Seasonal and Circadian Variation in Salivary Testosterone in Rural Bolivian Men

Testosterone (T) plays a key role in the increase and maintenance of muscle mass and bone density in adult men. Life history theory predicts that environmental stress may prompt a reallocation of such investments to those functions critical to survival. We tested this hypothesis in two studies of rural Bolivian adult men by comparing free T levels and circadian rhythms during late winter, which is especially severe, to those in less arduous seasons. For each pair of salivary \( T_{AM}/T_{PM} \) samples (collected in a \(~12\)-h period), circadian rhythm was considered classic (CCLASSIC) if \( T_{AM} > 110\% T_{PM} \), reverse (CREVERSE) if \( T_{PM} > 110\% T_{AM} \), and flat (CFLAT) otherwise. We tested the hypotheses that mean \( T_{AM} > \) mean \( T_{PM} \) and that mean \( T_{LW} < \) mean \( T_{OTHER} \) (LW = late winter, OTHER = other seasons). In Study A, of 115 \( T_{PM}-T_{AM} \) pairs, 51% = CCLASSIC, 39% = CREVERSE, 10% = CFLAT; in Study B, of 184 \( T_{AM}-T_{PM} \) pairs, 55% = CCLASSIC, 33% = CREVERSE, 12% = CFLAT. Based on fitting linear mixed models, in both studies \( T_{OTHER-AM} > T_{OTHER-PM} \) (A: \( p = 0.035, B: p = 0.0005 \)) and \( T_{OTHER-AM} > T_{LW-AM} \) (A: \( p = 0.054, B: p = 0.007 \)); \( T_{PM} \) did not vary seasonally, and T diurnality was not significant during late winter. T diurnality varied substantially between days within an individual, between individuals and between seasons, but neither T levels nor diurnality varied with age. These patterns may reflect the seasonally varying but unscheduled, lifelong, strenuous physical labor that typifies many non-industrialized economies. These results also suggest that single morning samples may substantially underestimate peak circulating T for an individual and, most importantly, that exogenous signals may moderate diurnality and the trajectory of age-related change in the male gonadal axis. Am. J. Hum. Biol. 00:0–0, 2009.
have concluded that diurnal variation in T is blunted with age, possibly as a consequence of an age-related decline in circulating T (Diver et al., 2003 and references therein).

Most studies find that T levels decline by 30–50% over the course of adulthood (Liu et al., 2005), and the reasons for this change have become a principal focus of research on male aging (e.g., the ensemble model of the male gonadal axis, and the associated biomathematical constructs, developed by Veldhuis and coworkers (Keenan and Veldhuis, 2001; Keenan et al., 2006; Liu et al., 2005). This ensemble model accords well with empirical data and suggests that the precise physiological mechanisms underlying such declines are likely to be multifactorial. However, current understanding of circadian or age-related variation in T is predicated almost entirely on data from industrialized populations. Little is known of circadian variation in T among community-dwelling men in non-industrialized settings, and the scant data on age-related variation suggest that there is significant inter-populational variation for unknown reasons (Ellison et al., 2002).

Both evolutionary and medical sciences have much to gain from comprehensive studies of men in non-industrialized populations. Especially in rural economies unencumbered by fixed schedules, the frequently strenuous activities of adult men may vary daily and/or seasonally yet change little throughout a lifetime (Kaplan, 1994). The influence on T levels of less structured albeit demanding quotidian routines, relatively decoupled from age per se, bears directly on whether classic diurnality and/or an age-related decline in T are inherent features of the male gonadal axis or are entrained by extrinsic factors. This question bears, in turn, on the generalizability of current models of the male gonadal axis.

Based on data collected from Bolivian men living in rural agropastoral communities south of La Paz, we tested the hypotheses that T will be lower during late winter, an especially severe season in this environment, and that T diurnality and variation with age are comparable in this population to that typically observed in industrialized populations.

MATERIALS AND METHODS

Study participants

Protocols in Studies A and B were approved by the Institutional Review Boards at Case Western Reserve University and the University of California (Riverside), respectively. All participants, recruited from dispersed agropastoral communities in Provincias Murillo and Aroma, south of La Paz, Bolivia, were nominally healthy and gave informed consent. In Study A, subjects in a larger investigation of genetic variation in respiratory function (Beall et al., 1999) were invited to participate in this study of T; 115 men, aged 21–59 years (mean = 38.9, standard deviation = 10.8), volunteered and self-collected samples on a single night and following morning at some time during late May through late August (approximately late autumn through late winter), 1994.

