An Approach to Studying Circadian Rhythms of Adolescent Humans

Mary A. Carskadon,*,1 Christine Acebo, *
Gary S. Richardson,†
Barbara A. Tate,† and Ronald Seifer*

*Department of Psychiatry and Human Behavior, Brown University School of Medicine,
E. P. Bradley Hospital Sleep and Chronobiology Research Laboratory,
East Providence, RI 02915;
†Department of Medicine, Brown University School of Medicine,
Miriam Hospital Neurobiology Laboratory, Providence, RI 02906

Abstract The "long nights" protocol was designed to evaluate sleep processes and circadian rhythm parameters in young humans. A total of 19 children (10 boys, ages 11.2 to 14.1 years [mean = 12.7 ± 1.0], and 9 girls, ages 12.2 to 14.4 years [mean = 13.1 ± 0.7]) took part in the study. Sleep/wake initially was assessed at home using actigraphy and diary for 1 week on each child's self-selected schedule followed by an 8-night fixed light-dark (LD) condition, while sleeping from 22:00 to 08:00 h and wearing an eye mask to exclude as much light as possible. Phase measurements included 4-night mean actigraphically estimated sleep onset and offset as well as 1-night dim light salivary melatonin onset (DLSMO) phase at the end of each condition. Subjects then lived in the laboratory for 6 consecutive cycles: Day 1 LD = 14:10 h, lights out 22:00 to 08:00 h; Days 2-4 LD = 6:18 h, lights out 18:00 to 12:00 h; Days 5-6 = constant routine in continuous dim light (about 20 lux); Night 6 = 14 h recovery sleep. Phase markers (sleep onset, sleep offset, DLSMO) were significantly less dispersed after the fixed LD as compared to the self-selected condition, indicating efficacy of the LD protocol. Phase markers were correlated at the self-selected assessment (sleep onset vs. sleep offset $r = .72$; DLSMO vs. sleep onset $r = .82$; DLSMO vs. sleep offset $r = .76$) but not on the fixed schedule, probably due to restricted range. The constant routine provided additional phase markers, melatonin offset and midphase. Offset phase of melatonin secretion was significantly correlated with age ($r = .62$) and Tanner stage ($r = .62$). In conclusion, these preliminary data indicate a relationship between adolescent development and circadian phase. Thus, the long nights protocol is a feasible way in which to assess circadian parameters in young humans as well as to examine intrinsic sleep processes.

Key words adolescent humans, salivary melatonin, circadian rhythms, sleep, constant routine

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1. To whom all correspondence should be addressed, at Sleep and Chronobiology Research Laboratory, E. P. Bradley Hospital, 1011 Veterans Memorial Parkway, East Providence, RI 02915.

INTRODUCTION

One of the most consistent changes in sleep patterns of children passing into adolescence is a delay in the timing of sleep; adolescents tend to go to sleep later at night and, if undisturbed, to sleep later in the morning than do preadolescents. For many years, an assumption was made that the impetus for this change in sleep patterns at adolescence was the altered psychosocial milieu that accompanies maturation (Anders et al., 1978; Kirmil-Gray et al., 1984; Carskadon, 1990) including increased striving toward independence, growth in the number and nature of social opportunities, and changed relationships with parents, particularly with regard to negotiating limits around behaviors such as bedtime. Recent interest has focused on biological factors that also may influence the adolescent sleep phase delay, such as changes in circadian timing mechanisms or alterations of central sleep-regulating processes, as well as the relationship of such factors to pubertal maturation (Carskadon et al., 1993; Andrade et al., 1993). We have hypothesized that the phase delay of adolescents may reflect developmental alterations in these processes, and, as a result, we have begun to study them in a systematic way.

Several major concerns arise when attempting to assess circadian rhythm parameters in adolescents (not the least of which is acceptance of complicated research protocols by prospective volunteers and their families). Studies of circadian parameters in adult humans typically require relative social isolation and invasive procedures, whether in conditions of temporal isolation (cf. Wever, 1979), constant routine (Mills et al., 1978; Czeisler et al., 1985), or forced desynchrony (Czeisler et al., 1990). Simultaneous evaluation of sleep and its homeostatic regulation adds further complexity to circadian assessments. In an initial attempt to pursue such evaluations in adolescents, we wished to focus primarily on a single circadian parameter—entrained phase—and on the sleep response to sleep loss, both of which seemed accessible using a variant of the constant routine protocol.

As we began to plan an experiment to examine the relationship of these measures to puberty, however, potential confounds arose, particularly concerning how to contextualize the constant routine procedure. One approach might be to measure phase markers with a constant routine embedded into the adolescents' usual routines. Our preliminary evidence using dim light salivary onset determinations in teenagers

showed that this parameter was significantly correlated with usual summertime sleep schedule (with sleep onset: $r = .68, p = .03$; with sleep offset: $r = .90, p = .0005$) (Tzischinsky et al., 1995). Our hypothesis that usual sleep schedules in adolescents are affected by many nonbiological factors, such as parental limit setting, peer influences, and social opportunities and obligations, placed constraints on using adolescents' natural conditions to measure a purely biologically mediated marker of phase. Furthermore, adolescent sleep/wake schedules often are widely variable and frequently involve insufficient sleep (cf. Dahl and Carskadon, 1995), hence confounding the assessment of sleep homeostasis with assessments embedded into the "usual" sleep/wake matrix. Another option would be to maximize or optimize sleep schedules beforehand; doing so might provide a purer assessment of sleep response to sleep deprivation but also might confound the phase measure, depending on how sleep schedules are timed.

