996, Venners, et al., 2006).

Factor 3 has very high loadings (0.88 or greater) for total cycle length and follicular phase length, which confirms the close association between follicular phase length and overall cycle length that has been noted elsewhere (Fehring, et al., 2006, Waller, et al., 1998). The moderate negative loadings of the mid-cycle concentrations of LH and FSH on this factor are more surprising,

suggesting that factors associated with slow follicular growth (resulting in a longer follicular phase and longer total cycle length) may later result in poor steroid response in the luteal phase. Because this factor is independent of steroid concentrations themselves (Factors 1 & 2), even in the luteal phase, it may indicate that higher gonadotropin levels are required to stimulate a given amount of steroid production in cycles with longer follicular phases than in those with shorter follicular phases. This may again be consistent with variation in the ovarian responsiveness to gonadotropin stimulation rather than the level of that stimulation itself, an effect that might be moderated at the receptor level. Further study is needed to understand these unexpected relationships.

Factor 4 has strong loadings (0.80 or greater) for luteal FSH and LH concentrations. It also has moderate loadings for mid-cycle FSH (0.49) and the magnitude of the mid-cycle estradiol drop (0.41) It is noteworthy that the magnitude of the mid-cycle estradiol drop only clusters with luteal gonadotropins (albeit moderately) and not with any of the ovarian steroid measures. In fact, magnitude of the mid-cycle estradiol drop is the only steroid index that correlates significantly with levels of gonadotropin stimulation, although it is somewhat surprising that it clusters with luteal, rather than mid-cycle gonadotropin concentrations. Baseline gonadotropin concentrations tend to be very low across the entire luteal phase and pulsatile release of gonadotropins occurs at low frequency (Hall, 2004, Johnson and Everitt, 2000), so it is unclear why they should be associated with the magnitude of the estradiol drop. Additional research is needed to confirm and better understand this unexpected observation.

Factor 5 has high loadings (greater than 0.70) for early follicular and mid-cycle LH, with moderate loadings (0.5-0.7) for early follicular and mid-cycle FSH. This indicates that gonadotropin concentrations in the first half of the cycle are closely associated even though the two have distinct functional differences, with FSH stimulating further development of the antral

follicle while LH promotes ovarian steroid production and eventually, ovulation (Hall, 2004, Strauss and Williams, 2004). Nevertheless, given that both are produced and secreted by a common source (the pituitary gland) and that both are responsive to fluctuating ovarian steroid concentrations (through negative feedback), it is not surprising that FSH and LH concentrations would load on the same factor. More surprising, perhaps, is how weak the ovarian steroid loadings are on this factor, which may indicate that across women, there is little relationship between early follicular and mid-cycle gonadotropins and ovarian steroid concentrations. Once again, this suggests that it may be sensitivity to gonadotropin stimulation (for instance through receptor densities or the effect of co-gonadotropins), and not absolute gonadotropin concentrations that are most important for regulating ovarian steroid production.

Finally, only luteal phase length loads on Factor 6 (0.97), indicating that it is virtually independent of the other hormone and cycle characteristics considered in this analysis. It is not surprising that luteal phase length did not cluster with the other cycle length variables given that the literature suggests that while total cycle length and follicular phase length are tightly correlated, luteal phase length tends to be less variable and show only moderate correlations with both (Fehring, et al., 2006, Waller, et al., 1998). In fact, one study found that only three percent of the variance in total cycle length was attributable to variation in luteal phase length, whereas follicular phase variation explained over 84 percent (Waller, et al., 1998). At least one study has identified differences in urinary ovarian steroid concentrations in relation to luteal phase length, however those differences were in comparisons of cycles with short (≤10 days), average (11-14 days), and long (≥15 days) luteal phases and did not consider luteal phase lengths continuously within the normal range (Windham, et al., 2002). In general, little is known about predictors or determinants of luteal phase length and additional research is needed to understand the existing variation and how it is related to other cycle indicators of ovarian function.