Study B was conducted within the framework of Project REPA (Reproduction and Ecology in Provincia Aroma) (Vitzthum et al., 2004); 65 men, aged 21–59 years (mean = 33.2, standard deviation = 7.43), volunteered and self-collected 6 samples during a single week at some time from late May through late October (approximately late autumn through spring), 1996.

Saliva sample collection

In Study A, each man self-collected a 5-ml saliva sample shortly before retiring (about 8:00–9:00 pm) and then upon awakening (about 6:00–7:00 am). In Study B, on each of 3 days (Monday, Wednesday, Friday), an assistant visited a participant’s home at about 8:00 am and again at 8:00 pm, and waited while the man self-collected a 5-ml saliva sample according to an established protocol that included rinsing the mouth with cool clean water and waiting at least 20 min before collecting saliva, procedures that mitigated contamination from food, drink, blood and coca-chewing (Vitzthum et al., 1993). In both studies, samples were maintained at ambient temperature until shipped to Emory University where they were frozen at −26°C until assayed.

Hormone assays

Saliva samples were assayed for T using an 125I radioimmunoassay method detailed and comparatively assessed in previous reports (Beall et al., 1992; Dabbs et al., 1995). Assay precision for the low (mean 0.44 ng/dl (0.015 nmol/l)) and high (5.11 ng/dl (0.177 nmol/l)) controls was intra-assay 12.3% and 9.1% and inter-assay 11.0% and 6.6%, respectively.

Statistical analyses

Statistical analyses were performed with SPSS version 16.0 (Chicago, IL). For all analyses, T observations were log2 transformed and men's ages were centered at 30 years (i.e., centered age = subject's age – 30). Centering on a value near the mean or median of an age (or other term’s distribution) is widely recommended for both simple and more complex regressions because it reduces the correlations between the different powers (linear, quadratic, cubic) of the term in fitted models (for additional technical advantages of centering, see West et al., 2008). Correlations of TAM–TAM and TFM observations in Study B were determined.

To evaluate patterns in circadian rhythms, each TAM–TFM pair was plotted (see Fig. 1). The circadian rhythm of each pair was defined in this study as classic (CCLASSIC) if TAM > 110%TFM, reverse (CREVERSE) if TFM > 110%TAM, and flat (CFLAT) otherwise. Any cut-off is necessarily arbitrary; the chosen threshold was purposefully conservative to favor the null hypothesis. T diurnality for each man was defined in Study A as (log2TFM – log2TAM) and in Study B (which had three days of observations for each man) as [(log2TFM-MON + log2TFM-WED + log2TFM-FRI)/3] – [(log2TAM-MON + log2TAM-WED + log2TAM-FRI)/3)]. Late winter—when temperatures are severely cold, days are short, food scarcity is common, and physical activity is typically less arduous than the preceding harvest and upcoming planting seasons—was defined as July 15 through August 31.

Linear mixed models (LMM) were fitted to evaluate circadian, seasonal, between day, and age-associated variation in T variables. The protocols for fitting and evaluating models followed West et al. (2007); individuals were treated as random effects in all final models. Age
variation was parameterized by various combinations of linear, quadratic, and cubic powers of centered age. Reflecting the \textit{a priori} expectation that $T_{AM} > T_{PM}$, $T_{OTHER} > T_{LW}$, and $T$ decreases with increasing age, significance tests for these hypotheses were one-sided. Tests for significant differences in $T$ variables among days of week were two-sided.

\section*{RESULTS}

Descriptive statistics for $T$ variables are in Table 1. Plots of each $T_{AM} - T_{PM}$ pair on a $\log_2$ scale are in Figure 1. Histograms of $T$ diurnality are in Figure 2.

In Study A, the Pearson correlation between $\log_2 T_{PM}$ and next morning $\log_2 T_{AM}$ was $0.301$ ($P < 0.001$).

