Daily salivary cortisol profile: Insights from the Croatian Late Adolescence Stress Study (CLASS)

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Abstract

Introduction: The aim of the study was to examine basal hypothalamic-pituitary-adrenal (HPA) axis activity and to determine associations of various covariates (gender, sleep-wake rhythm, demographic, academic, life style and health-related characteristics) with altered daily salivary cortisol profiles in late adolescence.

Materials and methods: The total analytic sample consisted of 903 Croatian secondary school students aged 18 - 21 years (median 19 years). Salivary cortisol was sampled at home at three time points over the course of one week and its concentrations were measured by using the enzyme immunoassay.

Results: In comparison to males, female students had a higher cortisol awakening response (CAR) (median 4.69, IQR 10.46 and median 3.03, IQR 8.94, respectively; $P < 0.001$), a steeper ("healthier") diurnal cortisol slope (DCS) (median 0.51, IQR 0.55 and median 0.44, IQR 0.51, respectively; $P = 0.001$), and a greater area under curve with respect to ground (AUC_G) (median 206.79, IQR 111.78 and median 191.46, IQR 104.18, respectively; $P < 0.001$). Those students who woke-up earlier and were awake longer, had a higher CAR ($P < 0.001$), a flatter ("less healthy") DCS ($P < 0.001$), and a greater AUCG ($P < 0.001$), than students who woke-up later and were awake shorter. Less consistent but still significant predictors of salivary cortisol indexes were age, school behaviour, friendship, diet healthiness and drug abuse.

Conclusion: Gender and sleep-wake up rhythm were major determinants of the altered daily salivary cortisol profiles in late adolescence. The predictive power of other covariates, although less clear, has a potential for identifying vulnerable subgroups such as male drug users and females without a best friend.

Key words: psychological stress; cortisol; adolescence

Received: June 24, 2016 *Accepted: September 15, 2016*

Introduction

The hypothalamic-pituitary-adrenal (HPA) axis is one of the major stress-response systems in the body, reactive to both physical and psychological stressors and responsible for "'transducing subjective social-environmental experience into physiological changes relevant to health" (1). Activity of the HPA axis shows a robust circadian rhythm, controlled by the endogenous central pacemaker, the suprachiasmatic nucleus. Adrenocorticotropic hormone (ACTH) and cortisol are secreted in short pulses that occur approximately once an hour, with differences in amplitude of these pulses accounting for variation in cortisol concentrations across the 24-hour span (2). In healthy people with regular nocturnal sleep and daytime activity, cortisol increases in concentration upon wakening, reaches peak levels 30 to 45 minutes post wakening, declines during the day and attains nadir around midnight (3,4). This typical diurnal cortisol pattern may be altered in stressful situations and

cortisol levels may become chronically elevated or blunted. In fact, both stress-induced increases and declines in cortisol output can predispose individuals for pathogenesis and illness, depending on the type of medical condition and a number of other factors related to stressor and person features (5).

Saliva sampling is cost-effective, uncomplicated, minimally invasive and convenient for multiple sampling throughout the day for different population groups and in various clinical and non-clinical settings (6). Salivary cortisol parallels the free, biologically active plasma cortisol levels and it has been widely recognized as a reliable biomarker of HPA axis stress response (1,7). Major parameters of the diurnal cortisol rhythm typically measured in salivary research include the cortisol awakening response (CAR), the diurnal cortisol slope (DCS) and the area under the curve with respect to ground (AUC_G).

The CAR is the size of post-wakening surge of cortisol that occurs in the period of 30 to 45 minutes after awakening. The CAR might be a unique component of the diurnal rhythm associated with awakening, related to the number of pre-analytical factors (time of awakening, weekday vs. weekends, seasonal effects and light, health condition, smoking etc.) (8,9). The exact function of the CAR is not yet fully understood, but it is hypothesized that CAR might be associated with the anticipation of a series of upcoming demands for the particular day (10). The CAR may also function as a response to negative mood during the previous day or operate as a successful coping mechanism with the same-day daily stress (11,12). In the case that employed coping is inefficient in eliminating the feeling of stress over time, the heightened CAR (and possibly the inflexible or stiff CAR) may switch from signalling coping to signalling anticipation of stress on the next day (12). The function of the CAR has also been implicated in relation to the recovery from sleep inertia and the provision of an "energetic boost", as well as in cognitive function and regulation of the immune system (10).

The DCS is the degree of change (usually decline) in cortisol levels from early morning to bedtime. A steeper DCS is associated with better health outcomes whereas a flatter slope or less steep decline in cortisol during the day (decreased morning cortisol and/or increased evening cortisol) is associated with high chronic stress, perceived uncontrollability of the stressor, persistent fatigue, post-traumatic stress disorder, increased mortality of breast cancer, coronary calcification etc. (1,5).

Whereas the CAR and the DCS are key parameters of the diurnal cortisol change, the AUC_G is used to estimate the total cortisol secretion. The AUC_G is the total area under all cortisol measurements, which takes into the account sensitivity (the difference between measurements) and intensity (the distance of measurements from the ground) (13). In general, higher AUC_G has been associated with chronic stress, but different studies have reported inconsistent results, most likely because they did not consider the diurnal cortisol change (1).