Our attempt to obtain a relatively "uncontaminated" measure of phase and sleep regulation led to the development of the protocol described in the following, which we call the "long nights" protocol (Fig. 1). This procedure ultimately provides phase markers under conditions that are influenced by psychosocial factors, under conditions of entrainment of all subjects to one light-dark (LD) schedule, and, finally, under conditions that we attempt to free of these factors. In this article, we describe our experimental protocol and initial experience with a group of adolescents. With this approach, we were able to measure entrained circadian phase and sleep parameters. This approach enables us to examine our principal hypotheses: human adolescence (perhaps puberty) is associated with a developmentally mediated phase delay or a reduction in the rate of accumulation of the "sleep drive" or sleep/wake homeostatic process; and either of these processes, or a combination of both, contributes to the sleep phase delay that commonly appears during adolescence. The present article presents results of the phase assessments.

MATERIALS AND METHODS

Subjects

The experimental protocol was developed with feedback from the E. P. Bradley Hospital Institutional Review Board for the Protection of Human Subjects

(IRB), and all procedures were approved by that committee. Subjects were recruited through flyers given to 5th, 6th, and 7th graders at local schools inviting interested students and parents to attend an "open house" at the laboratory for a complete description of the study. A laboratory tour, demonstrations, a question-answer interchange, and an initial screening were accomplished at two open house sessions. Our selection criteria were explicitly not intended to have "super normal" children in the study; rather, they were intended to have healthy children within normal limits on a number of measures. Exclusion criteria were a self-reported sleep schedule that varied by greater than 3 h across a week or that indicated a pattern of chronic insufficient sleep accompanied by overt signs of excessive sleepiness (e.g., falling asleep inappropriately); current sleep problem or sleep disorder noted in self- or parental report such as enuresis, sleepwalking, night terrors, sleep apnea syndrome, insomnia, or narcolepsy; family history of diagnosed narcolepsy or obstructive sleep apnea syndrome in a first-degree relative; chronic major illness such as cancer, diabetes, or kidney disease (mild unmedicated asthma was not an exclusion); current illness, fever, or symptoms of respiratory infection or allergy; current use of psychoactive agents or other drugs that may affect the sleep/wake cycle, alertness/sleepiness, or circadian parameters; history of head trauma or brain injury; physical handicap that interferes with testing (e.g., blindness, deafness); evidence of learning disabilities such as enrollment in special education classes, diagnosis of learning disability, or mental retardation; and family history (in a first-degree relative) of diagnosed major depressive disorder, bipolar depression, or schizoaffective disorder.

A total of 19 children (10 boys, ages 11.2 to 14.1 years [mean = 12.7 ± 1.0], and 9 girls, ages 12.2 to 14.4 years [mean = 13.1 ± 0.7]) who passed the screening were able to fit the study dates into their summer schedules. Of these children, 18 were Caucasian and 1 was of mixed African American/Native American descent. Four sessions were scheduled for 4 subjects and a backup volunteer each (two sessions had the same backup subject). The backup subjects fulfilled all parts of Conditions I and II (see Procedures) and came to the laboratory at the start of Condition III prepared to stay if another subject was unable to complete the protocol. Sessions were run during the summer of 1995. Each child's parents signed informed consent, and children cosigned to indicate their assent to participate. Each child was compensated with a $150 cash payment and a $100 U.S. savings bond for his or her participation.

All 19 subjects completed Conditions I and II. One 12-year-old male subject was noncompliant with the Condition II schedule (in bed at the appropriate times but attempting to sleep later than the scheduled time), and his data (Subject 11) have been dropped from the Condition II analysis. A backup subject completed the study in his stead. A total of 14 subjects completed all three phases of the protocol. An 11-year-old boy (Subject 12) and a 12-year-old girl (Subject 13) were dropped during Condition III because of discomfort attributed to homesickness. Unfortunately, subjects were not prescreened for previous experience sleeping away from home, which was lacking in these 2 volunteers and in 2 others (an 11-year-old boy [Subject 15] and a 12-year-old girl [Subject 10]) who experienced homesickness, but to a lesser degree.

Procedures

During Condition I (see Fig. 1), subjects were instructed to keep their usual summertime schedules. The only constraints were to sleep at home each night and to avoid "all-nighters," that is, staying awake all night. We call this condition "self-selected" and presume that the youngsters were exposed to a multiplicity of biopsychosocial influences on their sleep/wake schedules and that sleep was the principal influence on the LD cycles through the exclusion of retinal light exposure while asleep. Each subject received written assurance of good health from his or her personal physician and was seen by a research physician who confirmed good health. Two research physicians examined each child independently to rate Tanner stage (Tanner, 1962). Discrepancies between raters were resolved by consensus, and the pubic hair rating was used as our marker of pubertal development because this rating has somewhat greater reliability than do breast or genital ratings.

During Condition II (see Fig. 1), each subject was instructed to sleep in a darkened room wearing an eye mask between 22:00 and 08:00 h. The instructions specifically urged subjects to "exclude as much light as possible from your eyes" on these nights. We call this condition "entrainment," and we anticipated that sleep/wake schedules and circadian phase would show adaptation to this imposed LD regimen.

