Daily life stress and the cortisol awakening response over a 13-months stress period - findings from the LawSTRESS project

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Abstract

The LawSTRESS project is a controlled prospective-longitudinal study on psychological, endocrine, central nervous and genetic predictors of responses to long-lasting academic stress in a homogenous cohort. In this first project report, we focused on the association between daily life stress and the cortisol awakening response (CAR). The CAR, a distinct cortisol rise in the first 30 to 45 minutes after morning awakening, is a well-established marker of cortisol regulation in psychoneuroendocrinology.

Law students from Bavarian universities (total *n* = 452) have been studied over a 13-months period at six sampling points starting 12 months prior exam. The stress group (SG) consisted of students experiencing a long-lasting and significant stress period, namely the preparation for the first state examination for law students. Law students assigned to the control group (CG) were studied over an equally long period without particular and sustained stress exposure.

To investigate stress related alterations in the CAR, we examined a subsample of the LawSTRESS project consisting of 204 students with 97 participants from the SG (69.1% female, mean age = 22.84 ±1.82) and 107 from the CG (78.5% female, mean age = 20.95 ±1.93). At each sampling point, saliva samples for cortisol assessment were collected immediately upon awakening and 30 as well as 45 minutes later. Perceived stress in daily life was measured by repeated ambulatory assessments (about 100 queries over six sampling points).

The time course of perceived stress levels in the two groups differed significantly, with the SG showing an increase in perceived stress until the exam and a decrease thereafter. Stress levels in the CG were relatively stable. The CAR was not significantly different between groups at baseline. However, a blunted CAR in the SG compared to the baseline measure and to the CG developed over the measurement timepoints and reached significance during the exam. Remarkably, this effect was neither associated with the increase in perceived stress nor with anxiety and depression symptoms, test anxiety and chronic stress at baseline.

The present study successfully assessed multidimensional stress trajectories over 13 months and it documented the significant burden, law students preparing for the first state examination are exposed to. This period was related to a blunted CAR with presumed physiological consequences (e.g., on energy metabolism and immune function). Mean psychological stress levels as well as the CAR returned to baseline levels after the exam, suggesting a fast recovery in the majority of the participants.

Keywords: chronic stress, longitudinal and experimental design, cortisol awakening response, ambulatory assessment

1 Introduction

While the occasional experience of moderate stress is assumed to constitute an inevitable element of everyday life with no negative health consequences in most individuals, chronic stress is a significant risk factor for several disorders, including depression, anxiety, sleep disorders, cardiovascular diseases as well as diseases resulting from dysregulated immune functions (Chrousos, 2009; Cohen et al., 2007). A dysregulation in the hypothalamic-pituitary-adrenocortical (HPA) axis seems to be a key factor in mediating the stress-disease relationship (e.g., O'Connor et al., 2021; Tsigos and Chrousos, 1994). However, while there is ample evidence for this association between stress and malady, the biopsychological mechanisms mediating this link are not fully understood. In our view, studies could possibly make a useful contribution to human psychobiological stress research, if they combine a prospective-longitudinal design - including an appropriate assessment of baseline levels of stress related variables - with a research cohort of healthy participants that will be exposed to a long-lasting and significant stress period in a clearly predictable future period and an appropriate control group. Additionally, the application of state-of-the-art biopsychological laboratory methods and ecologically valid assessments of the participants' experience and behavior in everyday life should be feasible in such a cohort.

These requirements are met to a high degree by (the preparing for) the first state examination for German law students. This state examination is commonly considered one of the most stressful academic exam periods in the German university system. It consists of six (in Bavaria) written exams of several hours each within eight days (and an oral exam at a later date). The failure rate is about 24% to 30%, the exam can be repeated only once and the final mark is of major importance for the future career. Usually, the students prepare intensively for this exam for about one year. Although in general, it can surely be assumed that university students constitute a relatively healthy part of the population, academic stress was shown to be a severe burden for many of them. Increased depression and anxiety scores were found in medical students (Burger et al., 2014) and, in a review paper, it was reported that about 50% of the students develop significant burnout symptoms in the course of their university studies (Ishak et al., 2013). Moreover, academic stress was found to be related to salivary and hair cortisol levels (Koudela-Hamila et al., 2020; Stetler and Guinn, 2020) as well as to changes in immune functions (Maydych et al., 2017). It should be noted that the stress periods in previous studies have been significantly shorter and / or less continuous than in the present project. In the LawSTRESS project, we studied law students over a 13-months period. Students preparing for the state examination have been assigned to the stress group (SG), while law students, who did not experience this specific stress period, constituted the control group (CG; see https://doi.org/10.5283/epub.51920 for additional information).

