



Published in final edited form as:

*Psychoneuroendocrinology*. 2016 November ; 73: 79–90. doi:10.1016/j.psyneuen.2016.07.216.

## Diurnal Coupling between Testosterone and Cortisol from Adolescence to Older Adulthood

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### Abstract

The hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes are typically conceptualized as mutually inhibitory systems; however, previous studies have found evidence for positive within-person associations (i.e., *coupling*) between cortisol and testosterone. One developmental hypothesis is that positive testosterone-cortisol coupling is unique to the adolescent period and that coupling becomes attenuated, or even switches direction, in adulthood. This study used a lifespan sample ( $N = 292$ , ages 11 to 88) to test for age-related differences in coupling between cortisol and testosterone in daily life. Participants provided salivary hormone samples at waking, 30 minutes after waking, and during the evening for two days. Hierarchical linear modeling was used to test the within-person and between-person associations between testosterone and cortisol. Within-person associations were further decomposed into associations due to coupled diurnal change versus coupled variability around diurnal change. Results indicated positive associations between cortisol and testosterone at all levels of analysis. Additionally, positive coupling was evident across the lifespan, even in older adults who are no longer expected

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The authors have no conflicts of interest to declare.

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to reproduce, but further investigation of developmental differences with a larger sample is necessary. Potential mechanisms and functions for positive coupling are discussed.

## Keywords

cortisol; testosterone; dual axis; coupling; hierarchical linear modeling; lifespan

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## 1. Introduction

### 1.1 The HPA and HPG Axes across the Lifespan

Cortisol and testosterone are the most commonly studied end-products of the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes, respectively. Testosterone, which is produced by the testes and ovaries, and, to a lesser extent, by the adrenal cortex, is necessary for the development of reproductive maturity and secondary sex characteristics in males, and is an anabolic steroid that promotes protein synthesis in both sexes (Rubinow & Schmidt, 1996). Cortisol is the primary hormone released as part of the biological stress response (Dickerson & Kemeny, 2004; Miller et al., 2007). Cortisol and testosterone have been extensively studied as individual biomarkers in relation to social behavior, psychiatric illness, and physical health (e.g., Ford et al., 2016; Vreeburg et al., 2009). Increasingly, a dual axis approach that considers the interaction between HPA and HPG output is recognized as critical for understanding the predictive value of either hormone.

The functioning of the HPA and HPG axes changes dynamically across the lifespan. The HPG axis is active during fetal development, but then is quiescent during childhood, when sex hormones function in a negative feedback loop to inhibit HPG axis activity. The HPG axis is then reactivated at puberty as the inhibitory effect of sex hormones diminishes (Sisk & Turek, 1983). Similarly, human and animal research suggests that the HPA axis is significantly less reactive in early childhood (Sapolsky & Meaney, 1986; Gunnar & Cheatham, 2003) than in adolescence, when average cortisol levels increase with puberty in both sexes (Apter et al., 1979). Sex hormone levels decline in older adulthood, and older non-human animals exhibit a hyper-reactive cortisol response in comparison to young adult animals (Sapolsky & Meaney, 1986). Overall, there are lifespan changes in HPA and HPG functioning, and the two axes show parallel developmental trends, at least in early life.

### 1.2 Evidence for Positive Testosterone-Cortisol Coupling in Adolescence

The HPA and HPG axes are typically conceptualized as mutually inhibitory systems: Stress impedes reproductive function, and gonadal hormones dampen the stress response (Rivier & Rivest, 1991; Toufexis et al., 2014; Viau, 2002). This conceptualization is supported by experimental animal research in which one hormonal system is directly manipulated. Research using rats has shown, for example, that gonadectomy increases the HPA response to acute stress (Viau & Meaney, 1996) and that injecting an antagonist to corticotropin-releasing factor directly into an animal's brain can reverse the dampening effects of electric shocks on HPG axis function (Rivier et al., 1986). Moreover, correlational research in humans has suggested that testosterone and cortisol may inhibit the behavioral effects of the

other hormone. For example, at low levels of cortisol, testosterone exhibits the strongest associations with social dominance (Edwards & Casto, 2013; Mehta & Josephs, 2010; Mehta et al., 2008), aggression (Popma et al., 2007), psychopathy (Glenn et al., 2011; Tackett et al., 2014), and callous-unemotional traits (Johnson et al., 2014).

Given experimental evidence for mutual inhibition of the HPA and HPG axes, one might expect that concentrations of cortisol and testosterone would be inversely associated. Indeed, one study of adult humans found that baseline levels of testosterone were negatively correlated with cortisol response to an acute in-lab stressor in men (but not in women; Stephens et al., 2016), and another found that baseline levels of cortisol were negatively correlated with testosterone response to acute stress (Bedgood et al., 2014). A recent series of papers, however, presented evidence that cortisol and testosterone showed positive within-person associations, i.e., were positively coupled, in several independent samples of adolescents (ranging in age from 11 to 18). Interestingly, positive coupling between cortisol and testosterone was observed across different time frames and different research paradigms, including endogenous (i.e., naturally-occurring) change across the course of a normal day (Marceau et al., 2015a), change across the course of a stressful laboratory day (Dismukes et al., 2015b), and longitudinal change from early- to mid-adolescence (Ruttle et al., 2015).

Overall, these papers presented results that appear discrepant from what is known about the antagonistic actions of the HPA and HPG axes on each other. To explain this apparent discrepancy, Marceau and colleagues (2015a) presented a developmental hypothesis – that positive coupling may be unique to the adolescent period, when activity in both axes is resurging following a period of childhood quiescence. Similarly, Ruttle and colleagues (2015) surmised (p. 697-698): “...initially both the HPA and HPG axes are developing and maturing, resulting in positive coupling but, as the axes mature over time, their cross-talk develops into a more mutually inhibitory pattern.” These studies, however, did not include any adult participants and did not directly test developmental differences.

### 1.3 Competing Hypotheses regarding Developmental Specificity

Other studies have questioned the idea that positive testosterone-cortisol coupling is unique to the adolescent period. In two independent samples of pre-/early-pubertal boys and girls ( $M$  age = 9.4) and young adult men ( $M$  age = 21.1), testosterone and cortisol responses to acute social stress were positively coupled, leading Turan et al. (2015) to conclude that “the positive coupling of cortisol and testosterone found in previous research is not limited to adolescence” (p. 85). This study, however, did not present any data on adult women or on adults in middle or older adulthood. Additionally, they focused on testosterone and cortisol responses to an experimentally-induced social stressor, and this design does not resolve whether coupling between endogenous, naturally occurring changes in testosterone and cortisol would be evident in developmental periods other than adolescence.

More generally, the expectation that testosterone and cortisol will be negatively associated in adults may be based on an overly simplistic conceptualization of the relationship between the HPA and HPG axes as purely antagonistic. Both testosterone and cortisol follow a predictable diurnal rhythm, with concentrations peaking in the morning and declining through the afternoon and evening (Matchock et al., 2007; Smyth et al., 1997), and “these

parallel circadian changes suggest that the two hormones may routinely serve complementary rather than antagonistic functions under day-to-day conditions” (Gettler et al., 2011). Coincident increases in both testosterone and cortisol have been observed in female collegiate (i.e., young adult) athletes following athletic competition (Edwards & Casto, 2013), in middle-aged men and women following a laboratory social stress test (Lennartsson et al., 2012), and in adult male subsistence hunters following a successful kill (Trumble et al., 2014). Yet another study found that young men (ages 21-23) who were “mating-oriented” (i.e., neither fathers nor in a pair bonded relationship) were more likely than men who were fathers to have high levels of both cortisol *and* testosterone (Gettler et al., 2011). Additionally, in a wide variety of non-human species, biologists have observed situations in which activity in the HPG axis is maintained or even increased during periods of intense stress (Sapolsky, 1982; Wingfield & Sapolsky, 2003). These studies support the perspective that coupled activation of both axes facilitates the ability of an organism – even in adulthood – to respond to adaptive challenges, such as threats to social status (Turan et al., 2015) or mating opportunities (Gettler et al., 2011).