An intensive orientation session was held at the start of Condition I with each child and at least one
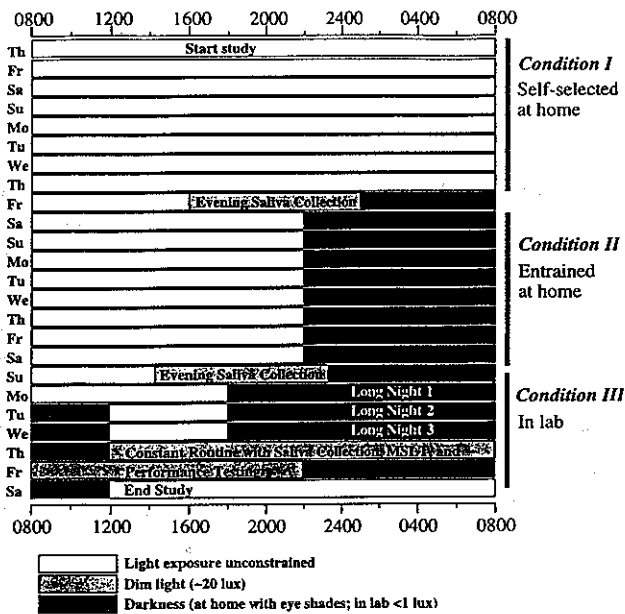
Figure 1.   Study protocol. The entire study protocol is outlined in this figure. Each run began with an orientation session attended by a subject and a parent. Subjects wore wrist actigraphs and kept their own schedules at home during Condition I (self-selected). A Friday evening session that included saliva collection in dim light was followed by Condition II (entrainment), which included 8 nights during which subjects tried to sleep and wore eye masks to exclude as much light as possible between 22:00 and 08:00 h. Subjects came to the laboratory for the subsequent 6 consecutive cycles that included evening saliva collection, 3 long nights, a constant routine, and a recovery night. See Fig. 2 for details of the in-laboratory session.

parent in attendance. During this orientation, the entire protocol was reviewed and subjects were instructed in the procedures they were required to follow during Conditions I and II. Subjects wore wrist actigraphs (Mini-Act, AMA-32, AMI, Ardsley, NY) throughout the study (exchanged as necessitated by battery life). Actigraph data during Conditions I and II were used to verify diary reports and adherence to the Condition II entrainment schedule. Sleep onset and offset times were estimated using the validated scoring algorithm of Sadeh et al. (1994) to score portions of the record for which self-reports indicated nighttime sleep.[2]

Subjects also completed the Social Rhythm Metric (Monk et al., 1990) and the Pittsburgh Sleep Diary (Monk et al., 1994), which we adapted for adolescent use (Carskadon et al., 1996). Morning telephone calls

to a computerized data collection system were made daily by subjects to indicate bedtime, rise time, diary completion, and that the actigraphs continued to function. Follow-up telephone calls were made by research staff to subjects who failed to telephone the laboratory and at midweek regardless of compliance. These procedures are very important components for field studies of adolescents, for whom frequent feedback is useful to help them remain on task.

Subjects came to the laboratory for saliva collection at the end of Condition I to determine self-selected circadian phase using the dim light salivary melatonin onset (DLSMO) phase. The time of the nocturnal increase of plasma melatonin in dim light conditions has been confirmed as a reliable circadian phase marker (Lewy and Sack, 1989; Van Cauter et al., 1994). Dim light salivary melatonin also is useful; unpublished data from our laboratory show that DLSMO phase has a significant within-subject reliability in adolescents across a 4-month interval, $r = .55$, $df = 26$, $p = .003$. Saliva was stimulated by chewing a small piece of Parafilm, and 2 ml was collected into a small tube for each sample. Ten samples were collected at 30-min intervals, the times of which were determined based on the call-in bedtimes on the first 6 nights of the self-selected protocol. The last scheduled saliva time was set at the nearest quarter of an hour after the mean reported bedtime. Thus, a child with a mean reported bedtime of 23:20 h had the first sample collected at 19:00 h, repeated each half hour until 23:30 h. Samples were collected while subjects were seated for at least 5 min remaining in dim light (about 20 lux) from arriving in the laboratory at approximately 17:00 h throughout the saliva collection evening.