Salivary cortisol has been progressively more integrated in developmental science and investigations of bio-behavioural processes in children and adolescents (14). Adolescence is a period of transition between childhood and adulthood during which several key developmental experiences occur (e.g. identity formation, psycho-physical maturation, acquisition of skills needed to realize adult relationships and roles), but also a time of considerable risks due to powerful influences of novel social environments and encounters. The transition from late adolescence to early adulthood (emerging adulthood) is a particularly turbulent period of adjustment characterized by major life changes involving a series of closely spaced and formative life events (e.g. entering into university, leaving parental home, starting careers and families) (15). However, very little research analysed salivary cortisol activity in healthy adolescents in their everyday environments although detailed knowledge about typical functioning of the HPA axis is a necessary prerequisite for identification of risky or abnormal cortisol profiles leading to adverse health outcomes (16).

Accordingly, to contribute to new knowledge about basal HPA axis activity in late adolescence in natural environments, this study aims to: 1) exam-

ine daily salivary cortisol rhythm in the context of everyday lives of Croatian secondary school seniors from the four largest cities in the country (Zagreb, Split, Rijeka and Osijek), and 2) determine covariates (gender, sleep-wake rhythm, demographic and academic characteristics, lifestyle, health-related practices) associated with altered salivary cortisol levels and stress-related vulnerability during late adolescence. The following hypotheses guided this study:

- Due to sex differences in the HPA axis response to stress, a strong effect of gender was expected. Therefore, all analyses of the diurnal cortisol profile were planned separately for females and males.
- It was expected that sleep-wake rhythm would be associated with diurnal cortisol profile, given the circadian rhythm of cortisol secretion and relationship of the cortisol awakening response and time of wakening.
- It was expected that health-related practices would be associated with diurnal cortisol profile, either as a direct influence on the body's physiological stress reaction or as a consequence of the stress reaction.
- Regarding demographic, academic and lifestyle characteristics, it was expected that low living standard, poor academic success and the lack of friendship or romantic relationship could act as chronic everyday stressors contributing to gradual wear and tear of the organism and elevated or blunted secretion of salivary cortisol.

Material and Methods

Subjects

Data for the current cross-sectional study are drawn from a two-phase anthropological research project carried out among students enrolled in the third and fourth year of secondary education, in two major types of public schools (gymnasiums and vocational schools) in the four largest cities in Croatia (Zagreb, Split, Rijeka and Osijek). To gather a representative sample of the target population, we applied a probabilistic two-stage cluster sample (17) stratified according to the type of school (gymnasium/vocational) and the city where the school was located. For the first sampling stage in Phase I, within each city and school type, schools were sampled with probabilities proportional to their size from the list of all public schools. Thus, schools were drawn into the sample with regard to their size, and students from each and every school had an equal probability of finding themselves in the chosen sample. In total, 26 public secondary schools were sampled: 2 gymnasiums and 3 vocational schools from Split, 2 gymnasiums and 3 vocational schools from Rijeka, 2 gymnasiums and 3 vocational schools from Osijek, 5 gymnasiums and 6 vocational schools from Zagreb.

The third grade secondary school students (juniors) participated in Phase I (from April until December 2014) conducted to analyze social and cultural contexts of late adolescents preparing for the transition from secondary education to universities/work market. The fourth grade secondary school students (seniors) participated in Phase II (from January12th to March 24th 2015) or the Croatian Late Adolescence Stress Study (CLASS). The CLASS involved saliva collection, anthropometric and cardiovascular measurements (height, weight, waist and hip circumference, blood pressure and electrocardiogram - ECG) as well as self-administered, anonymous paper-and-pencil questionnaires (Figure 1). Anthropometric and cardiovascular measurements were performed by anthropologists and medical doctors, according to the NHANES and ESH and ESC guidelines (19,20). Administrations of questionnaires lasted one school lesson (45 minutes), but if needed participants were allowed to take more time to complete the questionnaire. A field researcher (anthropologist) was present in the classroom at all times, to explain the instructions and to answer additional questions if needed.

Juniors were randomly sampled to participate in Phase I and they represented all social and cultural groups and categories typical of secondary school culture. All seniors of each participating school were invited to participate in the second sampling stage in Phase II. A total of 673 juniors participated in Phase I (age range 16-21, median 18) and a total

I. SOCIO-DEMOGRAPHIC DATA
Age
Gender
School type
Socio-economic data
Family structure
Academic performance
School behavior
Religiosity
Migration history

II. CULTURAL ORIENTATIONS IN EVERYDAY LIFE
Education
Work
Family
Intimate relationships
Health and well-being
Leisure time
Mobility
Political and social participation
Material possessions

III. HEALTH AND WELL-BEING
Diet
Physical activity
Diagnosed illnesses
Prescribed medications
Use of contraceptives
Satisfaction with life
Friendship
Romantic relationships
Alcohol consumption
Drug abuse
Smoking

IV. STRESS PERCEPTION AND COPING STRATEGIES
Problem Questionnaire (18)
School-related stress
Future-related stress
Parents-related stress
Peers-related stress
Leisure-time-related stress
Opposite-sex-related stress
Self-related stress