Finally, how the relationship between the HPA and HPG axes changes as adults lose reproductive capacity is unknown, as previous research on testosterone-cortisol coupling has focused primarily on adolescents or adults under 50. For organisms nearing the end of their reproductive lifespan, it would be adaptive to maintain reproductive functions even in the face of acute stress, as an opportunity deferred is potentially an opportunity lost forever (Wingfield & Sapolsky, 2003). However, for older adults who are unable or very unlikely to have any further offspring, co-elevated cortisol and testosterone may no longer be functional. In addition, unlike in the early lifespan when the HPA and HPG axes follow parallel trends (i.e., fetal activity, childhood quiescence, adolescent reactivation), developmental trends seem to diverge in older adulthood, when levels of gonadal hormones decrease but cortisol reactivity increases. These lines of evidence suggest that perhaps the biggest developmental shifts in testosterone-coupling occur after mid-life, rather than after adolescence.

#### 1.4 Goals of the Current Study

The current study presents results of the first investigation to use a lifespan sample to examine potential age-related differences in the sign and strength of within-person testosterone-cortisol coupling. Over the course of two days, adolescents and adults, ages 11 to 88 years, provided six salivary hormone samples (each day at waking, 30 minutes after waking, and in the evening). Samples were taken at home to assess awakening responses and naturally fluctuating hormone concentrations in daily life. Data were analyzed using hierarchical linear modeling to account for the nested data structure (hormonal assessments nested within persons). In particular, we tested the developmental hypothesis that adolescents would demonstrate positive testosterone-cortisol coupling, whereas adults would demonstrate no coupling or negative coupling.

## 2. Method

### 2.1 Participants and Procedure

Participants were part of the longitudinal Multi-Method Ambulatory Assessment project (MMAA, e.g., Riediger et al., 2009), which was designed to study affective and motivational processes in daily life. A fieldwork agency (TNS Infratest) recruited participants from three metropolitan German regions (Berlin, Duesseldorf, and Munich) based on predefined criteria to stratify the sample regarding age, gender, and educational level, and to ensure the independence of the participants (i.e., prohibiting members of the same family to take part in the study). The Ethics Committee of the Max Planck Institute for Human Development approved the study.

**2.1.1. Hormonal Measurements**—Hormone data were collected during the 2010 wave of the MMAA project. Given the original project design and aims, there were pragmatic constraints on how much hormonal data could be collected, and only testosterone and cortisol were measured. Participants were instructed to drool passively through a straw into a plastic tube at three times of day: immediately upon waking up, 30 minutes after awakening, and at 1930 h. Test tubes were color and time-coded. Furthermore, they were told to avoid eating, drinking (anything except water), smoking, and brushing their teeth 30 minutes before taking the saliva samples and to write down the time and occurrence of any deviations in instructions (e.g., taking sample late). Samples were kept at ambient temperature at home. On the day after sample collection was completed, participants sent all six saliva samples directly to the assay laboratory (Labor Krohne, Bad Salzuflen, Germany) using a pre-paid envelope.<sup>1</sup> At the end of the study, participants received a reimbursement of 10 € or 25 € (approximately US\$12 and US\$30 in 2010 dollars) if they provided complete samples for one or both days, respectively.

Samples were frozen at the laboratory at  $-20^{\circ}\text{C}$  prior to testing. For testing, samples were thawed and then mixed and centrifuged 10 min at 2000-3000 x g to remove particulate material. Hormone concentrations were determined using enzyme-linked immunosorbent assay (ELISA). The reportable ranges of hormonal concentrations using this method were 0.015 – 4.0  $\mu\text{g/dL}$  for cortisol and 2 – 760  $\text{pg/mL}$  for testosterone. The intra-assay and inter-assay coefficients of variation were 4.2% and 9.3% respectively for cortisol, and 6.9% and 12.3% for testosterone.

**2.1.2 Participant Characteristics**—Of the 400 targeted individuals,  $n = 319$  provided at least 1 saliva sample, and 293 (92% of participating individuals) had complete data for cortisol and testosterone at all 6 occasions. In a few cases, participants did not provide enough saliva for both hormones to be assayed, resulting in  $i = 1887$  usable cortisol values and  $i = 1871$  testosterone values. Individuals and samples were excluded from subsequent analyses because of pregnancy or lactation, night shift work, very early or late waking times, or failure to adhere to instructions regarding when to provide the samples (see Supplement

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<sup>1</sup>The lab did not record information on date of receipt, so we are unfortunately unable to assess whether delays in returning the samples were related to hormonal measurements. Additionally, having the samples kept at ambient temperature at home may have contributed to greater error variance in measurements (Granger et al., 2004).

for more information). The final analytic data set, therefore, used 1644 cortisol samples and 1629 testosterone samples from 292 people who were sampled on 561 days.

In the final analytic sample, age ranged from 11 to 88 years (median = 36.5 years). Participants were approximately equally distributed across four age groups (see Figure S1 in Supplement for histogram of age distribution): (1) *adolescents*, ages 11-19, which is the typical age range in which average circulating levels of both cortisol and testosterone are still increasing ( $n = 37$  males, 39 females)<sup>2</sup>; (2) *younger adults*, ages 20-39, which is often considered the peak reproductive years<sup>3</sup> ( $n = 33$  males, 37 females); (3) *middle-aged adults*, ages 40-59, who have largely finished reproducing ( $n = 27$  males, 48 females); and (4) *older adults*, age 60 to 88, who are past their reproductive years ( $n = 32$  males, 39 females). Twenty-one percent of the sample ( $n = 62$ , all adolescents) were still in secondary school. Of the remaining participants who had completed or left secondary school, 35% had received their *Abitur*, the high school graduation certificate that permits admission to university (44% of younger adults, 32% of middle-aged adults, and 33% of older adults).

Three female participants were excluded because they were pregnant or lactating. Of the remaining  $n = 163$  female participants,  $n = 11$  (7%) did not respond to questionnaire items regarding menstrual cycle. Of the females with non-missing data,  $n = 5$  (3%) reported that they were pre-menarcheal;  $n = 51$  (31%) reported that they were experiencing regular menstrual cycles and not using hormonal contraceptives;  $n = 53$  (33%) reported that they had ceased menstruating for at least 3 months due to menopause<sup>4</sup>;  $n = 43$  (26%) reported they were using oral contraceptives or were not experiencing a normal menstrual cycle for another reason.

Participants reported whether they had recently had any illness or flu, had a chronic disease, currently took any type of medication, or had any inflammation or irritation in their mouths. Over half of the sample ( $n = 151$ , 52%) reported at least one of these things, as might be expected from a community sample that includes substantial numbers of middle-aged and older adults. The remaining  $n = 141$  participants will be referred to below as the *very healthy* sub-sample and comprised 49 adolescents [64% of total adolescent sample], 43 younger adults [61% of total], 35 middle-aged adults [47% of total], and 14 older adults [20% of total]).

## 2.2 Analytic Plan

Hormone concentrations were log-transformed to reduce positive skew. Outliers were identified by inspecting the (log-transformed) distributions of each hormone, separately by age group, sex, and time of day; values that were greater or less than 3 *SDs* from the mean

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<sup>2</sup>See the Supplement (Table S2) for more information about variation in pubertal development among adolescents.

<sup>3</sup>The mean age at first birth in Germany is 29.2 years. Less than 3% of live births in Germany are to mothers ages 40 or older; 93.5% of births are to women ages 20 to 39 (Eurostat, 2015).