In the present manuscript, we focus on the cortisol awakening response (CAR) and perceived stress in daily life. The CAR represents a distinct increase of cortisol levels in the first 30 to 45 minutes after morning awakening (Pruessner et al., 1997; Stalder et al., 2016). The regulatory mechanisms of the CAR are not yet fully understood but they differ from the basal diurnal secretion pattern, since it is evoked by the morning awakening and superimposed upon the circadian rhythm (Wilhelm et al., 2007). Amongst others, the CAR was found to be related to various stress related disorders, including the risk of developing a major depression (Adam et al., 2010), posttraumatic stress disorder (Wessa et al., 2006) or systemic hypertension (Wirtz et al., 2007). Studies examining the association between

the CAR and perceived chronic stress and related concepts reported mixed results (Chida and Steptoe, 2009). An increased CAR was linked to chronic work overload and worrying (Schlotz et al., 2004), whereas a blunted CAR was found in subjects reporting burnout symptoms (Oosterholt et al., 2015) and in parents taking care of mentally ill children (Barker et al., 2012). However, in a recent review it has been concluded that studies with more reliable methodologies predominantly found chronic stress to be related to an attenuated CAR (for review see Law and Clow, 2020). These methods include, e.g., a sampling time verification, elaborate statistical analyses including relevant confounding variables (Stalder et al., 2016) and a longitudinal design (O'Connor et al., 2021). Results regarding the association between academic stress phases and the CAR are, as well, not fully consistent (Duan et al., 2013; Weik and Deinzer, 2010).

As saliva samples for the later assessment of the CAR can easily be collected and temporarily stored outside a laboratory, this measure is well suitable for ambulatory settings. Daily life research methods, known as ecological momentary assessment, experience sampling method or ambulatory assessment (AA), cover a wide range of methods, from momentary self-report up to physiological methods, aiming at capturing experience and behavior over the course of an individual's everyday life. The potential advantages of AA are higher reliability due to real-time measurements, higher ecological validity due to real-life measurements and an increased precision due to repeated measurements within individuals (Trull and Ebner-Priemer, 2014). A combination of self-administered salivary cortisol assessments with an AA design offers the opportunity to investigate variance in circulating cortisol and covariance with self-reported stress in daily life. For example, salivary cortisol levels collected throughout the day were shown to be associated with momentary negative affect in several AA studies (Jacobs et al., 2007; Schlotz, 2019). These and other encouraging findings support the view that reliable associations between indicators of different stress response levels (here: momentary stress ratings and cortisol) can be found when appropriate methods are applied, although a lack of significant covariation of stress indicators is a well-known phenomenon (Campbell and Ehlert, 2012; Fahrenberg, 1979).

For the present study, we hypothesized that long-term stress exposure, defined as preparation for the first state examination for German law students, results, on average, in an increase of perceived stress and other stress related psychometric variables during the preparation phase and a decrease thereafter, while non-exam students would stay relatively stable in these variables. Moreover, we expected a blunted mean CAR in this period in the stress group compared to the control group. Across all measurements over the observation period we assumed a significant negative association between the CAR and the perceived stress levels. As interindividual differences can certainly influence the CAR, the predictive value of psychometric variables recorded at the first sampling point, namely anxiety and depression symptoms, test anxiety and chronic stress, on the time course of the CAR over the observation period was tested.