Coping Across Situations Questionnaire (18)
20 coping strategies used in eight problem domains: school, future, parents, peers, leisure time, opposite-sex, work and self

Figure 1. Four domains of the self-administrated, anonymous, paper-and-pencil questionnaire used in the Croatian Late Adolescence Stress Study (CLASS).

of 1833 seniors (age range 17 - 22, median 19) participated in Phase II (CLASS). In line with the recommendations for assessing salivary cortisol in epidemiological research (1,14), exclusion criteria included the use of steroid-based medications (e.g. asthma and allergies), oral contraceptives, illness on the days of testing, presence of endocrine disorder, documented incompliance with the saliva collection protocol as well as samples with salivary cortisol concentrations and awakening or bedtime exceeding three standard deviations (SD) above the study mean.

This study was approved by the Ethics Committee of the Institute for Migration and Ethnic Studies (the Institution where the principal investigator was employed at the time of applying for the project), the Ethics Committee of the University Hospital Centre Split and the Ministry of Science, Education and Sports of the Republic of Croatia. All study participants signed the informed consent (separately for Phase I and Phase II) and those of minor age (below 18 years) were requested to also provide signatures of their parents or legal guardians.

Saliva collection

Saliva samples were collected according to the protocol for non-stimulated and passive drool, without use of saliva flow stimulants or cotton-based absorbent materials and with minimal disruption of participants' typical daily routines (1,14). On day 1, in schools, students received saliva-collecting packs in the zipper-lock bags labelled with 5-character participant codes constructed in such a way that the first two characters identified the city and the school of the study participant, whereas remaining 3 characters were randomly generated, not revealing the gender, school class or other information that could be used to identify a particular student. Each zipper-lock bag contained three labelled 2 mL polypropylene vials and three straws (Nal von Minden, Moers, Germany), saliva collecting protocols with instructions and the space in which participants could record sampling times, illnesses, medications, oral contraceptive use and the day of their menstrual cycle (for female participants). The designated team members explained to students face-to-face, individually or in small groups, the importance of complying to the saliva-collecting protocol for the success of salivary analyses and warned them to precisely re-

cord collecting times. On day 2 (weekdays only) students were asked to collect saliva samples in their homes. They were instructed to slowly move their jaws in a chewing motion to allow saliva to pool in the mouth and to force the specimen through a straw into a vail (14). In accordance with the recommendations for collecting a "minimal protocol" (1), the first sample was collected immediately upon awakening, as soon as participants awoke either naturally or with an alarm clock and were ready to get up (awakening cortisol at time zero, SCC_0), the second sample was collected 30 to 45 minutes post awakening (SCC_{30-45}) and the third sample was collected at bedtime ($SCC_{bedtime}$). Before collecting morning samples, students were instructed to rinse their mouths with water 5 minutes prior to the saliva collection and to refrain from brushing teeth, eating, drinking, smoking and chewing gum. At bedtime, students were asked not to exercise, brush teeth, eat, drink, smoke and chew gum at least 2 hours before collecting samples. A minimum volume of a 1 mL saliva sample was collected per each vial. Students were advised to keep saliva samples in their domestic refrigerators (at 2 - 8 °C) and to bring them to school on day 3 (samples stored at room temperature do not show a storage effect for up to 20 days, but due to bacterial growth long-term storage at room temperature is not recommended in the salivary literature). On day 3, samples were transported in portable refrigerators from schools to collection points in each city and kept at - 20 °C. Samples were sent by courier on dry ice for analyses to the Department of Medical Laboratory Diagnostics of the University Hospital Centre Split and stored at - 20 °C until assays were performed. The storage period lasted 4 to 8 weeks before assays were performed (cortisol stability during storage period at - 20 °C was documented to be up to one year).

Salivary cortisol measurement

The analyses of salivary samples, calibration and quality control were done by following manufacturer's recommendations (Nal von Minden, Moers, Germany). To precipitate mucins, samples were thawed and centrifuged at 1500 x g for 15 minutes. Cortisol concentration was measured by using the LUCIO-Medical ELISA Salivary Cortisol Kit (Nal von Minden, Moers, Germany). Briefly, 100 µL of 7 standard samples (0 - 220.69 nmol/L), 2 control samples (low: 9.05 - 16.77, mean concentration 12.91 nmol/L, and high: 93.79 - 174.34, mean concentration 134.07 nmol/L) and saliva samples in duplicate were analyzed on the Elysis Duo Instrument (Human, Wiesbaden, Germany). Cortisol concentrations were measured at 450 nm, within 10 minutes. Intra- and inter-assay variability for cortisol ranged between 1.5 and 4.5% (for concentrations from 2.59 to 48.28 nmol/L) and 5.8 and 7.5% (for concentrations 112.69 and 67.00 nmol/L), respectively, according to the manufacturer's data. The lowest detectable concentration that could be distinguished from the zero standard was 1.48 nmol/L at 95% confidence limit. The assay linearity ranged from 1.48 - 212.41 nmol/L and assay dynamic ranged from 1.48 - 220.69 nmol/L. The percentage of cortisone cross reactivity according to the available manufacturer's data was 3.00%, and no hook effect was observed in the test. Reference values of the LUCIO-Medical ELISA Salivary Cortisol Kit (Nal von Minden, Moers, Germany), based on the study of 109 healthy adults, are available only for morning concentrations of salivary cortisol (3.31 - 40.55 nmol/L).