<sup>4</sup>The criterion for menopause is often given as one year without a menstrual period, so this measure may overestimate the number of women who are menopausal. However, the median age of women in this group was 62.5 years, and over 90% of women in this group were older than 52 years old. For comparison, previous research has found that 75% of women reach menopause by age 52.4 years, and 95% of women by 54.7 years (Weinstein et al., 2003). Given their age, we are thus reasonably confident that these women had actually ceased menstruating due to menopause.

were winsorized to  $\pm 3$  *SD*. Less than 1% of cortisol ( $i = 13$ ) values, and 1.1% of testosterone values ( $i = 16$ ) were winsorized.

We conducted four sets of analyses. First, we estimated mean cortisol and testosterone levels by time of day, sex, age group, reproductive life-stage, and health status (Section 3.1). Second, we estimated the *between-person correlations* among the hormone samples (Section 3.2). For these analyses, each participant contributed one data point for each of the variables in question: Do people who have higher levels of cortisol at waking on day 1, for example, also show higher levels of testosterone at waking on day 1? Third, we estimated the *within-person correlations* between testosterone and cortisol (Section 3.3): Does an individual at a given time show an increase in testosterone, relative to his or her average value, if he or she also shows an increase in cortisol? Here participants contributed multiple data points for each of the variables in question and therefore, each person was treated as her own unique data set, whereby the two column variables were testosterone and cortisol and the rows were defined by the six occasions of measurement. The analysis of both within- and between-person correlations is important, because, as Molenaar and colleagues have described in their work on (non)ergodicity (Molenaar, 2004; Molenaar & Campbell, 2009), the relationship between *inter-individual* (i.e., between-person) variation in two variables is not informative regarding the relationship between *intra-individual* (i.e., within-person) variation in those same variables. Fourth, we formally tested age and sex differences in the between-person and within-person associations between testosterone and cortisol using hierarchical linear modeling (HLM; Section 3.4). All HLMs were fit using the *lme4* package (version 1.1-10, Bates et al., 2015) in *R* (R Core Team, 2015).

### 3. Results

#### 3.1 Descriptive Means

The mean cortisol and testosterone levels across the day for each sex and age group are illustrated in Figure 1 and summarized in Table 1. (See Supplement Figures S2 and S3 for scatterplots of cortisol and testosterone as a function of continuous age.) As expected, average levels of cortisol increased after waking (i.e., the cortisol awakening response) and then declined over the course of the day (Wake versus Wake + 30:  $b = .28$ ,  $SE = .03$ ,  $t = 8.34$ ,  $p < .001$ ; Wake versus Evening:  $b = -1.19$ ,  $SE = .03$ ,  $t = -35.19$ ,  $p < .001$ ). There were no significant sex  $\times$  age group interactions predicting average levels of cortisol ( $F = 1.79$ ,  $df = (3, 279.3)$ ,  $p = .15$ ).<sup>5</sup> Compared to young adults ages 20 to 30, older adults (ages 70 to 88) of both sexes had higher average cortisol ( $b = .16$ ,  $SE = .06$ ,  $t = 2.55$ ,  $p = .01$ ). These results are consistent with some previous research showing age differences in cortisol (Almeida et al., 2009), although results in this area have been mixed (reviewed in Fries et al., 2009).

Average levels of testosterone declined across the day (Wake versus Wake + 30:  $b = -.20$ ,  $SE = .03$ ,  $t = -6.85$ ,  $p < .001$ ; Wake versus Evening:  $b = -.69$ ,  $SE = .03$ ,  $t = -23.62$ ,  $p < .001$ ). Compared to young adult men ages 20 to 30, adolescent boys ( $b = -.58$ ,  $SE = .12$ ,  $t =$

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<sup>5</sup>Effects of age group, sex, reproductive life stage, and time of day on hormones were estimated using a hierarchical linear model with a random intercept to account for nesting within person. *P*-values and *F*-values were estimated using the package *lmerTest* (Kuznetsova et al., 2015), which uses the Satterthwaite approximation for degrees of freedom.

–4.65,  $p < .001$ ), middle-aged men ( $b = -.35$ ,  $SE = .14$ ,  $t = -2.56$ ,  $p = .01$ ), and older adult men ( $b = -.46$ ,  $SE = .13$ ,  $t = -3.53$ ,  $p < .001$ ) had lower testosterone concentrations. These age-related differences in testosterone were not as pronounced for females (sex  $\times$  age group interaction:  $F = 5.72$ ,  $df = (3, 282.22)$ ,  $p < .001$ )

Among women, as expected the average levels of cortisol did not vary significantly by reproductive life stage (pre-menarcheal, menstruating, contracepting / amenorrheic, or post-menopausal;  $F = .22$ ,  $df = (3, 856)$ ,  $p = .88$ ; all mean differences between groups  $bs < .10$ , all  $ps > .50$ ). Compared to menstruating women, all other reproductive life stage groups had lower average testosterone levels, but none of these differences were significant, likely because the number of women in each group was small (pre-menarcheal:  $b = -.32$ ,  $SE = .24$ ,  $t = -1.35$ ,  $p = .18$ ; contracepting/amenorrheic:  $b = -.16$ ,  $SE = .11$ ,  $t = -1.53$ ,  $p = .13$ ; post-menopausal:  $b = -.12$ ,  $SE = .10$ ,  $t = -1.15$ ,  $p = .25$ ).

Average levels of cortisol and testosterone did not differ between the very healthy subsample and the remaining participants (cortisol:  $t = 1.22$ ,  $p = .22$ ; testosterone:  $t = -1.16$ ,  $p = .25$ ).

### 3.2 Between-Person Correlations

Table 2 summarizes the between-person correlations among the hormone samples, separately for males and females. Four descriptive patterns are apparent. First, the correlations among cortisol samples taken on the same day are generally weaker (mean  $r = .20$ ) than the correlations among testosterone samples taken on the same day (mean  $r = .51$ ). Second, the correlations between cortisol samples taken at the same time on different days (mean  $r = .40$ ) are weaker than the correlations between testosterone samples taken at the same time on different days (mean  $r = .62$ ). Third, the correlations between cortisol and testosterone samples taken at the same time are consistently positive and increasing in magnitude across the day. Fourth, the pattern of correlations is similar between males (below the diagonal) and females (above the diagonal).

### 3.3 Within-Person Correlations

The within-person correlations between cortisol and testosterone are illustrated in Figure 2. Figure 2A shows the scatterplot of cortisol by testosterone for  $n = 100$  randomly selected individuals in the sample. Each cell shows data for one person, and a line of best fit *for that person* is drawn. Individuals are ordered by the strength of the cortisol-testosterone relationship. Although some individuals showed negative or minimal cortisol-testosterone correlations, the preponderance of within-person correlations were strongly positive.

Similar to Figure 2A, Figure 2B illustrates the within-person correlation, except now data from all individuals are combined into a single graph. In this scatterplot, both cortisol and testosterone concentrations have been centered within-person. That is, the mean cortisol and testosterone values for each person across the 6 sampling occasions were calculated, and these person-specific means were subtracted from the individual sample concentrations. This scatterplot reveals that there is, on average, a positive association between within-person variation in the two hormones (represented by the black line) – when an individual increases



(or decreases) in testosterone, relative to his or her average value, he or she also increases (or decreases) in cortisol.

Figure 2C plots the distribution (by age group) of the within-person correlations for all individuals who contributed at least 5 samples (see Supplement Figure S4 for plot of within-person correlations as a function of continuous age). The median within-person correlation was 0.67, and only 9% of individuals had a within-person correlation that was less than or equal to zero. The mean within-person correlation and the variability in within-person correlations were similar across age groups (age 11-19:  $M = .50$ ,  $SD = .45$ ; age 20-39:  $M = .59$ ,  $SD = .31$ ; age 40-59:  $M = .59$ ,  $SD = .34$ ; age 60-88:  $M = .55$ ,  $SD = .36$ ).