2 Methods

2.1 Sample

In cooperation with Bavarian faculties of law, 470 students were recruited via social media, flyers and presentations in university as well as commercial law school courses and lectures. In total, 452 law students from the universities of Regensburg (*n* = 154), Passau (*n* = 115), München (Munich; *n* = 85), Erlangen-Nürnberg (Nuremberg; *n* = 49), Würzburg (*n* = 28) and Augsburg (*n* = 21) completed at least the first sampling point. The whole study protocol was completed by 415 participants. Reasons for dropping out were the postponement of the exam to a timepoint after study ending (*n* = 19), no reactions to contact requests (*n* = 15), quitting without reasons (*n* = 16) or other reasons (*n* = 4).

Participants were recruited in two different cohorts. Cohort A comprised of 204 students mainly from the University of Regensburg. Cohort B consisted of 248 law students from the other Bavarian universities who underwent a modified examination protocol that did not include laboratory visits in Regensburg. Each cohort consisted of a stress group (cohort A: *n* = 97 and cohort B: *n* = 129), experiencing a long-lasting and significant stress period, namely the preparation for the first state examination for law students, and a control group (cohort A: *n* = 107 and cohort B: *n* = 119). It is

important to note that CG participants had a typical workload for law students in the mid phase of their study program.

Individuals who met any of the following (self-reported) criteria were excluded: current psychiatric, neurological, or endocrine disorders, treatment with psychotropic medications or any other medication affecting central nervous system or endocrine functions, regular night-shift work. The study was approved by the local ethics committee. All participants provided written informed consent and received monetary compensation as well as a feedback report on their individual study results.

2.2 General procedure

The study protocol provided six sampling points (t1 – t6) over 13 months. T1 for the SG was scheduled one year before the exam; the remaining appointments were three months (t2) and one week (t3) prior exam, at the weekend during the eight-days exam period (t4), as well as one week (t5) and one month (t6) thereafter. The same procedure, except the exam at t4, applied to the CG. Data collection lasted three years from March 2018 until April 2021. Adjusted to the dates of the state examination, the SG started each March or September, with the last group initiated in March 2020. The CG participants started interleaved to the SG each May or November. An additional CG was assessed in July 2019 (see supplementary Figure S1 for a description of the nested data collection in cohorts A and B). In the SG, 36.9% of the students postponed their examination date after t1. Consequently, t2 to t6 were adjusted accordingly in these participants to fit the new exam dates, only the baseline measure at t1 could not be repeated.

At t1, written informed consent was obtained and exclusion criteria were checked. An online questionnaire battery was submitted via SoSci Survey (https://www.soscisurvey.de/; Leiner, 2014) to assess baseline data, psychometrics, physical health, health behavior and university studies related variables. Moreover, a buccal swab for later DNA analysis as well as a hair sample were collected. The material for the first AA was handed out along with a detailed instruction. Furthermore, 124 participants of cohort A were examined using functional magnetic resonance imaging (fMRI; results

not presented here). At t2 (-3 months), t3 (-1 week), t4 (exam), t5 (+1 week) and t6 (+1 month) only the AA and a trajectory questionnaire were administered except for t4, where only the AA was conducted. At t6 a second hair sample was collected. Cohort B had the same study design as cohort A but they did not take part in the fMRI examination and they ran through a slightly less detailed AA (see section 2.3 Ambulatory assessment). In the present manuscript, only AA data (including the CAR) and questionnaire data are presented (Figure 1).