Overall, the results of this factor analysis suggest that although there is some consistency of menstrual function across domains, the particular cycle measures considered here also show considerable independence from one another across women. Perhaps of greatest interest are the associations between estradiol and progesterone indices, which clustered onto two distinct factors, but also showed minor loadings on each other's primary factors, suggesting some inter-dependence between the two. This finding is interesting in light of previous work suggesting that follicular estradiol concentrations are higher in conception cycles than non-conception cycles (Lipson and Ellison, 1996). One explanation is that high estradiol concentrations better stimulate the developing oocyte and prime the endometrium for proliferation, thus increasing the odds of a successful conception. Our results suggest a second explanation should be considered as well, namely that there may also be correlated luteal phase effects, including endometrial secretions and support for implantation that is necessary for successful conception. The associations between estradiol measures (which are primarily follicular) and progesterone measures (which are luteal) is clinically important, moreover, in that it further supports the idea that luteal phase defects are actually product of problems with follicular development earlier in the cycle (DiZerega and Hodgen, 1981).

At the same time, estradiol and progesterone measures also showed a degree of independence from one another, and the fact that ovarian steroid concentrations were not associated with variation in gonadotropin concentrations or in cycle and phase lengths, moreover, suggests that it may be mediated by tissue sensitivity, perhaps reflecting differences in receptor expression or variation, or other physiological, genetic, developmental, or constitutional factors. Such a mechanism would be consistent with findings of a study of adult Bangladeshi migrants to the UK, that indicated that progesterone, but not estradiol, was related to individual developmental history (Nunez-De La Mora, et al., 2008, Nunez-de la Mora, et al., 2007). Indeed, at least one study found that progesterone levels tend to be predictable within individuals over intervals of as much

as one year, whereas estradiol levels may vary dramatically within individuals over the same time period (Chatterton, et al., 2005). The dissociation of estradiol and progesterone profiles observed in such studies suggests that developmental history may exert long-lasting influence on some aspects of ovarian steroid production, whereas other aspects are more responsive to acute cues in the immediate environment.

There are some limitations to the interpretation of results from this study. First, the current study assessed only inter-individual effects, finding, for instance, that women who have high estradiol levels tend to have high progesterone levels and vice versa. Because hormone levels were only measured for the duration of a single cycle in this study, we are unable to examine whether the same trend holds between cycles within individual women. Additional research following women longitudinally over time is needed to determine whether, within a given woman, high estradiol cycles are likely to also feature high progesterone levels, while low estradiol cycles tend to have low progesterone levels. Similarly, our results do not address whether other cycle characteristics tend to covary within individual women across multiple cycles.

Because we measured estradiol concentrations only in samples collected on reverse cycle days -5 to -24 (i.e. from 5 to 24 days before the start of the next menstrual bleeding), our estradiol indices may be inaccurate for any women with extremely long cycles. Although the recruitment criteria generally excluded women with atypical cycles, in practice, approximately 5 percent of subjects had cycles longer than 35 days. In a 35 day cycle, for instance, by assaying estradiol concentrations only in days -5 to -24 of the cycle, our calculated estradiol indices are artificially truncated, omitting concentrations in the early follicular phase. By contrast, in women with shorter cycles, nearly the entire follicular phase would be captured in our estradiol indices. Similarly, given that progesterone concentrations were only assayed in sample from reverse cycle days -1 to -14 (i.e. from 1 to 14 days before the start of the next menstrual bleeding), in women