The positive within-person correlations shown in Figure 2B may be driven by the fact that cortisol and testosterone both have a diurnal rhythm, with levels declining over the day, i.e., morning values are higher than evening values. To examine the role of diurnal rhythm, we then re-plotted the individual-level data from the same 100 people as shown in Figure 2A, but omitted the evening samples; only the four morning samples are included (Figure 2D). The pattern of within-person correlations between cortisol and testosterone was no longer as consistently positive.

For Figure 2E, we plotted data from all samples (morning and evening) in all individuals, but centered cortisol and testosterone within person *and* within time of day. Recall that each person gave hormone samples on two consecutive days and thus have two samples for each time of day. For each of the three times of day (Wake, Wake+30, and Evening), we calculated each person's average hormonal concentrations *at that time*, and then subtracted this person-specific, time-specific mean from the observed sample concentrations. For example, the resulting person-and-time-centered values for the Wake samples quantify whether a person had higher or lower cortisol (or testosterone) at waking on Day 1 than he or she had at waking on Day 2. As shown in Figure 2E, within-person variability in testosterone and cortisol remained, overall, positively associated, even after accounting for diurnal rhythm, but the strength of this relationship was attenuated relative to the within-person association that did not take time of day into account.

Finally, we calculated the *within-person partial correlations*, controlling for the person-specific effect of time of day, using all six measurement occasions (both morning and evening samples). The distribution of these within-person partial correlations, by age group, is illustrated in Figure 2F (See Supplement Figure S5 for plot of within-person partial correlations as a function of continuous age.) The mean within-person partial correlation remained positive (0.30), but there was more substantial heterogeneity across people, with 28% of people showing a *negative* within-person partial correlation. Again, the mean within-person partial correlation was fairly consistent across age (age 11-19:  $M = .23$ ,  $SD = .62$ ; age 20-39:  $M = .26$ ,  $SD = .64$ ; age 40-59:  $M = .35$ ,  $SD = .57$ ; age 60-88:  $M = .34$ ,  $SD = .59$ ).

### 3.4 Hierarchical Linear Modeling (HLM)

**3.4.1 Model Specification**—HLM was used to test the between- and within-person associations between testosterone and cortisol, and to test whether these associations differed across age groups. The DV for all models was cortisol (un-centered), and

testosterone was the predictor variable (although, in this case, designating one variable as the outcome is arbitrary). Variation in cortisol was modeled at three nested levels: Level 1 variation is *within time of day* (two observations for each of the three times of daily measurement); Level 2 variation is *within individuals*, and Level 3 is *between individuals*.<sup>6</sup> Level 1 captures *intra-individual variability* around the diurnal rhythm, e.g., is an individual higher or lower at waking on one day than another day? Level 2 captures *intra-individual change* due to the diurnal rhythm, e.g., is an individual higher or lower in the morning compared to the evening? Level 3 captures *individual differences*, i.e., is an individual higher or lower than other people?

The fixed and random effects included in the HLMs are listed in Table 3, and model equations are provided in the Supplement. Model 1 included the following covariates: time of day (dummy-coded variables for Wake+30 and Evening, with Wake as the reference category), wake time<sup>7</sup>, female sex, and age. Age was centered at age 40 and divided by 10, such that the coefficient corresponds to the effect of a decade difference in age. Model 2 added interactions between time of day × wake time, female sex × age, female sex × time of day.

Model 3 added testosterone as a covariate. Testosterone values were centered *within person*. Centering testosterone values within person removes between-person variation in testosterone. The regression coefficient of person-centered testosterone (labeled *Within-Person Testosterone* in Table 3) on cortisol, therefore, is a direct estimate of the within-person association between the two hormones.<sup>8</sup> The effect of between-person variation in testosterone was also included in Model 3 by entering, for each person, his or her mean level of testosterone across all 6 samples (*Person-Average Testosterone*, grand-mean centered). Model 3 also included a Within-Person Testosterone × Time of Day interaction, to test whether coupling between testosterone and cortisol was more pronounced at certain times of day.

Model 4 further decomposed within-person variation in testosterone into two levels -- variation due to diurnal rhythm versus variability around the diurnal rhythm. For Level 2 (variation due to diurnal rhythm), testosterone was centered *within-person*, averaging across the two samples collected for each time of day. For Level 1 (variability around the diurnal rhythm), testosterone was centered *within-person and within-time* (as in Figure 2F): For each of the three times of day (Wake, Wake+30, and Evening), each person's average hormonal concentrations *at that time* was calculated, and this person-specific, time-specific

<sup>6</sup>An alternative way to conceptualize the structure of the data is as time nested within day nested within persons (versus days nested within time nested within persons). However, preliminary analyses indicated that clustering of cortisol within days was minimal, as has been observed in similar studies (Marceau et al., 2015b). Additionally, modeling days as Level 1 and time of day as Level 2 allows us to decompose the within-person testosterone effect into Level 1 and Level 2 components, as is described in Section 3.4.1.

<sup>7</sup>Time elapsed between Sample 1 and Sample 2 was not significantly associated with cortisol or testosterone concentrations at Sample 2. The exact time of day at Sample 3 was not associated with cortisol or testosterone concentrations for that sample. Because participants all gave their third sample during the same narrow time frame, time at waking and time *since* waking at Sample 3 were highly correlated,  $r = -.98$ .

<sup>8</sup>Some previous HLM analyses of testosterone-cortisol coupling have analyzed hormonal data in the raw metric, rather than centering with respect to person (Dismukes et al., 2015a; Marceau et al., 2015b; Ruttle et al., 2015). If testosterone values are uncentered (or centered with respect to the grand mean), the regression coefficient of testosterone on cortisol is *not*, in fact, an estimate of the within-person association between testosterone and cortisol, as has been claimed, but is rather “an uninterpretable blend” of the between-person and within-person regressions (Raudenbush & Bryk, 2002, p. 138, see also Enders & Tofghi, 2007).

mean was subtracted from the observed sample concentrations. These quantities are illustrated in Figure 3. Model 4, therefore, entered three testosterone terms: *Person-Average Testosterone* (Level 3, grand-mean centered), *Within-Person/Between-Time Testosterone* (Level 2), and *Within-Person/Within-Time Testosterone* (Level 1). Finally, Model 5 entered additional interaction terms to test whether age group or sex moderated the effects of testosterone on cortisol (at all three levels).

For all models, four random effects were estimated: (1) a random intercept reflecting variance in cortisol level between people<sup>9</sup>, (2) a random slope reflecting variance in the cortisol awakening response between people, (3) a random slope reflecting variance in the diurnal decline in cortisol from morning to evening, and (4) residual (error) variance within day and within person. For Model 3, a random slope reflecting variance in the within-person effect of testosterone was also estimated. For Models 4 and 5, random slopes reflecting variance in the within-person/between-time and within-person/within-time effects of testosterone were estimated.

**3.4.2 Model Results**—Results from Model 1 (summarized in Table 3) indicated that cortisol levels increased immediately after waking and then declined. Results from Model 2 indicated that the effect of waking time on cortisol depended on the time of day that cortisol was measured (and, conversely, that the effect of time of day on cortisol was moderated by waking time). Waking time was unrelated to cortisol at waking, but had a significantly more negative association with cortisol at Wake+30 and significantly stronger effect on cortisol at Night. Put differently, later wake times were associated with an attenuated cortisol awakening response and a flatter diurnal slope. The cortisol awakening response also differed by sex, as females showed a significantly greater increase in cortisol from Wake to Wake+30 than males. Among males, there was a positive association between age and cortisol; however, there was a significant sex difference in the association between age and cortisol, with females showing an attenuated association. Model 2 fit the data significantly better than Model 1 ( $\chi^2 = 45.90$ ,  $df = 5$ ,  $p < .001$ ).