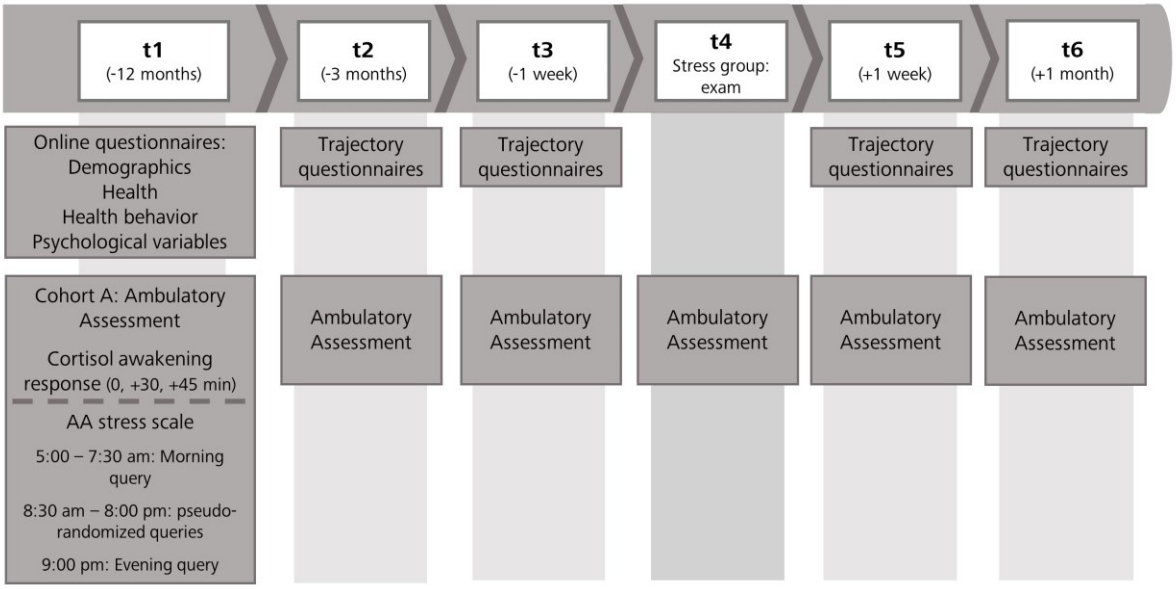

Figure 1. Timing of the data collection for the ambulatory assessment and questionnaires. *Note.* Trajectory questionnaires comprised health, health behavior and psychological variables. For an overview of the entire study procedure of the LawSTRESS project see https://doi.org/10.5283/epub.51920).

2.3 Ambulatory assessment

The AA for cohort A consisted of an assessment of current perceived stress via the AA stress scale, a short morning and evening questionnaire and the collection of saliva samples after awakening for later assessment of the CAR.

The AA was carried out via the combined smartphone app and web platform movisensXS (Version 1.3.2 to 1.5.13; movisens, Karlsruhe, Germany). At measurement timepoints t1, t2, t5, and t6, ten queries appeared on two consecutive working days. Queries were announced by an acoustic and

vibration alarm. To limit the study related burden at the timepoints close to the exam (t3 and t4), queries were presented on one day only. T4 in the SG (not in the CG) was scheduled at the weekend in the middle of the eight-days exam period. The first daily query took place immediately at the individually chosen awakening time between 5:00 and 7:30 a.m. and the last one at 9:00 p.m. The remaining queries were presented at pseudo-randomized times between 08:30 a.m. and 8:00 p.m. with a minimum interval of 60 minutes between two queries. Across all measurement points, we collected 100 queries per participant. Those who did not have a compatible smartphone were equipped with a device provided by the institute (Motorola G4, Motorola Play G4, Motorola Play G6). The CAR assessment was based on three saliva samples, obtained on the first day of each AA phase. Only at t1, we assessed the CAR on both sampling days. Saliva samples were collected using cortisol Salivettes (Sarstedt, Nümbrecht, Germany) immediately after awakening as well as 30 and 45 minutes later. Participants were instructed not to eat, drink (except from water), smoke or brush their teeth during this period. To increase compliance and sampling accuracy, functional and non-functional ('fake') electronic monitoring devices to verify times of sample collection (MEMS caps, AARDEX Ltd., Zug, Switzerland) were used in 57.6% – 75.4% (varying over sampling points) of the measurements (Broderick et al., 2004; Kudielka et al., 2003). Moreover, at each saliva sampling the participants were instructed to transfer a random three-digit code to the sampling tube, that was briefly presented via smartphone.

Saliva samples were stored at −20°C until analysis. Samples were assayed in duplicate using a time-resolved fluorescence immunoassay with fluorometric end-point detection (DELFIA) at the biochemical laboratory of the University of Trier (Dressendörfer et al., 1992). The intra-assay coefficient of variation was between 4.0% and 6.7%; inter-assay coefficients of variation were between 7.1% and 9.0%.