559 with extremely long luteal phases, the progesterone indices might not capture the earliest days of
560 the luteal phase. For several reasons, however, we believe that this potential error is unlikely to
561 affect our results. First, we conducted a sensitivity analysis (not shown) restricting the analyses
562 to subjects with cycle lengths ranging from 24-34 days and found that although there were slight
563 differences in the exact factor loadings, the patterns and relationships that emerged were
564 unchanged from those found using the whole cohort. Second, the fact that ovarian hormones and
565 cycle phase lengths load on different factors in our analysis suggests the two are largely
566 independent of one another. If there were significant confounding of these variables, there would
567 have been strong loadings of ovarian hormone and cycle length characteristics on the same
568 factors, which there was not. Ultimately, if there were bias due to improper calculation of
569 hormone indices in long cycles, it would be for cycles with longer follicular phases to have higher
570 average estradiol levels (since it would be the early follicular levels, which are typically low, that
571 were omitted from the calculated indices), and we do not see any evidence of that. Any bias in
572 progesterone levels due to cycle length would be similar, but there is strong evidence from many
573 studies, including these data, that variation in luteal phase length is minimal (Matsumoto, et al.,
574 1962, Vollman, 1977). Thus we suggest that any bias in this regard is negligible.
575
576 Another limitation is our subject population, which was specifically recruited to be ages 25-35
577 and self-identifying as having regular cycles. Our population's cycle length and cycle phase
578 lengths were typical of healthy women in this age range, however we cannot necessarily
579 extrapolate our findings to address this question in other groups of women (Chiazze, et al., 1968,
580 Treloar, et al., 1967, Vollman, 1977). In particular, women with less typical cycles (who would
581 have been excluded from participation in the current study) might show different patterns of cycle
582 hormones and characteristics, as might the 14 subjects whose hormonal profiles did not allow us
583 to readily identify an estradiol drop day (and hence were excluded from analysis). Whether these
584 results also apply to younger and older women (whose cycles may tend to be more erratic and

585 have lower hormone levels) remains unknown (Chiazze, et al., 1968, Lipson and Ellison, 1992,
586 Treloar, et al., 1967). Further research is also needed to determine whether these patterns hold
587 true in non-Western populations in which the level of ovarian function (as evidenced by estradiol
588 and progesterone concentrations) is typically lower (Ellison, et al., 1993). Given the results of
589 migrant studies (Nunez-De La Mora, et al., 2008, Nunez-de la Mora, et al., 2007), it may be of
590 particular interest to examine women whose current environment differs radically from the
591 environment in which they were born and raised. Perhaps under such conditions, there will be
592 even weaker relationships across domains, with different cycle components reflecting
593 developmental and current conditions.
594
595 Finally, our findings on the relative independence of gonadotropin concentrations from other
596 measures of cycle quality should be interpreted with caution. As discussed, one possibility is that
597 although serum gonadotropin concentrations may not be directly associated with ovarian steroid
598 concentrations or cycle length characteristics, other indicators of gonadotropin activity (such as
599 ovarian receptor densities) may be. It is also possible, however, that because gonadotropins are
600 released in approximately hourly pulses (Kazer, et al., 1987, Moret, et al., 2009), our
601 measurement techniques (based on single serum samples at three points in the cycle) may have
602 been too imprecise to capture circulating concentrations and have resulted in additional "noise" in
603 our data. Gonadotropin concentrations would be better quantified by repeated blood sampling at
604 short intervals (approximately five minutes) during an extended time period followed by pulse
605 detection analysis (Moret, et al., 2009). Even with that improved methodology, however, we
606 would not be able to address whether the serum gonadotropin concentrations reflected the
607 concentrations in the follicle, which are ultimately of greatest relevance and interest.
608
609 In conclusion, this study is the first to directly address the extent to which multiple components of
610 the menstrual cycle and ovarian function are inter-related in healthy, cycling, Western women.

We have found that there is a significant degree of coordination of ovarian steroid production across the cycle, however estradiol and progesterone production also show considerable independence from one another. We have determined, furthermore, that across women, circulating gonadotropin concentrations and cycle length characteristics are almost entirely unrelated to ovarian steroid concentrations, suggesting that these aspects of cycle quality are independent of one another. Contrary to the textbook depiction of the menstrual cycle, cycle quality is not uniform across measures. Even in healthy, cycling women, different components of the cycle (ovarian steroids, gonadotropins, and cycle phase lengths) do not necessarily covary in a straight-forward, predictable manner. Future research may look at not only how these measures of ovarian function are related to (or independent of) one another within women, but also how additional aspects of ovarian function, such as follicular development, follicular gonadotropin levels, or endometrial proliferation fit into this complex system.

Authors' Roles:

E.B. analyzed and interpreted the data and drafted the manuscript. P.E. directed hormone analyses in the study and helped with data interpretations and manuscript drafting. S.L. conducted the hormone analyses in the study and edited the manuscript for intellectual content. A.F. helped to design the study, implement it, and critically reviewed the manuscript. I.T. designed the study, directed implementation and data collection, and critically reviewed the manuscript.

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Conflicts of interest:

The authors have no conflicts of interest to declare.

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