Cortisol concentrations were measured in nmol/L. The CAR was calculated as the difference between the waking cortisol level and the 30 to 45 minutes after waking cortisol ($CAR = SCC_{30\text{-}45min} - SCC_0$). The DCS was estimated as the difference between waking cortisol level and bedtime cortisol level, divided by the time (hours) between these samples: $DCS = (SCC_0 - SCC_{bedtime}) /$ time between waking and bedtime. The AUC_G was calculated using the trapezoid formula (13):

$$AUC_G = \frac{(m_1 + m_2)t_1 + (m_2 + m_3)t_2}{2}$$

where m_1 to m_3 represent salivary cortisol concentrations at three occasions during the day, t_1 and t_2 represent time in hours elapsed between two measurement points.

Demographic, academic, life style and health-related measures

For the purpose of this study, demographic, academic, life style and health-related variables were extracted from the questionnaire. Demographic variables included age, gender, secondary school type (gymnasium/vocational school) and self-assessed family living standard (low, average, high). For school achievement we used school grades (ranging from 1 to 5; 1 marking the poorest academic progress and 5 marking excellent academic progress) and descriptive school behaviour grades (misconduct, good and exemplary behaviour) from the official school records. For assessing their social life, participants were asked to indicate whether (yes, no) they had a best friend and a boyfriend/girlfriend at the time of the study. Participants also used a five-point scale to rate their health (1 - very poor, 2 - poor, 3 - fair, 4 - very good and 5 - excellent) and diet healthiness with respect to having regular and diverse meals (1 - never, 2 - rarely, 3 - sometimes, 4 - most of the time, 5 - always). Additionally, participants were asked to rate how often they exercised (not at all, rarely - up to once a week, regularly - two to three times a week, every day). Lastly, participants were asked to indicate how many sexual partners they had and to assess their alcohol consumption (slight – up to several times a year, moderate – up to several times a month, and heavy – several times a week or more), smoking status (never or almost never, rarely – up to several times a week, frequently – daily) and drug abuse history (never or just once, several times a year or more).

Statistical analysis

All statistical analyses were performed using SPSS/PASW version 20 (IBM Corp., NY, USA). Due to skewed distributions of salivary cortisol concentration, we applied non-parametric statistics. Mann-Whitney's U and Kruskal-Wallis tests were used to test whether samples of male and female students and groups of students characterized by different sleep-wake rhythms belong to the same distribution regarding salivary cortisol indices. To identify participants with different sleep-wake rhythms on the day of salivation we used a two-step cluster analysis included in SPSS/PASW (21). To determine the possibility of prediction and differences between relative contributions of demographic, academic, life-style and health-related factors on cortisol secretion indices, separate linear regression analyses were run with the CAR, DCS, and AUC_G as dependent variables (and separate analyses for gender subsamples). Given that the ordinary R^2 is sensitive to the number of included predictors and capitalizes on chance with adding even non-significant ones, in addition to it we also used the adjusted R^2 to obtain a more realistic picture of explanatory power of the applied regression model. In all analyses we considered P values < 0.05 to be statistically significant.

Results

Characteristics of the analytical sample

From the total sample of 1833 CLASS participants, 1097 collected and returned at least one saliva sample (the response rate was 59.8%) and 736 did not return any saliva sample (40.2%) (Figure 2). Out of 1097 study participants who returned at least one sample, 194 were excluded from the analysis based on the exclusion criteria typically applied in salivary cortisol research and 903 (or 49.3%) were included in the analysis (1,14).

A summary of basic demographic, life style and health-related variables of the total analytic sample of students (N = 903, age range 18 - 21 years, median 19 years), as well as males (N = 357) and females (N = 546) is shown in Table 1. A higher percentage of males (M) attended vocational schools and a higher percentage of female (F) students attended gymnasiums (P = 0.021). The majority of students perceived their family living standard as average. Female students had higher school achievement than male students both with respect to the academic success ($P < 0.001$) and school behaviour ($P < 0.001$). A large proportion of students (93%) reported having a best friend and 36% of students reported having a romantic relationship. The frequency of physical exercise was higher among male students ($P < 0.001$). In addi-