Condition III (Figs. 1 and 2) of the protocol occurred in the sleep research laboratory and began after the evening saliva collection for determination of Condition II (entrainment) DLSMO phase. Entrainment saliva collection for all subjects began at 17:30 h and continued half hourly until a final sample at 21:57 h, right before bedtime. During this evening, subjects received further orientation and practiced performance tests, and electrodes were applied for sleep monitoring.[3] Subjects slept in individual darkened bedrooms from 22:00 to 08:00 h on the first in-laboratory night and remained in the laboratory under moderate lighting conditions (about 20 lux) on the baseline day. The baseline day included meals served at 08:00, 12:30, and 16:30 h; 20-min performance testing batteries at 10:00, 12:00, 14:00, and 16:00 h; and the Multiple Sleep Latency Test (MSLT) (Carskadon et al., 1986) at 09:30,
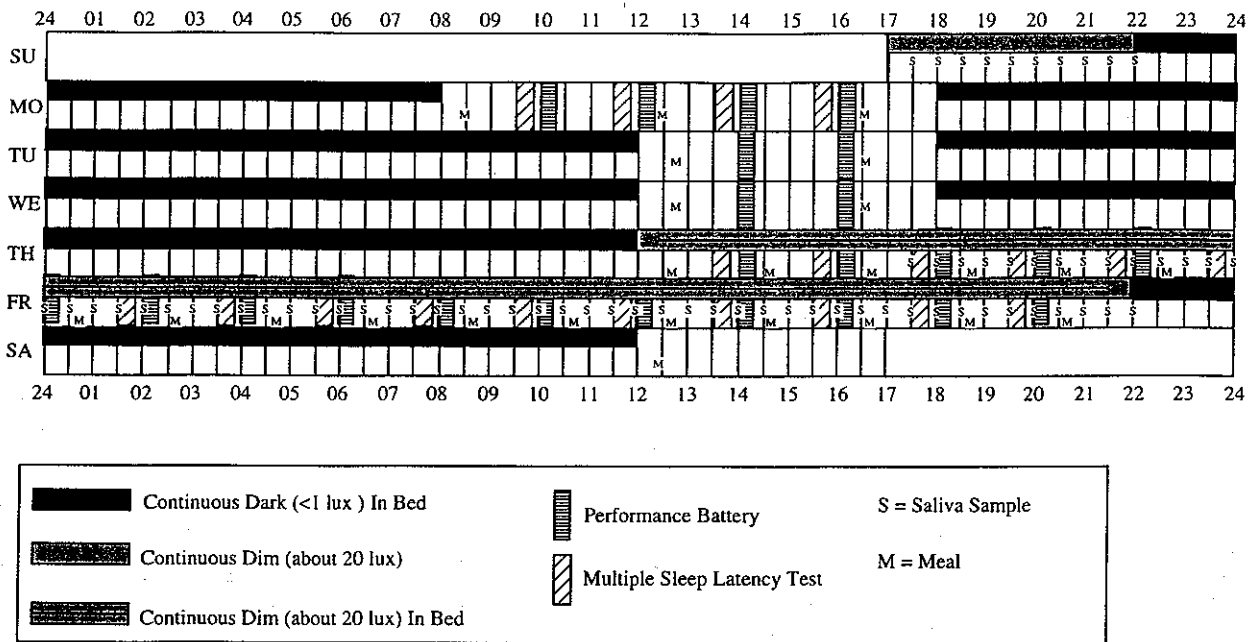
Figure 2.   In-laboratory protocol. Details of the activities during the in-laboratory phase (Condition III) of the study are illustrated in this figure. Times are indicated by the two-digit numbers above and below the schematic of the protocol, for example, 24 = midnight and 12 = noon. Lighting conditions, testing schedule, meals, and saliva samples are indicated by the key.

11:30, 13:30, and 15:30 h. Each MSLT on the baseline day was terminated after 20 min whether or not subjects slept.

The first of three "long nights" began at 18:00 h and lasted until noon the following day. The goal of the long nights was to free subjects from constraints on their sleeping and light exposure patterns, thus minimizing psychosocial phenomena that typically affect sleep and circadian patterns in humans, and to avoid light input to subjects' retinas during the photosensitive portions of the phase response curve (PRC). (Findings reported after this study was run indicate that the human PRC has no "dead zone" for light [Jewett et al., 1996; Hashimoto et al., 1996].) Only three long nights were given to minimize drift of the photosensitive phase of the PRC into the daytime portion of the protocol, as might occur if subjects free run on the long nights. At the request of the hospital's IRB that we not have the children in a silent dark room for 18 h, very dim (< 1 lux) lighting was provided and a continuous loop audiotape of frog sounds (Baldwin, 1990) was played just above each child's waking auditory threshold. The lighting was below the level of sensitivity thought to influence the human circadian system but sufficient so that the dark-adapted eye can see shapes

and even read letters approximately one-half inch high. The audiotape was sufficient to provide a comforting presence when the children were awake but was unlikely to affect sleep given that the sound was relatively constant and the sound level was below the sleeping auditory threshold (Busby and Pivik, 1983).

The subjects were monitored throughout the long nights by polysomnography and video. Subjects were given instructions to remain in bed, to relax and lie quietly trying to sleep, or to "think about fun or interesting stuff" and to call for assistance if hungry or requiring a rest room trip. If a child called, the time was not revealed and an attempt was made to keep the interaction brief, on average shorter than 10 min. Each long night interaction included an 11-item large-font questionnaire that could be read in the < 1 lux ambient light (including eight visual analog scales for mood and alertness), oral temperature, and saliva sample—always in < 1 lux. Subjects requested meals on only six occasions (of a total of 42 nights), and they were required to remain in bed in < 1 lux while eating. Rest room trips were kept brief and in < 1 lux. On the first long night, each child received a "comfort visit" by a staff member if he or she lay awake for 60 min before any sleep or for 30 min after at least an hour of sleep.

This procedure was included to ensure that subjects knew how to ask for assistance and in general to ensure their well-being. During a comfort visit, the child was asked "Is everything okay?" and told that he or she was doing fine. [After the initial settling phase, the average number of visits per subject per long night was 1.8, principally for rest room trips (73%)].

During the two "short days" between long nights, subjects were permitted daylight exposure and spent about 3 h in a well-lit (approximately 200-400 lux) playroom or outdoors. A shower was permitted at wake-up (12:00 h). Performance test batteries were taken twice (14:00 and 16:00 h) in individual bedrooms, and breakfast (12:30 h) and supper (16:30 h) were served family style in the playroom. Subjects' family members were encouraged to visit during mealtimes on these short days and were not permitted to visit at other times. The remainder of time on the short days was spent playing games. As on baseline, each subject was attended by a student research apprentice throughout the short days.

A modification of the constant routine protocol (Mills et al., 1978; Czeisler et al., 1985) began following a wake-up rest room trip at the end of the 3rd long night. During the constant routine, subjects remained in bed (except for rest room trips) in dim light (about 20 lux), with the head of their beds at a 45° angle. Although time cues (watches, television, etc.) were eliminated from the environment, social interactions with laboratory staff and other subjects were encouraged. A research apprentice remained with each subject throughout the constant routine except during testing, and subjects were in communication with one another through a voice intercom system. A single video player was connected to video monitors in each bedroom so that subjects in each session shared a common video-watching experience. The subjects also played board or card games with the research apprentices to help encourage wakefulness. Performance testing batteries, the MSLT,[4] and meals occurred at 2-h intervals (Fig. 2). Meals were limited to small, fixed portions, although subjects were given some choices at each mealtime. If a subject declined a meal, then he or she was nonetheless presented a "default" meal. Fluids in the amount of 500 ml were available every 2 h, 250 ml with the meal and 250 ml fluid in the ensuing half hour.

