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COMMENT

Variation in Levels of Serum Inhibin B, Testosterone, Estradiol, Luteinizing Hormone, Follicle-Stimulating Hormone, and Sex Hormone-Binding Globulin in Monthly Samples from Healthy Men during a 17-Month Period: Possible Effects of Seasons

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To obtain information on the scale of the intraindividual variation in testicular hormone, blood samples for inhibin B determination were collected monthly in 27 healthy male volunteers during a 17-month period. In addition, the traditional reproductive hormones FSH, LH, testosterone, estradiol, and SHBG were measured. The intraindividual variation in inhibin B over the study period was, on the average, 10%, corresponding to the assay variation of the inhibin B assay, indicating that most of the observed day to day variation in inhibin B levels in men could be explained by assay variation.

A seasonal variation was observed in LH and testosterone levels, but not in the levels of the other hormones. The seasonal variation in testosterone levels could be explained by the variation in LH levels. The seasonal variation in LH levels seemed to be related to the mean air temperature during the month before blood sampling, but not to the length of daylight or the hours of sunshine.

In conclusion, our data showed that day to day levels of inhibin B are relatively constant in men and do not seem to be influenced by seasonal factors. In contrast, we found a seasonal variation in LH and testosterone levels in men. The peak levels of both LH and testosterone were observed during June-July, with minimum levels present during winter-early spring. Air temperature, rather than light exposure, seems to be a possible climatic variable explaining the seasonal variation in LH levels. (*J Clin Endocrinol Metab* 88: 932–937, 2003)

AN INDIVIDUAL'S SERUM levels of reproductive hormones fluctuate, and an estimation of the magnitude of this fluctuation, expressed as the intraindividual variation, may be relevant in both research and clinical settings. The diurnal and individual day to day variations in testosterone, estradiol, FSH, LH, and SHBG levels are well documented (1–7). We recently published data on the diurnal variation in the testicular peptide hormone inhibin B in normal men (8). However, information on the individual day to day variation in serum inhibin B levels in men is still lacking. Accordingly, this study was initiated to obtain an estimate of the intraindividual variation in inhibin B. During recent years a renewed interest in the role of inhibin in the male pituitary-gonadal hormone axis has emerged due to the development of new improved immunoassays that specifically measure inhibin B (9), which is the physiological relevant inhibin form in men (10, 11). Inhibin B is involved in the negative feedback regulation of FSH secretion and has been shown to be a serum marker of spermatogenesis in adult men (12–14).

In this study we measured the levels of inhibin B, LH, FSH, testosterone, estradiol, and SHBG in monthly serum samples obtained from 27 healthy men over a 17-month period. The design of this study allowed not only an estimate of the intraindividual variation in all six reproductive hormones, but also investigation of some possible components of this variation, including a circannual rhythm. A previous study of monthly inhibin levels in 16 healthy men using a less specific inhibin assay that comeasured biologically nonactive inhibin forms present in serum has shown significantly increased inhibin levels during the month of July (15), but no information on a possible circannual rhythm in the levels of the physiological relevant inhibin B form in men is available. Although longitudinal studies on the intraindividual variation in other reproductive hormones have been performed (3, 5, 7, 15–20), these studies are generally based on a small number of subjects, and their results are inconsistent with regard to circannual rhythms. Therefore, in this study we also tested for seasonal variation in serum levels of testosterone, estradiol, LH, FSH, and SHBG.

Subjects and Methods

Subjects

Men were recruited by advertisement at the medical school at University of Copenhagen and among men who had previously participated in semen quality control studies within the department. Participants were required to be between 20 and 40 yr old and have no history of urogenital operations (*e.g.* for cryptorchidism). Twenty-seven men fulfilling these criteria were enrolled and entered the study during a 2-month period (February 23 to April 29) in 1998. The median age of the

Abbreviations: CV, Coefficient of variation.

men was 24.4 yr (range, 20.0–37.2), and the mean body mass index [weight (kilograms)/height (meters)^2] was 23.0 (range, 20.1–30.4) at entry into the study. All men had hormone levels within the normal range. Five of the men had previously proven fertile, and none had been treated for infertility. During the study period participants had a blood sample drawn every month. The median number of blood samples delivered per individual was 16 (range, 11–17), and the median duration of participation was 457 d (range, 305–484). The study was approved by the local ethical committee.