\textbf{Fig. 1.} $T_{PM}$ plotted against $T_{AM}$ in 3 studies. A: Rural Bolivian men from Provincia Aroma (Study B); B: Rural Bolivian men from Provincia Murillo (Study A); C: U.S. men from Spratt et al. (1988). Both axes are $\log_2$ scale (nmol/l = 0.0347 ng/dl). Below the solid diagonal line, $T_{PM} < T_{AM}$ (classic circadian rhythm); above the line, $T_{PM} > T_{AM}$ (reverse circadian rhythm). The first pair of dotted lines on either side of the solid diagonal delineate $T_{AM} - T_{PM}$ pairs defined as “flat” ($T_{AM} - T_{PM} \pm 10\%$). The second pair of dotted lines delineate $T_{AM} = 2T_{PM}$ (below the solid diagonal) and $T_{PM} = 2T_{AM}$ (above the solid diagonal). $\dagger$, younger men, $\bullet$, older men (defined as above and below the median of each sample in Studies A and B; all men in C are $< 37$ years). In A, 3 points outside the plotted area are not included to conserve space and increase clarity.
The seasonal difference in diurnality arises from a larger proportion of the sample having T_{FM} > T_{AM} in late winter than during other seasons (A: 2.806 vs. 3.112 (P = 0.0005), in Study B: 2.931 vs. 3.338 (P = 0.007)).

There was striking heterogeneity in circadian rhythms, both between men and between days within an individual (Fig. 1, plots A and B: Fig. 2). In Study A, of 115 T_{AM}-T_{PM} pairs, 51% = C_{CLASSIC}, 39% = C_{REVERSE}, and 10% = C_{FLAT}. In Study B, of 184 T_{AM}-T_{PM} pairs, 55% = C_{CLASSIC}, 33% = C_{REVERSE}, and 12% = C_{FLAT}. In both studies, similar proportions of each diurnal pattern were observed in younger and older men and, in Study B, on each of 3 weekdays. Variation in T variables was not associated with age in either study.

In both studies, mean diurnality in late winter was substantially less than in other seasons (A: -0.008 vs. -0.252; B: -0.052 vs. -0.420). These differences in mean circadian rhythms reflected a shift in the distribution of diurnality (see Fig. 2), such that during late winter a smaller proportion of men exhibited classic circadian variation (i.e., either a rise in T during the night or a drop during the day). Even so, there was still marked heterogeneity in circadian patterns throughout the time spans of both studies.

Morning T levels (Table 1) were significantly lower during late winter than during other seasons (log2T_{PM-LW} < log2T_{PM-OTHER} in Study A: 2.814 vs. 3.112 (P = 0.0054); in Study B: 2.931 vs. 3.338 (P = 0.007)) but evening levels did not vary seasonally (log2T_{PM-LW} vs. log2T_{PM-OTHER} in Study A: 2.806 vs. 2.860 (P = 0.38); in Study B: 2.881 vs. 2.925 (P = 0.40). In other words, in both studies: T_{AM}\_OTHER > T_{PM-OTHER} \approx T_{AM-LW} \approx T_{PM-LW}. These patterns underlie the absence of circadian variation in late winter (log2T_{AM-LW} vs. log2T_{PM-LW} in Study A: 2.814 vs. 2.806 (P = 0.48); in Study B: 2.931 vs. 2.881 (P = 0.34), and significant diurnality in other seasons (log2T_{OTHER-AM} > log2T_{OTHER-PM} in Study A: 3.112 vs. 2.860 (P = 0.035); in Study B: 3.338 vs. 2.925 (P = 0.0005)).

### DISCUSSION

Together these two studies provide clear evidence of seasonality in both morning T level and T diurnality in a non-industrialized population. In these rural Bolivian
men, the mean levels of T across seasons and at morning and evening within each season suggest that during late winter, T fails to rise during the night to the level observed during other seasons, and that over the course of a late winter day mean T levels are about the same, in contrast to the decline in mean T observed during other seasons. The demonstration of parallel seasonality in two independent studies, one in 1994 and another in 1996, conducted in two separate sets of communities using different protocols to collect comparable data substantially mitigates any possibility that the observed seasonality is anomalous.