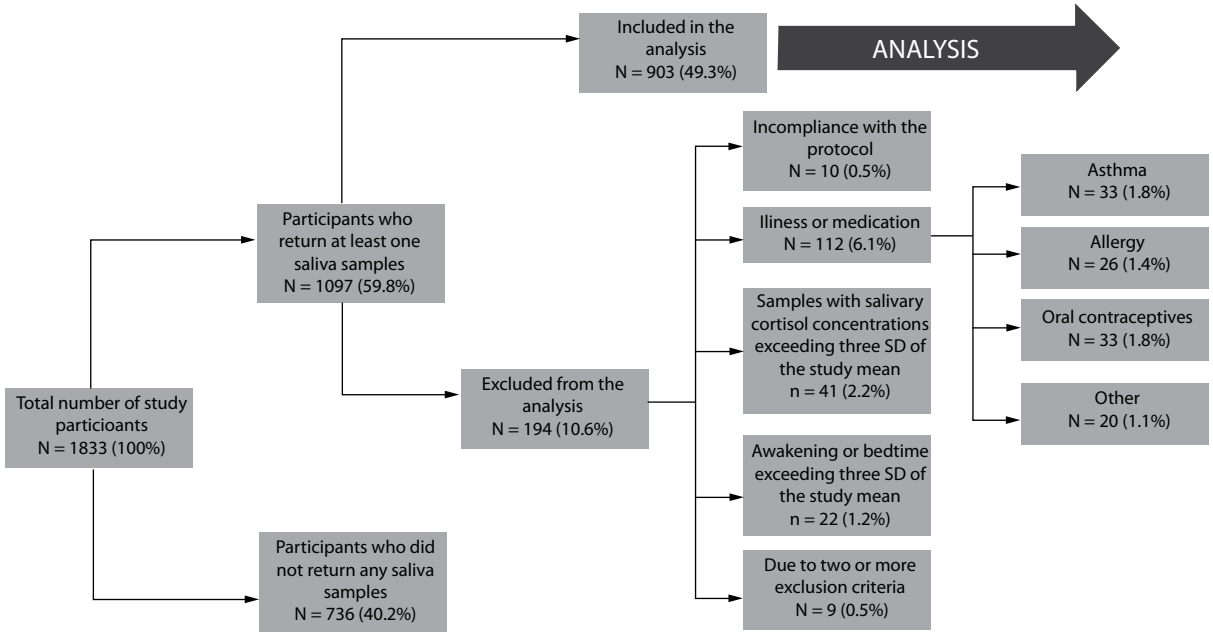

Figure 2. The flow chart of included and excluded participants in the Croatian Late Adolescence Stress Study (CLASS)

tion, males had higher number of sexual partners than female students ($P < 0.001$). Alcohol consumption and drug abuse were more frequent in males than females ($P < 0.001$ and $P = 0.007$, respectively), and smoking was more frequent in females than males ($P < 0.001$).

Gender differences

Median and interquartile range (IQR) of awakening time, wakefulness duration, salivary cortisol concentrations and cortisol secretion indexes are given for the total sample, males and females, as well as the results of Mann-Whitney U test (Table 2). The median awakening time for 875 participants was 7.48 hrs and duration of wakefulness 15.33 hrs. There was a statistically significant difference between males and females for all cortisol parameters except $SCC_{bedtime}$ (female students had higher values than males). As the interval between the first and the second morning saliva sample was allowed to vary between 30 and 45 minutes, we tested if the differences in the interval length influenced the CAR magnitude (1). The CAR showed a significant decreasing trend ($P < 0.001$) after the interval length exceeded 30 minutes, both in males and females (Figure 3).

Sleep-wake rhythm differences

Cluster analysis revealed five clusters of students based on their sleep-wake rhythm on the day of salivation (Figure 4): night birds (latest wake-up, late bedtime), sleepy heads (late wake-up, early bedtime), sleeping beauties (earliest bedtime), early risers (earliest wake-up time) and short sleepers (early wake-up and late bedtime). Table 3 shows median and interquartile range (IQR) of awakening time, wakefulness duration, salivary cortisol concentrations and cortisol secretion indexes for five sleep-wake rhythm clusters, as well as the results of Kruskal-Wallis test. Earlier median awakening time was characteristic for early risers 6.33 hrs and short sleepers 6.68 hrs (6:40 AM) and later awakening time for sleeping beauties 7.75 hrs (7:45 AM), sleepy heads 8.50 hrs and night birds 9.87 hrs. Longer median wakefulness time was characteristic for early risers (16.08 hrs) and short sleepers (17.33 hrs) and shorter median wakefulness time was characteristic for sleeping beauties (13.00 hrs), night birds (14.13 hrs) and sleepy heads (14.15 hrs). Five awakening-bedtime rhythm clusters differed significantly in all salivary cortisol parameters except $SCC_{bedtime}$.

Table 1. Demographic, academic, life style and health-related characteristics of the total sample and gender subsamples