Saliva collection began at 17:50 h and continued at 30-min intervals until 21:50 h on the second cycle—a total of 57 samples. Subjects were permitted out of bed at 21:25 h to use the rest room and prepare for bed. A 14-h recovery night of sleep began at 22:00 h.

Phase Determinations

Saliva samples were frozen (−20°F) within 4 h and subsequently shipped to Elias USA Inc. (Osceola, WI) for determination of melatonin using radioimmunoassay. The detection limit of the assay was 0.75 pg/ml, and the intraassay coefficients of variation ($n = 11$) were 12.1, 5.7, and 9.8% at mean concentrations of 16.5, 68.7, and 162.7 pg/ml, respectively. The interassay coefficients of variation ($n = 10$) were 13.2, 8.4, and 9.2% at mean concentrations of 17.3, 69.0, and 164.7 pg/ml, respectively. Melatonin was analyzed for all samples from the evening saliva sessions and for samples collected between 17:50 and 12:00 h on the first portion of the constant routine and between 17:50 and 21:50 h on the second portion.

Deacon and Arendt (1994), using simultaneous plasma and salivary melatonin, showed that the saliva values are approximately 40% of plasma levels. Lewy and Sack (1989) estimated that the saliva values are approximately one third the plasma levels. We selected 4 pg/ml as the threshold for melatonin onset and offset phase determinations, in parallel with the 10 pg/ml plasma level criteria commonly used. According to the method of Lewy et al. (1992), melatonin onset (DLSMO) was calculated as the first interpolated point above 4 pg/ml that was followed by a higher value. (In several cases, the only value above threshold was the last of a determination series, for example, on the self-selected or entrained nights. In these cases, the time of this suprathreshold value was used as the onset phase. If no value achieved threshold, then the data point was missing.) Melatonin offset phase (determined only during the constant routine portion of the protocol) was measured as the first interpolated point below 4 pg/ml that was followed by a lower value. Midphase of melatonin secretion during the constant routine was determined as the midpoint between the onset and offset phases for each subject. In addition to melatonin onset, offset, and midphase, we calculated the duration of melatonin secretion during the constant routine as the elapsed time from the onset phase until the melatonin offset phase for each subject. A sum of melatonin values during this interval also was calculated to provide information about levels of melatonin secretion for comparison across subjects.

Salivary cortisol levels were determined by Elias USA for the 17:50-11:50 h portion of the constant rou-
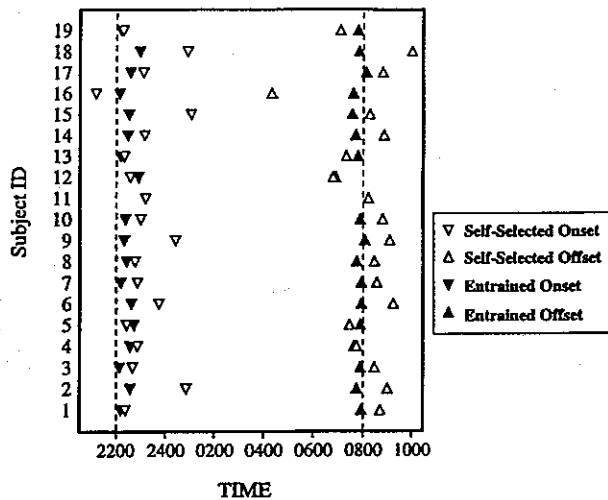
Figure 3.   Mean sleep onset and offset for Conditions I and II. The mean actigraphically estimated sleep onset and offset times averaged over the last 4 nights of each condition are displayed for every adolescent (Subjects 1-19). Sleep onsets are indicated by downward triangles, and sleep offsets are indicated by upward triangles. Data from the self-selected condition are displayed with open triangles, and data from the entrainment condition are displayed with filled triangles. The dashed lines at 22:00 and 08:00 h indicate the sleep schedule during the entrainment condition.

tine protocol, allowing determination of onset phase of cortisol on this cycle. Values were determined using a radioimmunoassay with intraassay coefficients of variation ($n = 20$) of 7.6, 8.2, and 6.5% at mean concentrations of 4.8, 13.9, and 19.1 µg/100 ml, respectively. The interassay coefficients of variation for the assay ($n = 100$) were 9.2, 9.6, and 9.7% at mean concentrations of 4.9, 13.7, and 20.9 µg/100 ml, respectively. Because cortisol values were available for only one cycle, cortisol was not used to determine phase.

Onset phase of sleep and wake were derived from actigraphy data during the at-home portions of the protocol. A mean of the actigraphically estimated sleep onsets and offsets was computed for each subject over the last 4 nights of the self-selected and entrained conditions. (A validated actigraphy algorithm was used, and we also computed within-subject Pearson correlation coefficients of sleep onsets and offsets estimated by actigraphy and from polysomnography across the 5 in-lab nights to confirm validity of the sleep onset and offset data in this sample. For sleep onset, correlation coefficients ranged from .585 to 1.0 [11 of 14 correlations > .80], and 80% of actigraph estimates were within 18 min of polysomnographic

data. For sleep offset, correlation coefficients ranged from .574 to 1.0 [10 of 14 correlations > .80], and 71% of sleep offsets estimated by actigraphy were within 18 min of polysomnography data.)