The reduction in mean TAM during late winter in these altiplano communities (altitude ~ 4,000 m) is most likely multifactorial. During this season, the days are short, mean low temperatures are typically several degrees below freezing, and wind/dust storms are not uncommon. People may sleep longer, but perhaps less comfortably as fuels are too precious for most to use for heating homes, apart from the heat generated by cooking. Food stores from the fall harvest are declining, and many families are unable to purchase more foodstuffs. Documented responses to seasonal nutritional stress in the Andes include reduced activity levels and fewer meals to reduce post-prandial energy loss (Leonard and Thomas, 1989). All these environmental and behavioral factors can be expected to modify T levels. Experimentally controlled short-term fasting in both primates and humans (reviewed in Cameron, 1996) and daylight fasting during Ramadan (Bogdan et al., 2001; Mesbahzadeh et al., 2005) are associated with reductions in T and shifts in T diurnality. Both sleep and activity patterns are also known to influence T levels and circadian rhythms (Axelsson et al., 2005; Kern et al., 1995; Luboshitzky et al., 2001). Additional research is needed to test these specific hypotheses in rural Bolivian men.

The pattern of seasonality in these Bolivians is distinct from that observed in Lese men (Bentley et al., 1993), the only other report of T seasonality in a non-industrialized population. In the Lese, TAM levels were comparable in both seasons, but TPM levels were lower in August than in June. Because energetic conditions had improved and the men had gained an average of 2 kg during the observation period, the reason for the seasonal decline in evening T was unclear.

In the present studies of Bolivians, all indicators of T levels and T diurnality were comparable for younger and older members. Most studies of industrialized populations (Axelsson et al., 2005; Diver et al., 2003; Keenan et al., 2006; Liu et al., 2005) have observed a 1–2% T decline/ year at older ages (40–90 years). Findings from the few reports for non-industrialized populations are mixed (Beall et al., 1992; Bentley et al., 1993; Bribiescas, 1996; Campbell et al., 2003; Ellison et al., 1989a, 2002), some observing an age-related T decline, others not, even within the same population. This inconsistency may be the result of some studies having relatively fewer men at much older ages, but 51 of the 115 participants in Study A were >40 years of age. However, all men were younger than 60 years of age in both of the present studies. Although it may be that T declines in rural Bolivian men at ages >60 years, no significant decline at younger ages was detected in these studies.

Compared to reports of up to two-fold differences between TPM and TAM levels (Axelsson et al., 2005), diurnal variation appears blunted in rural Bolivian men, especially in late winter. However, the sample means mask considerable heterogeneity in circadian rhythms. In both studies, about half of the TAM–TPM pairs did not conform to the expected circadian rhythm (see Fig. 1). Because the criterion for testing the null hypothesis was conservative (i.e., a TAM–TPM pair was considered classic if TAM > 110%PM), this estimate of the proportion of non-conforming pairs can be considered a minimum. Absence of classic diurnality is not equivalent, however, to an absence of circadian variation per se. In both studies, only 10–12% of the pairs had TAM values within TPM ± 10%; that is, only a small proportion of TAM–TPM pairs were consistent with the blunting of diurnality suggested by the TAM and TPM sample means. In the remaining pairs (39% in Study A, 33% in Study B), circadian variation was as pronounced as that observed among the classic diurnal pairs, but the timing of the observed peak and nadir was reversed. In Study B, the modest correlations among the post-waking TAM levels across 3 days and the absence of significant correlations among the TPM values indicate substantial intra-individual variation in both daily absolute T levels and T/circadian rhythms.

Notably, this marked within and between individual variation in diurnality is not fully explained by the severe conditions during late winter. In Figure 2, it is clear that the significant seasonal difference in mean circadian rhythm arises from a modest shift in the distribution of diurnality such that there are fewer TAM–TPM pairs exhibiting the classic pattern in late winter. Nonetheless, both classic and reverse diurnality are still commonly exhibited in all seasons.

These observations suggest that some factor(s) underlies an apparent modulation of T temporal patterning such that peak T does not always occur in these Bolivian men shortly after waking. As already noted, dietary, sleep and activity patterns are known to modify T levels and circadian rhythms. As sleeping styles typical in most industrialized countries are not, in fact, common to most of the world’s population (Worthman and Melby, 2002), circadian rhythms may also vary accordingly. Likewise, most men outside industrialized countries, and some within, engage daily in often strenuous physical labor that may stimulate T production during the day and hence blunt and/or shift the timing of diurnal variation. Such physiological responses have been documented among subsistence hunters; the typical drop in T from morning to evening observed in !Kung San men of Botswana was cut by half on days spent hunting for food (Worthman and Konner, 1987).