Model 3 introduced testosterone values as covariates. There was a positive between-person association between testosterone and cortisol. Additionally, there was a positive within-person association for cortisol at Wake. This positive within-person association was equivalently strong for cortisol at Wake+30 (i.e., there was no significant interaction between within-person testosterone and Wake+30), but was even more positive at Night. Model 3 fit the data significantly better than Model 2 ( $\chi^2 = 221.61$ ,  $df = 8$ ,  $p < .001$ ).

Model 4 further decomposed the within-person association into within-time and between-time components. At waking, there were positive associations between testosterone and cortisol, both within-person, between-time and within-person, within-time. These within-person effects were equivalently strong at Wake+30 and were even more positive at Night. To summarize, we found positive testosterone-cortisol associations at all three levels of analysis: (1) within-person variability in testosterone around the diurnal rhythm, (2) within-

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<sup>9</sup>It is also possible to include a random intercept for Level 2 (time nested within people); however, time of day was also included as a fixed effect at Level 2, and models including a Level 2 random intercept estimated the variance of this random effect to be zero.

person change in testosterone due to its diurnal rhythm, and (3) between-person differences in testosterone. Model 4 did not fit the data any better than Model 3, which collapsed the within-person effects ( $\chi^2 = 7.30$ ,  $df = 8$ ,  $p = .51$ ); this is evidence that the within-person, between-time and within-person, within-time associations between testosterone and cortisol were similar in magnitude.

Results from Model 5 indicated that there were no significant age group or sex differences in the magnitude of within-person coupling between testosterone and cortisol (neither within-time nor between-time), nor were there significant sex differences in the between-person association between testosterone and cortisol. Moreover, Model 5 did not fit the data better than Model 4, which did not include these interactions ( $\chi^2 = 5.68$ ,  $df = 6$ ,  $p = .46$ ).

Regarding the random effects, in Model 5, there was residual heterogeneity between-persons in cortisol level ( $\sigma^2 = 0.142$ ); 45% of the residual variance in cortisol levels was at the level of person [ $.142 / (.173 + .142) = 0.45$ ], while the remaining 55% was intra-individual variability (including measurement error). There was also significant variance in the random slope for time of day ( $\chi^2 = 120.71$ ,  $df = 9$ ,  $p < .001$ )<sup>10</sup>, with greater between-person heterogeneity in the extent of cortisol decline from morning to evening ( $\sigma^2 = 0.310$ ) than in the extent of the cortisol awakening response ( $\sigma^2 = 0.052$ ). There was also significant between-person variation in the slope of within-person/within-time testosterone on cortisol ( $\sigma^2 = 0.135$ ;  $\chi^2 = 30.36$ ,  $df = 5$ ,  $p < .001$ ) but not in the random slope for within-person/ between-time testosterone ( $\sigma^2 = 0.037$ ;  $\chi^2 = 8.69$ ,  $df = 5$ ,  $p = 0.12$ ).

**3.4.3 Bayes Factors for Age Interactions**—None of the three testosterone  $\times$  age interactions was significantly different from zero at  $p < .05$  (person-average interaction:  $p = 0.94$ ; within-person/between-time interaction:  $p = 0.34$ ; within-person/within-time interaction:  $p = 0.64$ ). However, non-significant  $p$ -values do not, in and of themselves, necessarily indicate strong support for the null hypothesis. To address this, we estimated Bayes factors ( $B$ ) for each of these age interaction effects using the online Bayes factor calculator provided by Dienes (2014).  $B$  compares an alternative hypothesis (in this case, that the testosterone-cortisol association differs by age) with a null hypothesis (in this case, that the testosterone-cortisol does not differ by age), and indicates how many times more likely the data are under the alternative rather than under the null. Values of  $B$  close to 1 suggest that the data is insensitive to discriminating between the hypotheses, whereas  $B$ s greater than 1 or close to 0 suggest strong evidence for the alternative or the null hypothesis, respectively. Following Jeffreys et al. (1939), Dienes (2014) suggests  $B < .33$  as “substantial evidence for the null.” Based on the hypothesis that testosterone-cortisol coupling becomes more negative with age, we represented the distribution of plausible values for the age interaction regression coefficient under the alternative hypothesis as a normal distribution with  $M = -.06$  and  $SD = .03$  ( $M/2$ ).  $M = -.06$  was chosen to characterize the distribution of plausible effects, because this is the effect we would expect if the strength of the testosterone-cortisol association in adolescence was as positive as observed in the current

<sup>10</sup>Significance of random effects tested using  $\chi^2$  difference tests comparing the original model with a nested model in which the random effect was omitted. In *lmer*, a random effect term with  $q$  components contributes  $q(q+1)/2$  parameters, because the covariances among the random effects are also estimated. For example, Model 5 estimated 5 random effects and 15 parameters, whereas a model omitting the random slopes for time of day estimated 3 random effects and 6 parameters, for a difference of 9 parameters.

study, but if this association declined over the decades such that it was equivalently negative by older adulthood. The corresponding  $B$  values for age moderation of the person-average, within-person/between-time, and within-person/within-time testosterone-cortisol associations were 0.13, 0.34, and 0.11, respectively. Based on a rule-of-thumb of  $B < .33$ , these estimates indicate strong evidence in support of the null hypothesis that the person-average and the within-person/within-time associations between cortisol and testosterone do not decline with age, and moderate evidence in support of the null hypothesis that the within-person/between-time association does not decline with age.

**3.4.4 Sensitivity Analysis in Very Healthy Participants**—As a sensitivity analysis, we re-estimated Model 5 with the very healthy sub-sample ( $n = 141$ ). Full parameter estimates from this model can be found in the Supplement (Table S3). Although standard errors were larger as a consequence of having a smaller sample, the results were essentially unchanged: Testosterone was positively associated with cortisol at every level of analysis, and this relationship was consistent across age and sex.

## 4. Discussion

### 4.1 Summary of Results

This study collected repeated salivary measures of cortisol and testosterone over the course of two days in an age-heterogeneous community sample spanning adolescence to older adulthood. We found consistent evidence for positive coupling between testosterone and cortisol at every level of analysis. First, within both sexes, people who were higher, on average, in testosterone were also higher, on average, in cortisol. Second, within-person declines in testosterone over the course of the day were, on average, associated with within-person declines in cortisol. Moreover, within-person, day-to-day variability in testosterone around its diurnal rhythm was associated with within-person variability in cortisol around its diurnal rhythm. Testosterone-cortisol associations were, on average, positive at all ages across the lifespan. None of the age  $\times$  testosterone interaction effects was significantly different from zero ( $p > 0.3$ ), and Bayes factors suggest that the data are more consistent with the null hypothesis that testosterone-cortisol associations do not change with age. Additionally, consistent with previous research (Dismukes et al., 2015b; Mehta et al., 2008; Mehta & Josephs, 2010), we found no significant sex differences in the magnitude of the testosterone-cortisol associations.

Given the popular conception of the HPA and HPG axes as mutually inhibitory, the current results may seem surprising. Certainly, the mutual inhibition model is based on compelling experimental evidence from the animal literature (Rivier & Rivest, 1991; Viau, 2002). As several previous authors have noted, however, the effects of an acute, massive, exogenous shock to one hormonal system (such as gonadectomy) on another hormonal system are not necessarily congruent with how naturally occurring variation in those systems are related (Marceau et al., 2015b). Indeed, our results are consistent with the majority of studies on cortisol-testosterone associations in humans, which have repeatedly found positive correlations between testosterone and cortisol, both between- and within-persons (Dismukes

et al., 2015b; Juster et al., 2016; Leining et al., 2010; Marceau et al., 2015b; Mehta et al., 2008; Mehta & Josephs, 2010; Popma et al., 2007; Ruttle et al., 2015; Turan et al., 2015).