RESULTS

Tanner Stages

A total of 7 boys and 4 girls were rated as Tanner Stage 1 or 2 (pre-/early pubertal), and 3 boys and 5 girls were rated as Tanner Stage 3 or 4 (midpuberty). None of the children received a rating of Tanner Stage 5 (mature). The sex-by-Tanner stage distribution reflects both the overall younger age of the boys and the tendency for girls to mature at an earlier age than boys.

Condition I and II Actigraphy Data

Figure 3 illustrates the individual means of actigraphically estimated sleep onset and offset phases for subjects averaged for the last 4 nights of the self-selected and entrainment schedules. Sleep onset phase during entrainment was significantly earlier (matched pair $t = 3.06$, $df = 17$, $p = .007$) than when the schedule was self-selected. Mean self-selected sleep onset phase was 23:09 h ± 61.8 min, and entrainment was 22:28 h ± 15.4 min. The standard deviations reflect a significant decrease in variability during entrainment, $t = 4.08$, $df = 17$, $p = .001$. Sleep offset phase did not change significantly during the entrainment condition, although on average subjects were waking approximately 25 min later during the self-selected condition (08:12 h ± 75.6 min) than during the entrainment condition (07:48 h ± 16.2 min). As with sleep onset phase, however, the variability of sleep offset phase was significantly decreased during entrainment, $t = 3.79$, $df = 17$, $p = .001$). For 15 of 18 subjects, entrainment was associated with an earlier sleep onset; for 13 of these 15, sleep offset also was earlier on entrainment than during the self-selected condition. The actigraphically determined sleep onset and offset phase positions during the self-selected and entrainment conditions were not correlated with age or Tanner stage.

Sleep duration as estimated by actigraphy did not show a significant group mean difference between the self-selected (483 ± 49 min) and entrainment (497 ± 36 min) conditions averaged for the last 4 nights of each condition. Individual differences were apparent, however, as 4 subjects averaged 15 min or less sleep during

Table 1. Actigraphically estimated total sleep on long nights (minutes).

Parameter	Long Night 1	Long Night 2	Long Night 3
Mean[a]	700	626	570
Standard deviation	86	80	86
Median	706	643	590
Minimum	566	464	346
Maximum	858	743	678

a. Significant night-to-night difference, $F = 26.36$, $df = 2, 26$, $p < .001$; significant linear decrease across nights, $F = 57.53$, $df = 1, 13$, $p < .001$.

entrainment (maximum sleep "deficit" = 44 min) and 9 subjects averaged 15 min or more sleep on average during entrainment than on the self-selected schedule (maximum sleep "extension" = 92 min).

Long Nights Actigraphy Data

Polysomnographic records have not yet been fully evaluated, and the data presented here are based on actigraphy. The accuracy of actigraphic estimates of sleep on long nights may be compromised, however, due to prolonged episodes of quiet wakefulness, which were likely to affect sleep onset and offset estimations (although correlations were quite good with polysomnography [see Materials and Methods]). For purposes of gross comparisons, we present the actigraphic total sleep time estimates in Table 1. Repeated-measures analysis of variance of these data indicated a significant night-to-night change in actigraphically estimated sleep, with a significant linear decrease across nights.

Condition I and II Melatonin

DLSMO phase was available for 16 subjects following the self-selected condition and for 12 following the entrainment session (Table 2). The difference between the self-selected and entrainment group mean DLSMO phases was not statistically significant, but dispersion of the values was significantly reduced at entrainment. Examination of DLSMO times for individual subjects at these two assessments shows that 2 subjects essentially did not change phase after entrainment versus self-selected DLSMO. Otherwise, those whose self-selected DLSMO phases were later than 21:15 h advanced to earlier phases after entrainment ($n = 6$), and those whose self-selected DLSMO phases were earlier than 21:15 h phase delayed after entrainment ($n = 4$).

Correlations among several phase markers were computed within and across the self-selected and entrained conditions. DLSMO phases did not show a statistically significant correlation across these two conditions. Within condition, correlations among self-selected phase markers were significant (self-selected DLSMO with sleep onset [$r = .82$, $df = 14$, $p < .001$] and sleep offset [$r = .76$, $df = 14$, $p = .001$] and sleep onset with sleep offset [$r = .72$, $df = 17$, $p = .001$]), whereas correlations among phase markers during entrainment were not. Correlations in the entrained condition were likely limited due to the restricted range of entrained phase positions, as shown in Fig. 3.

Our developmental hypothesis was not supported by the DLSMO phase data from the self-selected or entrainment conditions. Neither Tanner stage nor age were correlated with these DLSMO phases.

Constant Routine Melatonin and Cortisol

Overnight salivary melatonin and cortisol profiles were available for all 14 subjects who completed the constant routine. Figures 4 and 5 illustrate the group mean nocturnal profiles of salivary melatonin and cortisol with DLSMO as the reference phase (0 h). Table 2 lists the melatonin values during the constant routine. DLSMO phase at the constant routine evaluation was significantly correlated with the self-selected DLSMO phase ($r = .70$, $df = 11$, $p = .008$) and with the entrained phase ($r = .69$, $df = 8$, $p = .028$). Figure 6 displays the phase relationships of DLSMO for each subject across the three conditions.