Hormone assays

Blood samples for hormone measurements were drawn between 0815 and 1200 h, with 88.3% of the samples drawn before 1000 h, and 98.6% of the samples drawn before 1100 h. After centrifugation, sera were frozen and stored at –20 C until the end of the study when all samples were analyzed. Samples from each subject were assayed together in the same assay run to avoid interassay variation.

Serum inhibin B was measured in a double antibody immunoenzymemetric assay (Oxford Bio-innovation, Oxford, UK) using a monoclonal antibody raised against the inhibin β_B-subunit in combination with a labeled antibody raised against the inhibin α-subunit as previously described (9). The detection limit was 20 pg/ml, and the intraassay coefficient of variation (CV) was 10% at a concentration 244 pg/ml and 13% or less at concentrations of 64 and 71 pg/ml (the intraassay CV was calculated based on a minimum of 20 measurements). Serum FSH, LH, and SHBG were measured by time-resolved immunofluorometric assays (DELFIA, Wallac, Inc., Turku, Finland). The detection limits were 0.06 IU/liter, 0.05 IU/liter, and 0.23 nmol/liter, respectively. The intraassay CV in the LH and FSH assays was less than 3%, and that in the SHBG assay was less than 5% at the relevant concentrations. Testosterone was measured by time-resolved fluoroimmunoassay (DELFIA, Wallac, Inc.), with a detection limit of 0.23 nmol/liter and an intraassay CV less than 5% at the relevant concentrations. Estradiol was measured by RIA (Pantex, Santa Monica, CA) with a detection limit of 18 pmol/liter, and an intraassay CV less than 8%. Detection limit was defined as the dose corresponding to the value that is 2 SD above or below the mean of the zero standard measurement in the immunometric (immunofluorometric assay and immunoenzymemetric assay) and competitive (fluoroimmunoassay and RIA) immunoassays, respectively. The free androgen index was calculated as (testosterone/SHBG) $\times$ 100.

Climatic data

Climatic data for the study period in the Copenhagen area where the participants were living were obtained from Denmark's Metrological Institute. We used mean daily air temperature (centigrade), daily air maximum temperature (centigrade), and hours of sunlight per day. The daily weather data reflect measurements obtained from midnight to midnight. Data for day length were also used to test the effects of photoperiodicity. Copenhagen has a temperate coastal climate and, located at latitude 55°,46′N, a difference in day length between midsummer and midwinter of more than 10 h.

Statistical analysis

Hormone values were natural logarithm transformed to obtain homoscedacity and an approximate normal distribution. A statistical analysis of the individual hormones was carried out using a general linear model. In the statistical model a random subject-specific component was introduced that allowed adjusting for the interindividual variation in hormone levels. The model also allowed estimation of the intraindividual variation (the residual error variation) in hormone levels as well as the effect of the hour of sampling and the calendar time (used either as a continuous or a categorical explanatory variable; see below) on the variation in hormone levels. The hour of sampling was entered into the model as a continuous variable coded from 0825–1300 h, where, for example, 9.5 corresponds to 0930 h and 10.75 corresponds to 1045 h. When appropriate, the intraindividual variations in hormone levels were estimated, adjusted for the effects of hour of sampling and calendar time.

The effect of calendar time on LH and testosterone levels was estimated both using a categorical variable allowing for a separate level in each of the 17 months and using linear splines with knots for each month, *i.e.* a piecewise linear function with different slopes for each month as described previously (21). Day length, temperature, and hours of sunlight were included as explanatory variables to account for the observed calendar time effect on hormone variation. Estimates of the effect of temperature were obtained by entering temperature in the model as either a continuous or a categorical explanatory variable (the quartiles of mean air temperature, $\leq$2.5, 2.6–7.5, 7.6–13.3, and $\geq$13.4 C).

The fit of the general linear model was evaluated by testing the residuals for normality and by inspection of the residual plots. The Statistical Package for the Social Sciences (SPSS, Inc., Chicago, IL) for Windows (edition 10.0.7) was used for all statistical analyses.