#### 4.2 Potential Mechanisms and Functions for Positive Coupling

These results raise intriguing questions regarding the mechanisms underlying positive testosterone-cortisol coupling in humans. One possible mechanism is shared neural regulation (Dismukes et al., 2015b). The amygdala is a key limbic system structure that regulates the HPA axis, and stimulating the amygdala activates the HPA axis to increase cortisol output (Herman et al., 2005). Testosterone increases activity in the amygdala and decreases functional connectivity between the amygdala and the orbitofrontal cortex, which modulates amygdala activity to down-regulate affective responses to threat (Dolan, 2007; Lee et al., 2012). This effect of testosterone on the neural systems underlying threat responding has been observed not only for acute increases in testosterone via experimental administration (van Wingen et al., 2010), but also when examining individual differences in endogenous testosterone concentrations in adult men (Volman et al., 2011), as well as endogenous longitudinal increases in testosterone in young adolescents (Spielberg et al., 2015).

Additionally, HPA and HPG axis functioning may both be shaped by the same aspects of early rearing environments. For example, one animal model of the early rearing environment is maternal “licking and grooming” (LG) in rats. Compared to offspring of high LG mothers, the offspring of low LG mothers show increased corticosterone responses to acute stress (restraint) and lower sensitivity to negative-feedback regulation of the HPA axis (Liu et al., 1997). Interestingly, daughters of low LG mothers versus high LG mothers also show differences in HPG axis activity, with daughters of low LG mothers showing earlier puberty, elevated luteinizing hormone and progesterone levels, and greater sensitivity to the positive-feedback effects of estradiol on gonadotropin releasing hormone (Cameron et al., 2008). In humans, low socioeconomic status has been linked with both higher basal cortisol levels (Lupien et al., 2001) and with earlier pubertal timing (Deardorff et al., 2014). Indeed, positive HPA-HPG axis coupling may provide a potential hormonal mechanism for the acceleration of pubertal development among youth in stressful and unpredictable environments.

Finally, there are converging lines of research that suggest co-elevated levels of testosterone and cortisol serve a functional purpose. Specifically, studies using non-human primates (Dittami et al., 2008) and human participants (Gettler et al., 2011) have found that co-elevated levels of testosterone and cortisol occur during breeding seasons and in socially dominant males. These results have been interpreted to suggest that co-elevated rises in cortisol and testosterone would enhance mating preparedness (Lynch et al., 2002). The current paper shows that positive coupling between testosterone and cortisol is also evident in women and in older adults who are no longer expected to reproduce, suggesting that the functions of co-elevations in testosterone and cortisol are perhaps broader than facilitating mating in reproductively-able males.

### 4.3 Limitations

Results must be interpreted in light of four limitations. First, in order to capture naturally occurring hormonal changes over the course of the day, we used an in-home collection protocol that required participants to note when they collected their saliva samples and to send samples directly to the lab. Previous studies have found participants' self-reports of when they provided at-home saliva samples correspond highly ( $r_s = .75$ ) with electronically recorded times (Karlama et al., 2013), although one can never be perfectly confident that participants followed instructions or reported their compliance honestly. However, these participants were part of a larger, on-going study that involved multiple ambulatory assessments of mood and cognition (via cellphone) on several days over the course of three weeks (Riediger et al., 2014; Wrzus et al., 2016), and they, therefore, have an established record of being motivated participants who were accustomed to following instructions. Additionally, the endocrine data show expected diurnal patterns (cortisol awakening response, decline from morning to evening), which is evidence for their validity and reliability.

Second, this study did not involve adequate numbers of young adolescents for us to examine how coupling between testosterone and cortisol may change over the course of pubertal development. Although the sample ranged in age from 11 to 88, only 23 participants were under the age of 15, the average age at which boys and girls reach Tanner stage 5 (Hollenstein & Lougheed, 2013). That is, there was minimal variation in pubertal status for the vast majority of our participants, and participants were not young enough to capture the first hormonal changes of puberty (i.e., adrenarche). Future research that focuses on 6- to 16-year-olds would be informative regarding the contribution of pubertal development, rather than age *per se*, to testosterone-cortisol coupling. More generally, a larger sample would increase power to detect age-related differences in coupling, particularly non-linear age associations.

Third, the only output of the HPG axis that was measured in this study was testosterone; we do not have information on other gonadal hormones, including estradiol or progesterone. Coupling between estradiol and cortisol would be particularly interesting to examine in future research. Just as testosterone, which is typically considered a "male" hormone, is consequential for females, estradiol, which is typically considered a "female" hormone, is consequential for males (de Ronde et al., 2003). In fact, many of testosterone's effects in the brain and body occur via its aromatization to estradiol (de Ronde et al., 2003). Research with adolescents has found that high levels of estradiol are associated with higher levels of externalizing behavior problems only among youth who are also low in cortisol (Tackett et al., 2015), a finding that mirrors results from previous studies of testosterone  $\times$  cortisol interactions (e.g., Mehta & Josephs, 2010; Tackett et al., 2014). Some studies of cortisol reactivity to standardized acute stress have found evidence that estradiol dampens cortisol reactivity (Juster et al., 2016; Kajantie & Phillips, 2006; Kirschbaum et al., 1992, 1995), whereas another study found no association between baseline estradiol and cortisol reactivity (Stephens et al., 2016). Furthermore, estradiol was positively correlated with diurnal change in cortisol (specifically, the cortisol awakening response) among women

experiencing a menstrual cycle, but not among men (Juster et al., 2016). Overall, coupling between cortisol and other outputs of the HPG axis remain to be clarified.

Finally, although we controlled for major error sources in hormonal data (e.g., time, pregnancy, illnesses, shift work), the current analysis does not include further potentially relevant covariates, including menstrual cycle phase or irregularities for menstruating females, relationship status, physical exercise, sexual activity, sleep, acute or chronic stress, and diet and nutrition. Moreover, information about whether the participant was menstruating at all was missing for 7% of women. Any of these could contribute to the positive coupling observed between testosterone and cortisol, and considering these specific mechanisms as explanations for the current results seem to be important directions for future investigations.

#### 4.4 Conclusions

Although the average within-person correlation between cortisol and testosterone was positive, there were inter-individual differences in the strength of this correlation, and the distribution of within-person correlations indicated that testosterone and cortisol were negatively correlated in a minority of individuals. Identifying the biological and social sources, and the behavioral and/or experiential implications, of such individual differences in intra-individual coupling between the HPA and HPG axes is an interesting topic for future research. For example, early life stress might affect the pattern of testosterone and cortisol coupling over time (Ruttle et al., 2015), as well as the magnitude of this relationship (Dismukes et al., 2015b). Overall, these results add to a growing body of literature calling for a more nuanced understanding of the HPA-HPG relationship that considers species, social context, individual differences, and source of variation (inter-individual versus intra-individual, exogenous versus endogenous, acute versus chronic).

#### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

#### Acknowledgements

Data collection for this study was funded by the Max Planck Society for the Advancement of the Sciences and realized by the Max Planck Research Group “Affect Across the Lifespan” at the Max Planck Institute for Human Development. We thank TNS Infratest Sozialforschung, especially Andreas Stocker, for their help with the data collection. K.P.H. is a Faculty Research Associate of the Population Research Center at the University of Texas at Austin, which is supported by a grant, 5-R24-HD042849, from the Eunice Kennedy Shriver National Institute of Child Health and Human Development. Her work on this paper was supported by a grant, 1-R21-AA023322, from National Institute on Alcohol Abuse and Alcoholism, and by the Russell Sage Foundation Visiting Scholar program. This paper was further supported by the Focus Area DynAge of the Freie Universität Berlin and a Heisenberg stipend awarded to M.R. from the German Research Foundation.