Because of our interest in developmental changes in circadian rhythms, we computed correlation coefficients of the constant routine DLSMO parameters with age and Tanner stage to examine possible associations. The offset phase of melatonin secretion was significantly correlated with age ($r = .62$, $df = 12$, $p = .018$) and Tanner stage ($r = .62$, $df = 12$, $p = .02$). Figure 7 illustrates the relation with Tanner stage. Of the other parameters, a trend for a positive correlation with Tanner stage was present for the midpoint phase of melatonin secretion, $r = .50$, $df = 12$, $p = .10$.

DISCUSSION

The successful completion of Conditions I and II of this experiment by all subjects and of Condition III by 14 of 16 subjects indicates that adolescents are able to comply with the requirements of the study. The chief

Table 2. Salivary melatonin parameters.

| | Self-Selected Onset Time | Entrainment Onset Time | Constant Routine (post long nights) | | | | |
			Onset Time	Offset Time	Midpoint Time	Duration (minutes)	Mean per Sample (picograms/milliliter)
Mean	21:29	20:56	21:45	08:20	03:02	634	19.6
Standard deviation (minutes)	64[a]	25[a]	77	51	59	55	8.2
n	16	12	14	14	14	14	14

a. Variability in the time of melatonin onset phase on the entrained schedule was significantly reduced as compared to the variability of melatonin onset on the self-selected schedule, $t = 2.52$, $df = 11$, $p = .03$.
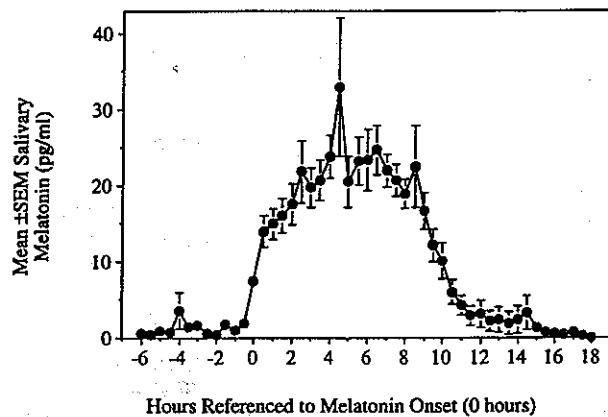
Figure 4. Salivary melatonin profile. The salivary melatonin profile for subjects during the constant routine portion of the in-laboratory protocol is displayed, with data referenced to melatonin onset phase. Group means and standard errors are illustrated ($N = 14$).
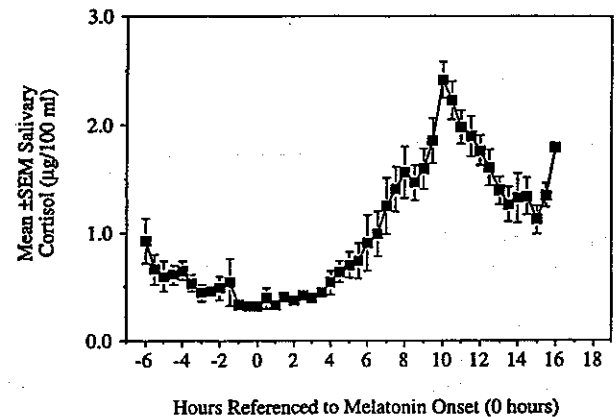

Figure 5. Salivary cortisol profile. The salivary cortisol profile for subjects during the constant routine portion of the in-laboratory protocol is displayed, with data referenced to melatonin onset phase. Group means and standard errors are illustrated ($N = 14$).

difficulties encountered were homesickness and the failure of 1 subject to comply with the entrainment schedule due to a misunderstanding of the requirements. Of interest was the temporal occurrence of the homesickness episodes, which were confined to the early evening hours of the long nights portion of the study. Subjects never manifested this discomfort at other phases, even though they often lay awake for lengthy bouts at the end of long nights. The association of this psychological discomfort with only the evening phase on long nights may represent a response to the somewhat abrupt separation from staff and parents that was forgotten by long nights' end. Conversely, a more biologically mediated influence may be involved, such as prolongation of prolactin secretion on

the long nights; Wehr and colleagues (1993) observed a lengthening of prolactin secretion in adults exposed to a short photoperiod and suggested that prolactin may allow for a pleasantly ruminative state.