Results

Intraindividual variation and effect of hour of sampling

The intraindividual variations in inhibin B, testosterone, estradiol, LH, FSH, SHBG, and free androgen index after adjustment for diurnal and calendar time variations are shown in Table 1. Inhibin B and testosterone levels were significantly affected by the hour of sampling even within the restricted time period in which the blood samples were obtained (0815–1300 h). The diurnal decrease in testosterone levels during the morning and midday hours was also reflected in a decreasing free androgen index during the day. The other hormones showed no diurnal variation in this material. Representative examples of unadjusted inhibin B monthly profiles are presented in Fig. 1.

The interindividual variations in inhibin B, testosterone, estradiol, LH, FSH, SHBG, and free androgen index are also shown in Table 1. It should be noticed, however, that the interindividual variation is based on only 27 selected healthy volunteers and therefore does not necessary reflect the interindividual variation in the general population.

Seasonal variation

A possible seasonal variation in hormone levels was investigated by entering the month of sampling as a categorical variable into the statistical model. LH and testosterone levels both showed a significant effect of calendar time ($P = 0.007$ and $P = 0.009$, respectively). None of the other hormones or the free androgen index showed any significant variation according to calendar time. For a more detailed description of the effect of calendar time, linear splines were used, which allowed the precise date of sampling to enter the model (see *Materials and Methods*). The estimated changes in LH and testosterone levels according to calendar time of sampling

TABLE 1. Intra- and interindividual variation (CV%) of reproductive hormones[a]

	Inhibin B	Testosterone	Estradiol	LH	FSH	SHBG	FAI
Average intraindividual variation	10%	12%	15%	22%	11%	14%	17%
Interindividual variation	25%	17%	13%	27%	49%	30%	25%

FAI, Free androgen index [(testosterone/SHBG) $\times$ 100].
[a] Adjusted for the effect of hour of sampling and month of sampling when appropriate.

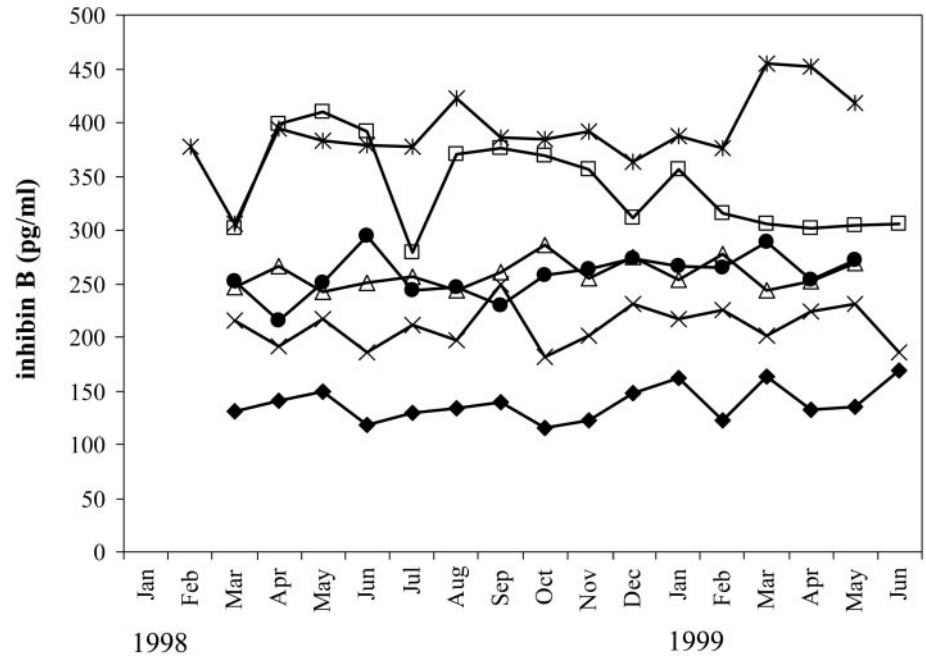

FIG. 1. Representative examples of individual unadjusted inhibin B monthly profiles of samples obtained in the morning (between 0815 and 1100 h).