Demography	Total (N = 903)	Males (N = 357)	Females (N = 546)	P
School type (%)				
Vocational high school	46.5	51.3	43.4	0.021
Gymnasium	53.5	48.7	56.6	
Living standard (%)				
Low	7.0	6.5	7.3	
Average	81.8	78.9	83.7	0.032
High	11.2	14.6	9.0	
School achievement				
School grades (1 - 5)	4.02 ± 0.69	3.90 ± 0.74	4.10 ± 0.65	< 0.001
School behavior (%)				
Misconduct	2.9	5.3	1.3	
Good	11.3	14.9	9.0	< 0.001
Exemplary	85.8	79.8	89.7	
Social life				
Having a best friend (%)	93.2	89.3	95.8	< 0.001
Having a boyfriend/girlfriend (%)	36.4	31.2	39.7	0.009
Health related practices				
Self-rated health (1 - 5)	3.96 ± 0.76	4.11 ± 0.77	3.86 ± 0.75	< 0.001
Self-rated healthiness of diet (1 - 5)	3.70 ± 0.83	3.72 ± 0.86	3.70 ± 0.81	0.738
Frequency of physical exercise (%)				
Doesn't exercise at all	19.0	7.0	26.7	
Rarely, up to once a week	26.8	20.2	31.1	< 0.001
Regularly, two to three times a week	34.7	41.6	30.2	
Every day	19.5	31.2	11.9	
Number of sexual partners so far	0.94 ± 1.36	1.38 ± 1.67	0.65 ± 1.01	< 0.001
Alcohol consumption (%)				
Slight (up to several times a year)	63.2	49.9	71.9	
Moderate (up to several times a month)	33.4	43.4	26.8	< 0.001
Heavy (several times a week or more)	3.4	6.7	1.3	
Smoking (%)				
Never or almost never	59.6	67.5	54.4	
Rarely (up to several times a week)	16.1	13.5	17.8	< 0.001
Frequently (daily)	24.3	19.1	27.8	
Drug abuse (%)				
Never or just once	76.3	71.6	79.4	0.007
Several times a year or more	23.7	28.5	20.6	

Data are expressed as percentage (%) and mean ± standard deviation (SD). Differences between males and females were calculated using the t-test and the χ^2 test. $P < 0.05$ was considered statistically significant.

Table 2. Awakening time, wakefulness duration and salivary cortisol measures for total sample and gender subsamples

	Total sample			Males			Females			P
	N	Median	IQR	N	Median	IQR	N	Median	IQR	
Awakening time (hrs)	875	7.48	2.50	341	7.25	2.50	534	7.50	2.50	0.937
Wakefulness duration (hrs)	864	15.33	2.67	339	15.50	2.83	525	15.20	2.50	0.197
SCC_0 (nmol/L)	903	14.47	8.03	357	13.71	6.87	546	15.23	8.44	0.003
SCC_{30-45} (nmol/L)	898	18.81	10.26	355	17.41	8.77	543	19.61	11.64	< 0.001
$SCC_{bedtime}$ (nmol/L)	897	6.73	3.70	354	6.65	4.14	543	6.76	3.42	0.846
CAR	898	4.18	10.05	355	3.03	8.94	543	4.69	10.46	< 0.001
DCS	859	0.49	0.53	337	0.44	0.51	522	0.51	0.55	0.001
AUC_G	855	199.80	105.01	335	191.46	104.18	520	206.79	111.78	< 0.001

SCC_0 – salivary cortisol concentration at awakening. SCC_{30-45} – salivary cortisol concentration at 30 to 45 minutes after awakening. $SCC_{bedtime}$ – salivary cortisol concentration at bedtime. CAR – cortisol awakening response, DCS – diurnal cortisol slope. AUC_G – area under the curve with respect to ground. IQR – interquartile range (the difference between the upper and lower quartile). Differences were calculated using the Mann-Whitney U test. $P < 0.05$ was considered statistically significant.

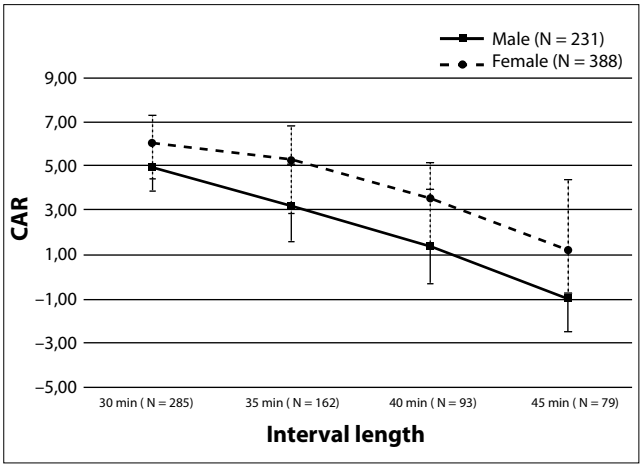

Figure 3. Median and 95% confidence intervals of the cortisol awakening response (CAR) in male and female students in relation to the time interval length between the two morning measurements.

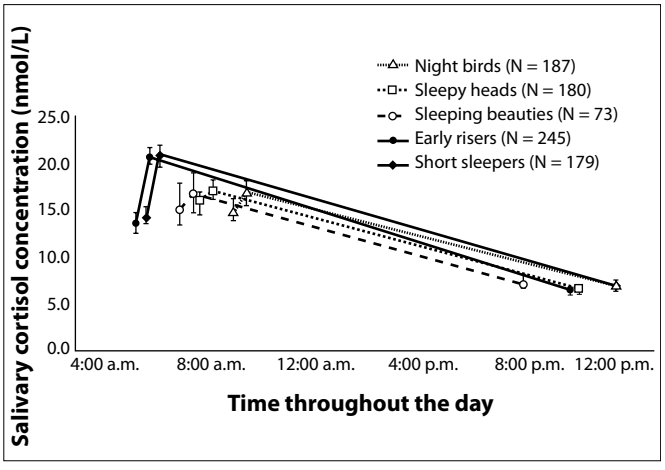

Figure 4. Median and 95% confidence intervals of cortisol concentrations from saliva samples collected at three points during the day for awakening-bedtime rhythm clusters.