We feel that one of the reasons for the success of the study was our use of relatively noninvasive procedures and the continued social interplay, even during the constant routine procedure. Subjects were able to draw moral support from one another as well as from the staff. Saliva samples were collected frequently and successfully. Few (1.1%) samples were missed, and those were chiefly during the early morning hours of the constant routine during the height of the melatonin secretory phase. By bringing subjects to the laboratory for all salivary melatonin sampling, we are confident that the measure was obtained at the appro-
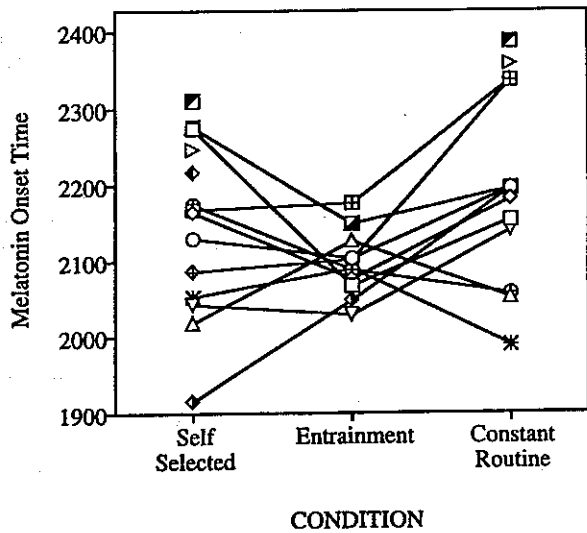
Figure 6. Dim light salivary melatonin onset (DLSMO) phase at three experimental conditions. Melatonin onset phase is displayed for each subject during the three experimental conditions. Lines connect the phase positions for those subjects for whom data were available at each assessment. Several subjects did not achieve melatonin onset before bedtime (22:00 h) on the entrainment assessment. Note the significant reduction in dispersion of DLSMO phase from the self-selected condition to the entrainment condition.
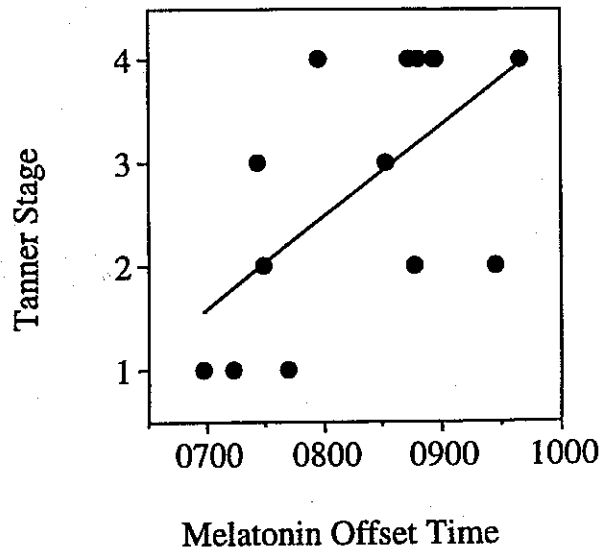
Figure 7. Melatonin offset phase versus Tanner stage. The melatonin offset phase determined during the constant routine following the long nights was correlated ($r = .62$, $p = .002$) with Tanner stage, as illustrated in this figure, which also displays the regression line.

priate intervals and in the proper lighting conditions. The introductory session and the Condition I saliva evening also gave subjects an opportunity to acquaint themselves better with one another and with the laboratory staff and environment.

Actigraphy provided a valuable measure for estimating sleep onset and offset phases during at-home portions of the study and for verifying compliance to the entrainment schedule. We have not yet confirmed its usefulness on the long nights; that will require verification with polysomnography. Particularly on the long nights, actigraphy may overestimate sleep duration and do a poor job of estimating sleep onset and offset.

The entrainment protocol was fairly simple for most subjects to accommodate, although the procedure modified sleep lengths as well as phase. For example, 9 subjects significantly extended sleep during the entrainment condition. On the other hand, 4 subjects obtained at least 15 min less actigraphically estimated sleep per night on entrainment than under self-selected conditions. Based on actigraphic esti-

mates of sleep on the long nights, however, it appears likely that all subjects became sleep sated given that estimated sleep time fell in a linear fashion across consecutive nights. Thus, the constant routine and recovery night components of this long nights protocol should provide an excellent opportunity to obtain an uncontaminated estimate of sleep homeostasis, including an MSLT estimate of sleep tendency across the constant routine and measures of the slow wave sleep components of the recovery sleep. These analyses currently are underway.

Among the protocol's shortcomings is the reliance on DLSMO as the phase marker for most analyses. Although this is a robust and reliable measure, it is only available during wakefulness and therefore was missed when the protocol called for sleep before the onset of melatonin secretion. Thus, only 10 subjects achieved melatonin onset threshold on the entrainment sample, and a second onset on the latter portion of the constant routine was available in only 5 subjects. Reliance on a single phase marker is limiting, and simultaneous monitoring of multiple overt rhythms is preferred for a number of reasons. In subsequent implementations of the protocol, we plan to place a constant routine procedure following the entrainment

condition and to add core body temperature monitoring, from which melatonin, cortisol, and temperature phase can be determined. This procedure also will permit examination of whether the long nights produce a lengthening of melatonin secretion. Wehr and colleagues (1993) showed lengthening of nocturnal melatonin in adult humans exposed to extended (14-h) nights for 4 weeks. We think it unlikely that the 3 long nights were sufficient to induce such a prolongation of melatonin secretion because the mean length of the salivary melatonin secretory phase on the constant routine for our subjects was 10.57 ± 0.9 h, which is more similar to the reported plasma melatonin phase in the subjects of Wehr et al. on short (8-h) nights (10.3 ± 1.3 h) than on long nights (11.9 ± 1.6 h).

Several findings from this study lend support to our hypothesis that adolescent maturation in humans is associated with a circadian phase delay. Most salient among the findings were the positive correlations of age and Tanner stage with melatonin offset phase during the constant routine. A trend for a positive association of Tanner stage with melatonin midsecretory phase also supports the hypothesis.

In conclusion, these results indicate that the long nights protocol will enable estimation of circadian and sleep homeostatic processes in young humans and ultimately will permit us to examine developmental changes in the two physiological processes controlling sleep: that due to circadian timing and that due to intrinsic sleep mechanisms (e.g., Borbely, 1982; Edgar et al., 1993). The data obtained in this initial small sample lend partial support to the hypothesis that circadian phase delays during pubertal development. Data regarding development of the sleep homeostatic process remain to be examined. When developmental changes are better understood, we can explore within this biological context important issues of behavioral factors that affect adolescent sleep patterns.

ACKNOWLEDGMENTS

NOTES

2. Specifically, the algorithm was applied during portions of the record encompassing 30 min before reported bedtime and 30 min after reported rising time.
3. Sleep, performance, and Multiple Sleep Latency Test data are not presented in this article.
4. MSLT sessions during the constant routine were terminated when the subjects achieved a threshold of three consecutive 30-sec epochs of sleep.

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