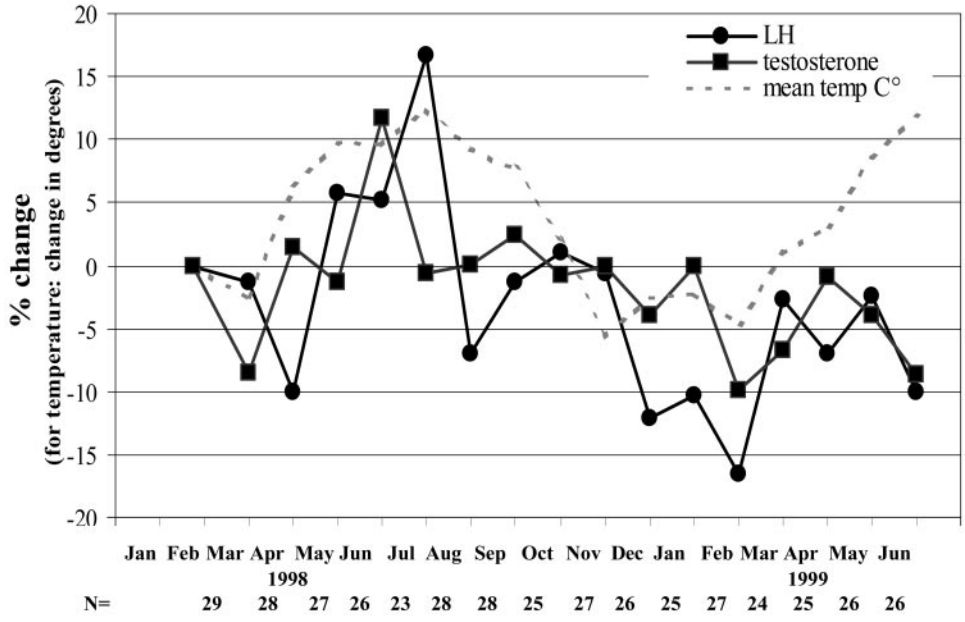

FIG. 2. Estimated mean variation in LH and testosterone levels according to calendar time. Changes are shown relative to the February 1998 level. For comparison, the seasonal changes in mean temperature in the Copenhagen area during the study period (data obtained from the Danish Metrological Institute and Institute of Astronomy, Copenhagen, Denmark) are also shown.

are shown in Fig. 2. LH and testosterone levels seemed to follow a similar general seasonal pattern of a nadir in spring, increasing levels during summer with peak levels during late summer, and subsequently gradually decreasing levels during autumn and winter. However, during the first calendar year LH and testosterone nadir levels seemed to be reached in April/May, whereas during the second calendar year the nadir was reached in February/March, *i.e.* 1–2 months earlier. LH levels exhibited the largest seasonal variation with, on the average, an approximately 30% difference between peak and nadir levels. Testosterone levels, on the average, varied approximately 20% between peak and nadir periods.

When inspecting the individual plots, this seasonal pattern was visually evident at the individual level in 12 of the subjects, whereas the remaining subjects showed no clear seasonal pattern. Seasonal variation only seemed to explain a small fraction of the observed intraindividual variation in LH levels even in subjects with an apparent circannual rhythm. This was also reflected by the fact that the average intraindividual variation in LH levels only decreased by 0.5% after adjusting for the effect of calendar time.

Climatic components of seasonal variation of LH and testosterone

Day length, hours of sunshine, and air temperature were entered into the statistical model as explanatory variables to assess whether they could account for the observed seasonal

variation in LH and testosterone levels. Seasonal changes in day length could not explain the seasonal variation in LH levels. Sun hours were entered in the model as either the sum of hours of sunshine during the last week or the last month before blood sampling, but neither could explain the seasonal variation in LH levels. In contrast, the mean air temperature a week or a month before blood sampling seemed to explain most of the seasonal variation in LH levels. Mean temperature and maximum temperature were closely related, and the mean maximum temperature during the week or month before blood sampling did not add more information about the variation in LH levels than that obtained from the mean temperature. The seasonal changes in mean temperature in the Copenhagen area during the study period is also shown in Fig. 2.