Other covariates

Predictors of CAR, DCS and AUC_G obtained though regression analyses are shown in Table 4. A greater CAR was associated with longer wakefulness duration ($P = 0.018$), younger age ($P = 0.045$) and drug abuse ($P = 0.002$) in males and earlier awakening in females ($P < 0.001$). A steeper DCS was significantly associated with shorter wakefulness duration ($P < 0.001$), exemplary school behaviour ($P = 0.035$), healthy diet ($P = 0.034$) and no-drug abuse status ($P = 0.040$) in males and later wake-up time in females ($P = 0.005$). A higher AUC_G was significantly associated with earlier awakening time in males ($P < 0.001$) and females ($P < 0.001$) and not having a best friend in females ($P = 0.008$). Considering the number of analyzed predictors, the level of explained total variance was rather low in the CAR (13% and 17%) and DCS (7% and 17%) and slightly higher in AUC_G (18% and 21%). When the number of predictors relative to the sample size was taken into consideration and adjusted coefficients of R^2

Table 3. Awakening time, wakefulness duration, salivary cortisol measures and statistical comparison of the sleep-wake rhythm clusters

	Night birds (N = 187)		Sleepy heads (N = 180)		Sleeping beauties (N = 73)		Early risers (N = 245)		Short sleepers (N = 179)		X^2	P
	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR		
Awakening time (hrs)	9.87	1.00	8.50	0.92	7.75	2.13	6.33	0.75	6.68	0.67	646.76	< 0.001
Wakefulness duration (hrs)	14.13	1.67	14.15	1.53	13.00	2.17	16.08	1.25	17.33	1.25	609.60	< 0.001
SCC_0 (nmol/L)	14.76	7.82	15.93	7.86	14.92	9.78	13.46	8.47	14.10	7.97	15.04	0.005
SCC_{30-45} (nmol/L)	16.87	8.99	16.94	9.52	16.61	11.26	20.52	11.05	20.63	10.95	47.51	< 0.001
$SCC_{bedtime}$ (nmol/L)	6.94	3.46	6.50	3.50	6.98	4.29	6.36	3.54	6.81	3.72	6.24	0.182
CAR	1.13	8.73	0.61	10.32	1.43	10.54	6.43	9.33	6.73	9.10	105.08	< 0.001
DCS	0.53	0.52	0.62	0.53	0.55	0.73	0.42	0.47	0.38	0.50	44.08	< 0.001
AUC_G	180.58	85.09	167.23	86.00	160.68	93.85	222.93	119.09	242.21	103.59	143.89	< 0.001

SCC_0 – salivary cortisol concentration at awakening. SCC_{30-45} – salivary cortisol concentration at 30 to 45 minutes after awakening. $SCC_{bedtime}$ – salivary cortisol concentration at bedtime. CAR – cortisol awakening response. DCS – diurnal cortisol slope. AUC_G – area under the curve with respect to ground. IQR – interquartile range (the difference between the upper and lower quartile). Differences were calculated using the Kruskal-Wallis test test. $P < 0.05$ was considered statistically significant.

Table 4. Ordinary least square regression: Predicting salivary cortisol indices CAR, DCS and AUC_G on the basis of demographic, academic, life style and health-related characteristics

	CAR		DCS		AUC_G	
	Males	Females	Males	Females	Males	Females
R^2	0.171	0.127	0.167	0.073	0.176	0.209
R^2 adjusted	0.127	0.098	0.123	0.042	0.135	0.184
Predictors (β weights)						
Wake-up time		- 0.241***		0.201**	- 0.356***	- 0.435***
Wakefulness duration	0.202*		- 0.376***			
Age	- 0.107*					
Gymnasium						
Standard of living						
School grades						
School behaviour			0.132*			
Having best friend						- 0.109**
Having boyfriend/girlfriend						
Self-rated health						
Self-rated healthiness of diet			0.124*			
Physical exercise						
Number of sexual partners so far						
Alcohol consumption						
Smoking						
Drug abuse	0.209**		- 0.139*			

*P < 0.05. ** P < 0.01. *** P < 0.001. Only statistically significant predictors are presented in the Table. All analyses were run separately for females (N = 520) and males (N = 304). CAR - cortisol awakening response. DCS – diurnal cortisol slope. AUC_G – area under the curve with respect to ground. R^2 – Coefficient of determination.

were used instead of the ordinary R^2, the level of explained variance dropped even further.

Discussion

Higher baseline morning salivary cortisol concentrations (SCC_0 and SCC_{30-45}) as well as a higher CAR, steeper DCS and larger AUC_G were found in healthy late adolescent females as compared to males. Bouma *et al.* measured higher salivary cortisol concentrations at awakening and 30 minutes post-awakening in adolescent females than males, but without a difference in the CAR (22). Since we did not measure the levels of gonadal sex steroids in the CLASS, we can only speculate that both estrogen and progesterone-driven effects could be responsible for higher levels of morning salivary cortisol in the CLASS females than males. However, it remains unclear why there is a sex difference in response and feedback-loop of the HPA axis only in the morning and not at bedtime in healthy late adolescents. Bedgood *et al.* demonstrated that men with lower basal cortisol levels had larger increases in testosterone, both at the baseline and after the social stress task (23). This interaction of cortisol and testosterone could be a part of male adaptive strategy in dealing with social stressors and a possible mechanism in preparedness for anticipated everyday stressors in the CLASS males. On the other side, the increased CAR could be considered a dominant mechanism for the CLASS females in preparing for the anticipated challenges of the upcoming day.