Few published studies include individual data that would permit an assessment of within-sample variation in circadian rhythms. A handful of agricultural and forager populations exhibit differences between mean TAM and mean TPM substantially less than twofold (Bentley et al., 1993; Bribiescas, 1996; Campbell et al., 2003; Ellison et al., 1989a). However, as only summary statistics were reported, it is unclear if the apparent blunting of diurnal variation in these samples holds for all study participants or if there is significant heterogeneity in circadian rhythms within each sample. Such variation is evident in the range (0.2–5.9) among individuals for the ratio of TAM/ TPM included in one report (Beall et al., 1992), and Ellison et al. (1989a) noted that 5 of 29 study participants had higher mean TPM than mean TAM.
The limited published data available from U.S. samples suggest that circadian rhythms may, in fact, be more heterogeneous in industrialized populations than is widely appreciated. Figure 1C plots serum TAM vs. TFM reported for 20 U.S. males aged 18–37 years (Spratt et al., 1988). Although participants were observed in the laboratory for 24 h and slept between 2300 and 0700 hours, they nonetheless exhibited substantial variation in T diurnality. Using the criteria defined here, 45% = CCLASSIC, 35% = CREVERSE, and 20% = CFLAT. The authors, noting two other studies in which some individuals did not exhibit classic T diurnality, also concluded that T levels need not necessarily reach a nadir during the evening in all men (Spratt et al., 1988 and references therein).

Both the reasons for heterogeneity in T diurnality and the consequences of such variation for aging, health and reproductive functioning merit further study. Likewise, as has been previously emphasized (Ellison et al., 2002), the apparent absence in some populations of an age-related T decline deserves greater attention. In particular, larger studies with frequent sampling and careful measurement of hypothesized covariates, including the timing and duration of behaviors relative to sampling, are called for. Until more is learned, we should not assume that any potential determinant has a constant impact on T levels over a 24-h period. For example, the effect of a missed evening meal may differ from that of a meal missed at midday.

The present studies of Bolivian men collected only 2 samples per individual for each 24-h observation period. Therefore, the greater of these observations may not have represented that man’s peak on that day nor would the lower value necessarily have been the nadir. Clearly a single sample, even one taken shortly after awakening, cannot be assumed to represent a given individual’s peak T and cannot be relied upon, alone, to characterize variation in T levels within or between populations. A significant clinical implication is that single morning samples may substantially underestimate peak circulating T for a patient.

Research predicated on an ensemble model of the male gonadal axis (Keenan and Veldhuis, 2001; Keenan et al., 2006; Liu et al., 2005) has yielded valuable insight into the mechanisms behind disrupted regulation during aging, but circadian linkages have yet to be fully investigated (Keenan and Veldhuis, 2001). This new evidence of varying T diurnality within and between individuals suggests the need for a more thorough examination of the relationship of circadian systems to regulation of testicular activity.

Likewise, life history theory has generated promising avenues of research into the proximate and evolutionary determinants of variation in T levels, and the consequences of these for male investment in somatic maintenance and reproductive effort (Bribiescas, 2001; Campbell et al., 2003; Ellison and Panter-Brick, 1996; Gray, 2003; Muehlenbein and Bribiescas, 2005; Muller et al., 2009; Wingfield et al., 1990). At the same time, findings from studies testing specific hypotheses, particularly in human males, are inconsistent. Accounting for seasonal and individual variation in circadian rhythms could help to resolve these apparent contradictions.

Most importantly, although formal evolutionary and clinical models provide powerful tools for analyzing complex systems, all physiological mechanisms are, in fact, integrated components of an organism functioning within a signal-generating milieu. The evidence of seasonal variation and the absence of an age-related T decline in these rural Bolivian males implies that the dynamic responsiveness of the male gonadal axis to behavioral, psychosocial, and/or environmental contexts may, at least to some extent, moderate the trajectory of age-related change in the axis. Should further research confirm this hypothesis, the implications for clinical practice and future studies of male evolutionary endocrinology would be substantial.

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LITERATURE CITED


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