Temperature was also the only climatic parameter tested that seemed to explain the seasonal variation in testosterone levels. For both LH and testosterone increasing mean air temperature was associated with increasing hormone levels (for estimated effects of air temperature, see Table 2). In May and June the second year the association between air temperature and the general LH and testosterone levels seemed to deteriorate, although in several of the individual men this association was kept at this time. As LH stimulates testosterone production, we tested whether the association between air temperature and testosterone levels was indirectly through LH. When both mean air temperature and LH levels were entered into the model, only LH remained as a significant explanatory variable. Thus, the observed effect of air temperature on testosterone is most likely indirectly via the observed effect of air temperature on LH levels.

Discussion

We here present for the first time data on the intraindividual long-term day to day variation in serum inhibin B levels in healthy men. The average intraindividual variation in inhibin B levels of 10% was significantly lower than the observed interindividual variation of 25% and was remarkably close to the intraassay variation in the inhibin B assay, indicating that most of the observed intraindividual variation in inhibin could be explained by assay variation. Thus, the individual inhibin B production seems, in general, to be remarkably stable over time. We have previously reported a diurnal rhythm in serum inhibin B levels, with peak levels in the morning and decreasing levels during the day (8). In the present study a significant effect of the time of blood sampling on inhibin B levels was also evident, with an estimated average decrease per hour of 2.1%, similar to the previously reported daytime decrease of 3%/h (8). Thus, even though all samples in this study were drawn around the same time of the day (99% of the samples drawn between 0815–1100 h), the intraindividual variation reported is adjusted for diurnal variation.

In the same individuals we estimated the interindividual variations in the reproductive hormones LH, FSH, testosterone, estradiol, and SHBG. The observed average intraindividual variation of 12% in testosterone levels is in accordance with previously reported intraindividual CV values for testosterone in men (1–3, 19). In our study assay variation may explain less than 5% of this observed intraindividual variation. For testosterone levels a significant effect of hour of blood sampling was observed, with an average decrease of 3.4%/h, in accordance with previous publications describing a diurnal variation in testosterone levels (1, 4, 22). Thus, as for inhibin B, the reported intraindividual variation in serum testosterone does not represent the total intraindividual variation because in this study the diurnal component of the intraindividual variation was adjusted for.

The observed average intraindividual variations in estradiol, FSH, and SHBG were of the same magnitude (11–15%) as the observed variation in testosterone, and as for testosterone, only about 2–5% of this variation could be explained by intraassay variation. In contrast, the intraindividual variation in LH of 22% was substantially higher than the variation in the other reproductive hormones. Less than 3% of this variation could be explained by assay variation. This high intraindividual variation in LH presumably reflects the episodic nature of LH secretion (23). Neither LH, FSH, estradiol, nor SHBG serum levels were affected by the time of the day of blood sampling in this study.

The design of this study makes it very suitable for investigating seasonal variations. We observed a significant effect of calendar time of blood sampling on LH and testosterone levels, indicating a seasonal variation in these two hormones. No effect of calendar time of blood sampling on the levels of the other four hormones was evident. For both LH and testosterone, a seasonal pattern, with peak levels during the summer and nadir levels during the early spring, was found. Previous reports on seasonal variations in LH and testosterone are not consistent, as some studies found a circannual

TABLE 2. Effect on LH and testosterone levels of the air temperature the month prior to blood sampling

Mean air temperature during month previous to blood sampling	LH		Testosterone	
	Estimate[a]	95% CI	Estimate[a]	95% CI
−0.5[b]–2.5 C	−11.4%	(−16.8 to −5.7%)	−5.2%	(−8.4 to −1.9%)
2.6–7.5 C	−5.7%	(−11.4 to −0.3%)	−4.5%	(−7.7 to −1.1%)
7.6–13.3 C	−1.8%	(−7.7 to 4.5%)	−2.0%	(−5.3 to 1.4%)
13.4–15.7[c] C	–	–	–	–
Temperature (change per C)	0.87%	(0.47–1.28%)	0.38%	(0.15–0.61%)

95% CI, 95% confidence interval.

[a] Estimate equaled percentage decrease in relation to the highest temperature category (>13.3 C) when mean temperature was entered in model as a categorical variable and equaled percentage increase in hormone level per increasing 1 C when mean temperature was entered in model as continuer explanatory variable.

[b] Lowest mean temperature (of 30-d intervals) during study period.