Clusters characterized by the early mean wake-up time and longer wakefulness duration (early risers and short sleepers) had lower SCC_0 and greater SCC_{30-45}, and therefore a more robust CAR, as well as a flatter DCS and greater AUC_G than students belonging to the clusters characterized by later wake-up time and shorter wakefulness duration (night birds, sleepy heads and sleeping beauties). In the stress literature, the association of the wake-up time has mostly been studied with respect to the CAR. As it was the case in the CLASS, early awakening was associated with a larger CAR and late awakening was associated with a steeper, "healthier" slope (24-26). Additionally, early wake-up time also predicted a larger CAR and flatter DCS in females, and an increased AUC_G both in males and females. Long wakefulness duration predicted a large CAR and flatter slope in males.

Another set of predictors was also revealed in the regression analysis, albeit very weak and thus requiring greater caution in the interpretation. The larger CAR was associated with younger age and drug abuse in males. The steeper ("healthier") DCS was positively associated with exemplary school behavior, healthy diet and no-drug abuse status in males. The increased AUC_G was negatively associated with having a best friend in females. Observed gender dissimilarities in the salivary cortisol indexes could be a consequence of the differences in the circulating sex hormones, but also due to different gender reactivity to psychosocial stressors in relation to sexual dimorphisms in brain limbic regions responsible for processing of psychological stress (27).

The association between not having a best friend and the increased overall cortisol daily output in females is in line with the hypotheses of the ameliorative effects of friendships in the context of stress (28). Likewise, substance use has also been associated with altered cortisol profiles in adolescence (29). In this study, drug abuse was associated positively with the CAR and negatively with the flattened ("less healthy") DCS, but only in male students. The positive association with drug abuse and the CAR might be related to the previously mentioned hypotheses explaining its increase in response to negative mood the day before (when drugs could have been used) or as a coping mechanism with same-day daily stress (when drugs are to be used) (11,12). One possible explanation for the observed associations only in male students could be a consequence of the finding that female students are more prone, according to our data, to alcohol consumption and smoking than drug abuse. On the other side, exemplary school behaviour of male students was associated with the steeper DCS and could be considered as a protective health factor for male late adolescents.

The results of this study should be considered in the light of the following methodological limita-

tions. To achieve minimal interference with regular daily activities and accurate approximation of natural cortisol secretion rhythm in the studied sample, saliva sampling was done at home. However, we did not use objective compliance monitors such as electronic track caps to obtain automatic date and time for each used saliva collecting container and actigraphy to monitor the wake-up time (movement monitoring). The importance of protocol compliance was therefore pointed out both in written instructions and face-to-face individually and in small groups. Also, when study participants handed in their salvia samples and filled-out protocols, researchers checked correctness of sampling and participants' experience of the sampling procedure. Saliva sampling included only three measurements per person on a single day ("minimum protocol"). This type of sampling was planned based on our consideration of student's schedule of daily school-related and leisure activities. In order to ensure a large and representative population-based sample and a pleasant experience of our study participants, we decided to include two samples in the morning and one sample before bedtime. Nevertheless, this protocol was a considerable burden for commuters (especially from more distant locations, such as islands) and/or students who started practical classes in the early morning (e.g. students of medical secondary schools started with practical classes in local hospitals at 7:00 AM). Due to the application of the minimum protocol, all estimated salivary cortisol features and especially the results of the regression analysis should be considered very carefully.

In conclusion, two major determinants of the daily salivary cortisol profile in healthy late adolescents in natural environments were revealed in the CLASS, gender and sleep-wake rhythm. CLASS females and males had different morning mechanisms of preparing for the challenges of the upcoming day. As expected in healthy populations, bedtime cortisol concentrations were low and did not differ between females and males. However, all other cortisol parameters (two morning concentrations and three indices) were significantly higher in females. Furthermore, CLASS students who woke-up earlier and were longer awake had a higher CAR, a flatter DCS and a greater AUC_G than students who woke-up later and were shorter awake. Finally, the importance of other covariates (demographic and academic characteristics, lifestyle, and health-related practices) was not equally clear in explaining the diurnal salivary cortisol rhythm. These covariates did not consistently appear as significant predictors and when they did, their predictive power was rather weak. However, these covariates provide grounds for identifying vulnerable subgroups of late adolescents with "risky" patterns of salivary cortisol activity, such as male drug users and females without a best friend.

Acknowledgements

This study (CLASS) was taken with the framework of the project "Modernity Stress, Youth and Modernization" (09.01/408) financed by the Croatian Science Foundation, awarded to IMK. We sincerely thank all study participants for taking part in this study as well as to school employees (principals, psychologists, pedagogues and professors) for helping in organizing the field work. Also, we are thankful to Professor Emma K. Adam for her helpful feedback on an earlier version of this manuscript.

Potential conflict of interest

None declared.

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