The funding sources did not have any involvement in the study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

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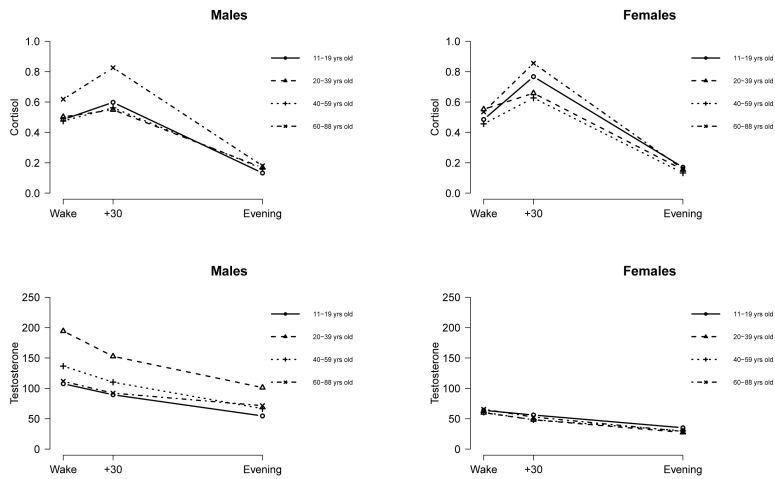
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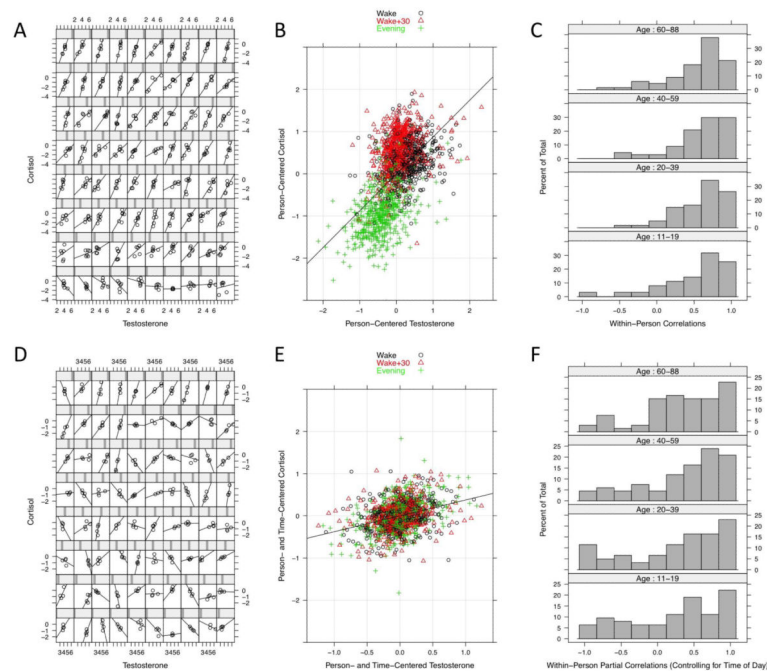
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**Highlights**

- Ambulatory cortisol (C) and testosterone (T) in saliva were measured on two days.
- C and T were, on average, positively correlated (coupled) within and between people.
- C-T coupling was, on average, positive in every age group from teenagers to older adults.
- Some people showed negative C-T correlations.
- The HPA and HPG axes are not always antagonistic.

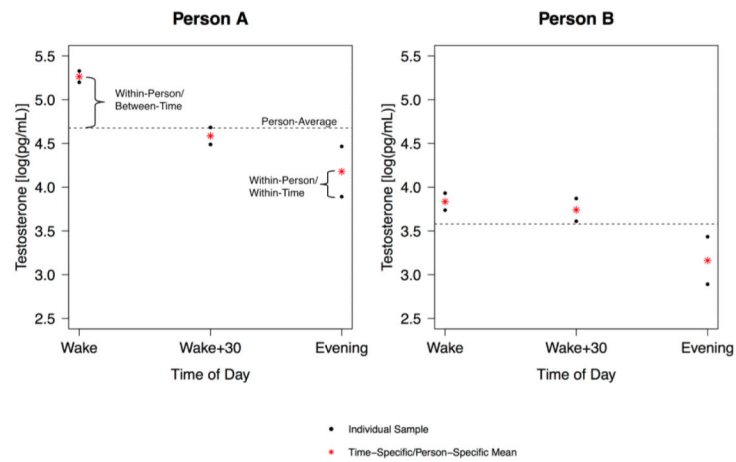


**Figure 1. Mean Cortisol and Testosterone Concentrations by Sex and Age Group**  
*Note.* Hormonal variables were log-transformed for all analyses, but means were exponentiated to show them in the original metric. Testosterone concentrations were measured in pg/mL; cortisol concentrations were measured in µg/dL. See Supplement for scatterplots of hormonal levels by age as a continuous variable and for Pearson correlations between hormonal levels and age. See web version of the article for color figure.



**Figure 2. Between-Person and Within-Person Correlations between Testosterone and Cortisol** (A) Scatterplot of cortisol and testosterone for  $N = 100$  randomly selected individuals, ordered by the strength of the within-person correlation. (B) Scatterplot of within-person variation in testosterone and within-person variation in cortisol for all individuals. Black circles = Wake, Red triangles = Wake +30, Green pluses = Evening. (C) Distribution of within-person correlations between testosterone and cortisol for all individuals who had at least 5 hormonal measures ( $N = 257$ ). (D) Scatterplot of cortisol and testosterone for  $N = 100$  individuals (same subset as panel A), omitting Evening samples. (E) Scatterplot of cortisol of within-person/within-time variation in testosterone and within-person/within-time variation in cortisol for all individuals. Black circles = Wake, Red triangles = Wake +30, Green pluses = Evening. (F) Distribution of within-person partial correlations between testosterone and cortisol, controlling for person-specific effect of time of day, for all individuals who had at least 5 hormonal measures ( $N = 257$ ).

**Note.** For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.



**Figure 3. Illustration of Testosterone Variation at Three Levels**

**Note.** Observed data from two individuals. Person A is an older adult male (age 66). Person B is an adolescent female (age 18). Individual samples are represented by black dots. The dotted line labeled “Person-Average” represents the person’s mean level of testosterone across 6 samples. The red stars represent the mean level of testosterone across the two days at that particular time of day. The bracket labeled “Within-Person/Between-Time” represents the difference between the Person-Average and the time-specific mean. The bracket labeled “Within-Person/Within-Time” represents the difference between the time-specific mean and the individual sample. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)



Table 1

## Summary Statistics

	Males						Females						
	<i>M</i>	<i>SD</i>	$e^M$	$e^{M-SD}$	$e^{M+SD}$		<i>M</i>	<i>SD</i>	$e^M$	$e^{M-SD}$	$e^{M+SD}$		
<b>Cortisol</b>													
<i>Day 1</i>													
Wake	-0.67	0.64	0.51	0.27	0.97		-0.70	0.54	0.50	0.29	0.85		
Wake + 30	-0.42	0.67	0.66	0.34	1.29		-0.33	0.53	0.72	0.42	1.22		
Evening	-1.87	0.68	0.15	0.08	0.30		-1.90	0.71	0.15	0.07	0.30		
<i>Day 2</i>													
Wake	-0.64	0.53	0.53	0.31	0.90		-0.67	0.54	0.51	0.30	0.87		
Wake + 30	-0.52	0.67	0.60	0.30	1.17		-0.34	0.52	0.72	0.43	1.20		
Evening	-1.82	0.75	0.16	0.08	0.34		-1.87	0.73	0.15	0.07	0.32		
<b>Testosterone</b>													
<i>Day 1</i>													
Wake	4.84	0.72	126.26	61.71	258.30		4.10	0.64	60.07	31.70	113.83		
Wake + 30	4.70	0.74	109.49	52.32	229.15		3.96	0.70	52.51	26.12	105.53		
Evening	4.26	0.66	70.95	36.77	136.89		3.37	0.74	29.07	13.80	61.22		
<i>Day 2</i>													
Wake	4.93	0.66	137.80	71.05	267.25		4.17	0.65	64.61	33.62	124.16		
Wake + 30	4.66	0.67	105.87	53.98	207.64		3.91	0.67	49.72	25.40	97.33		
Evening	4.27	0.72	71.26	34.55	146.99		3.44	0.68	31.28	15.81	61.90		

*Note.* *M*s and *SD*s are calculated from log-transformed values. Exponentiated values for *M* and  $M \pm 1SD$  are provided to describe the distribution of hormonal concentrations in their original metric ( $\mu\text{g/dL}$  for cortisol and  $\text{pg/mL}$  for testosterone).