[c] Highest mean temperature (of 30-d intervals) during study period.

rhythm, whereas others did not (5, 7, 15–20, 22, 24). Some of the reasons for this discrepancy may be that only small numbers of subjects are included in these studies, and very different statistical methods are employed, with some studies using stronger statistical tests than others. Considering the prominent intraindividual variation in LH levels, of which a seasonal variation contributes only marginally, it is clear that less strong statistical tests on a limited number of subjects may fail to detect this seasonal variation. Although there was no absolute agreement about when the highest levels of LH and testosterone were observed throughout the year among the studies detecting a seasonal variation (which all were carried out in the northern hemisphere), LH peaks were generally observed during the spring-summer (5, 15, 18, 20), and testosterone peaks were generally observed during the summer-autumn (7, 15, 16, 24). This is more or less in accordance with our findings.

In seasonally breeding animals, the controlling effect of photoperiodicity is well documented (25, 26). We also tested the effect of changing day length on LH and testosterone levels, but in our study day length did not explain the seasonal variation in LH levels. This is in accordance with studies in which no correlation between changes in daylight and the annual variation in reproductive hormones was found in geographical regions with large seasonal differences in photoperiod (18, 27). Also, in a study of men stationed in Alaska, the observed annual variation in testosterone levels (24) did not seem to be augmented compared with the annual variation in testosterone observed in regions with less extreme changes in daylight. The finding of normal levels and diurnal rhythms of gonadotropins and testosterone in blind men also indicate that light is not an important factor in the regulation of the pituitary-gonadal hormonal axis in men (28), and thus other seasonal factors seem to be responsible for the seasonal variation in reproductive hormones observed in several studies.

To our surprise we found that in our statistical model changes in climatic air temperature in the period up to blood sampling seemed to explain the observed seasonal variation in LH. During the last month of the study the association between LH levels and air temperature seemed to be deteriorating, as the general trend in LH levels went down as the air temperature increased with the onset of summer (June). However, in several of the men, an increase in LH levels was observed during this month, consistent with a positive association between air temperature and LH secretion. We cannot offer an explanation of how air temperature might affect LH secretion. Likewise we do not know why this seasonal pattern in LH secretion was obvious in some of the men and not in others. It may be argued that in our modern society with air-conditioning, warm clothing, and artificial lightning, it is questionable how much we are affected by climatic changes. Clearly our work, hobbies, and daily life determine how much we are exposed to climatic factors. Thus, it can be speculated that the men showing a seasonal pattern in reproductive hormone levels were those most exposed to weather, and those showing no obvious seasonal pattern were less exposed. However, we have no information about the daily activities of the subjects and thus cannot verify or dismiss this speculation. Of course, our results do not prove that it is air temperature *per se* that is causing the changes in hormone levels. Perhaps air temperature only indirectly affects LH secretion by affecting how much we are outdoors, how we are dressed, or how active we are. To our knowledge only one other study has looked at effect of air temperature on testosterone levels, and in this study no seasonal variation in testosterone levels was observed (19).

It is remarkable, however, that seasonal variation in reproductive hormones is recognized in man despite our modern way of living, which in many ways weakens the effects of climatic factors. The observed effect of season on LH levels was small compared with the general intraindividual variation, but it was nevertheless significant enough to lead to a corresponding seasonal variation in testosterone levels. The question is whether this seasonal variation in reproductive hormones has any biological consequences. Seasonal variation in the number of births has been documented, with the peak number in spring (March–May) and the minimum in late autumn (October–December) (29–31), consistent with peak and minimum conceptions in June, August, and January–March, respectively, thus coinciding with, respectively, the circannual peak and minimum levels in LH and testosterone observed in this study. The seasonal variation in births is presumed to be due mainly to seasonal variation in coital rates, although seasonal variation in female fertility cannot be excluded (32). Testosterone significantly affects libido, and circannual changes in male testosterone levels have been shown to positively correlate with sexual activity (5).

In conclusion, we have shown that the individual day to day level of serum inhibin B is very constant and does not seem to be influenced by seasonal factors. However, our data confirm previous studies showing a seasonal variation in LH and testosterone serum levels, with peak levels during summer and lowest levels during the winter/early spring. The biological significance, if any, of this seasonal pattern in LH and testosterone levels remains to be shown.

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