Table 2

## Between-Person Correlations

	Cortisol												Testosterone			
	Day 1				Day 2				Day 1				Day 2			
	C11	C12	C13	C21	C22	C23	T11	T12	T13	T21	T22	T23	T21	T22	T23	
<b>Cortisol</b>																
<i>Day 1</i> Wake	1	<b>.338</b>	.052	<b>.349</b>	<b>.162</b>	.046	.109	.070	-.059	-.041	-.075	-.037				
Wake + 30	<b>.532</b>	1	.096	<b>.202</b>	<b>.357</b>	.006	.061	<b>.213</b>	.116	-.002	.075	.065				
Evening	.093	-.086	1	.016	<b>.166</b>	<b>.393</b>	.038	.136	<b>.504</b>	<b>.155</b>	<b>.163</b>	<b>.321</b>				
<i>Day 2</i> Wake	<b>.303</b>	<b>.242</b>	.041	1	<b>.400</b>	<b>.188</b>	.003	.066	.004	<b>.204</b>	.093	.051				
Wake + 30	<b>.302</b>	<b>.432</b>	.041	<b>.411</b>	1	.059	-.037	.121	.091	<b>.261</b>	.147	.046				
Evening	.172	.081	<b>.593</b>	<b>.234</b>	.092	1	.044	.034	.132	.042	.023	<b>.406</b>				
<b>Testosterone</b>																
C11	C11	C12	C13	C21	C22	C23	T11	T12	T13	T21	T22	T23				
<i>Day 1</i> Wake	<b>.193</b>	<b>.183</b>	.065	<b>.267</b>	<b>.214</b>	<b>.187</b>	1	<b>.506</b>	<b>.294</b>	<b>.544</b>	<b>.341</b>	<b>.365</b>				
Wake + 30	.146	<b>.267</b>	.086	<b>.230</b>	<b>.220</b>	<b>.206</b>	<b>.638</b>	1	<b>.408</b>	<b>.504</b>	<b>.683</b>	<b>.434</b>				
Evening	.087	-.056	<b>.497</b>	.161	.034	<b>.355</b>	<b>.430</b>	<b>.394</b>	1	<b>.424</b>	<b>.459</b>	<b>.595</b>				
<i>Day 2</i> Wake	.100	.043	.158	<b>.385</b>	<b>.175</b>	<b>.177</b>	<b>.682</b>	<b>.605</b>	<b>.480</b>	1	<b>.615</b>	<b>.467</b>				
Wake + 30	.116	.087	<b>.202</b>	<b>.204</b>	<b>.264</b>	<b>.250</b>	<b>.586</b>	<b>.625</b>	<b>.512</b>	<b>.635</b>	1	<b>.501</b>				
Evening	<b>.240</b>	.099	<b>.344</b>	.162	.116	<b>.455</b>	<b>.471</b>	<b>.592</b>	<b>.605</b>	<b>.597</b>	<b>.628</b>	1				

*Note.* Correlations for females above the diagonal; males below the diagonal. Correlations significantly different from zero at  $p < .05$  are in bold font.

**Table 3**

Parameter Estimates from HLM Models

DV = Cortisol (uncentered)	Model 1		Model 2		Model 3		Model 4		Model 5	
	Estimate	SE	Estimate	SE	Estimate	SE	Estimate	SE	Estimate	SE
<i>Fixed Effects</i>										
Intercept	-0.692	0.037	-0.664	0.042	-0.834	0.048	-0.818	0.050	-0.859	0.057
Time of Day [reference = Wake]										
Wake + 30	0.290	0.030	0.224	0.045	0.275	0.046	0.261	0.050	0.266	0.052
Evening	-1.183	0.043	-1.185	0.065	-0.915	0.068	-0.897	0.073	-0.879	0.084
Wake Time (hr, grand-mean centered)	-0.025	0.013	-0.023	0.018	-0.003	0.017	-0.003	0.017	-0.003	0.017
Wake Time × Time of Day [reference = Wake]			-0.023	0.018						
Wake + 30			-0.084	0.022	-0.093	0.021	-0.094	0.021	-0.093	0.022
Evening			0.082	0.027	0.045	0.025	0.045	0.025	0.047	0.026
Female Sex [reference = Male]	0.020	0.044	-0.026	0.056	0.137	0.061	0.143	0.061	0.166	0.071
Female Sex × Time of Day [reference = Wake]										
Female: Wake + 30			0.133	0.060	0.138	0.058	0.132	0.058	0.123	0.062
Female: Evening			-0.004	0.086	0.042	0.079	0.038	0.078	0.008	0.106
Age [(age-40)/10]	0.020	0.011	0.042	0.015	0.042	0.014	0.039	0.014	0.041	0.015
Female × Age			-0.043	0.021	-0.041	0.020	-0.038	0.020	-0.039	0.023
Person-Average Testosterone			0.222	0.038	0.222	0.038	0.221	0.038	0.294	0.057
Person-Average Testosterone × Female									-0.138	0.078
Person-Average Testosterone × Age									-0.001	0.019
Within-Person Testosterone			0.262	0.054						
Within-Person/Between-Time Test.									0.196	0.076
Within-Person/Within-Time Test.									0.311	0.072
Within-Person Testosterone × Time of Day [reference = Wake]										
Wake + 30			0.021	0.076						
Within-Person/Between-Time Test.									0.057	0.119
Within-Person/Within-Time Test.									-0.013	0.099
Within-Person Testosterone × Time of Day [reference = Wake]										
Wake + 30									0.065	0.119
Within-Person/Between-Time Test.									-0.005	0.099
Within-Person/Within-Time Test.										

DV = Cortisol (uncentered)	Model 1		Model 2		Model 3		Model 4		Model 5	
	Estimate	SE	Estimate	SE	Estimate	SE	Estimate	SE	Estimate	SE
Evening			<b>0.299</b>	<b>0.073</b>						
Within-Person/Between-Time Test.										
Within-Person/Within-Time Test.										
Within-Person/Between-Time Testosterone × Female										
Within-Person/Between-Time Testosterone × Age										
Within-Person/Within-Time Testosterone × Female										
Within-Person/Within-Time Testosterone × Age										
<b>Random Effects</b>										
<b>Level 1: Within-Day, Within-Person</b>										
Residual Variance	0.226		0.220		0.178		0.173		0.173	
<b>Level 3: Between-Persons</b>										
Variance in Cortisol Level (Intercept)	0.099		0.102		0.143		0.142		0.142	
Variance in Cortisol Awakening Response (Slope of Wake to W+30)	0.018		0.018		0.041		0.054		0.053	
Variance in Cortisol Diurnal Decline (Slope of Wake to Evening)	0.283		0.282		0.365		0.314		0.311	
Variance in Testosterone Coupling (Slope of Within-Person Testosterone)					0.087					
Variance in Testosterone Coupling (Slope of Within-Person/Between-Time Testosterone)							0.034		0.037	
Variance in Testosterone Coupling (Slope of Within-Person/Within-Time Testosterone)							0.134		0.135	
<b>Fit Statistics</b>										
-2LL	2838.56		2792.66		2571.05		2563.75		2558.07	
AIC	2864.56		2828.66		2623.05		2631.75		2638.07	
parameters	13		18		26		34		40	

**Note.** Please see Section 3.4.1 for additional information on model specifications, and please see Supplement for model equations. All parameters in bold are significantly different from zero at  $p < .05$ .