In cohort B, the participants received a hyperlink including the AA stress scale and either the morning or evening questionnaire via SoSci Survey (SMS and e-mail) in the morning at 7:30 a.m. and in the

evening at 9:00 p.m. which had to be answered within 90 minutes. This resulted in 12 queries per respondent across all measurement timepoints.

2.4 Questionnaires

2.4.1 Questionnaires at t1

At t1, a survey was administered online via SoSci Survey. This battery included demographic variables (age, gender etc.), university studies related questions (e.g., academic study time, leisure time, career aspirations), health (behavior) related variables (including height, weight, smoking, alcohol and drug consumption, acute and chronic somatic complaints, disease history and medication use). Furthermore, sleep disturbances were measured with the Regensburg Insomnia Scale (RIS, Crönlein et al., 2013), psychosomatic symptoms with the somatization items from the Symptom-Check-List (SCL-90-R, Franke and Stäcker, 1995), test anxiety with the test anxiety questionnaire (Prüfungsangstfragebogen (PAF), Hodapp et al., 2011), anxiety and depression symptoms with the Hospital Anxiety and Depression Scale (HADS, Herrmann-Lingen et al., 2011), chronic stress with the Trier Inventory of Chronic Stress (TICS, Schulz et al., 2004) and coping behavior with the Stress and Coping Inventory (SCI, Satow, 2012). To explore child maltreatment retrospectively, the Childhood Trauma Questionnaire (CTQ, Bernstein et al., 2003) was administered.

2.4.2 Trajectory questionnaire

To examine the participants' experience and behavior across the study, some of the questionnaires used at t1 were also applied at subsequent timepoints. Besides the university studies and health related questions, the RIS and the HADS were used at t2, t3, t5 and t6. To reduce the burden of the study protocol there was no assessment at t4. The TICS was administered at t2 and t5.

2.4.3 AA questionnaire

To measure momentary perceived stress, a five-items AA stress scale was used, consisting of the following items: 'I am under time pressure', 'I am relaxed', 'I am tense', 'I am overstrained' and 'I am disappointed with my performance'. The factor analyses applied to construct this scale based on an

original 18-item version are described in the supplements (see supplementary Methods section AA stress scale and Table S1). Additionally, in the first query after awakening, four items related to sleep (e.g., 'The quality of sleep last night was ... ') and stress anticipation (e.g., 'I am confident that I can cope well with today's tasks') were added (Powell and Schlotz, 2012). In the last query, six extra items were asked regarding events of the day (e.g., 'I had an argument with someone today').

2.5 Statistical analyses

2.5.1 University studies and health related variables

To assess the impact of exam preparation on the participants´ health and behavior, several university studies and health related variables measured over the 13-months period have been used. Analyses of Variances (ANOVAs) for repeated measures with the relevant variables as within-subject factors were calculated using IBM SPSS Statistics (Version 25, IBM, Corp., Armonk, New York, USA). Group (SG vs. CG) was added as between-subject factor, Greenhouse-Geisser corrections were applied where appropriate and only adjusted results are reported. The entire study sample (n = 452) was included in this analysis.

2.5.2 AA stress scale and the cortisol awakening response

As the CAR was not assessed in cohort B, the association between the CAR and the AA stress scale was examined in n = 204 participants (cohort A). We computed hierarchical models using R (version 4.0.3; R Core Team, 2020). The models were estimated with Maximum Likelihood and the significance level was set at $\alpha = .05$. The explained variance of the final models was calculated via conditional R squared for the overall explained variance and via marginal R squared for the variance explained by the fixed effects (Nakagawa and Schielzeth, 2013).

The time course of the AA stress scale was calculated using generalized linear mixed models computed with the package glmmTMB (Brooks et al., 2017). In this two-level model, the variable *group* (0 = CG, 1 = SG), the variable *timepoint* as linear, quadratic and cubic trend and the interactions between these time trends and *group* were included. AA values were clustered in participants, hence random

intercepts and slopes for timepoint by participants were estimated to account for dependencies in the data.

To test for alterations in the CAR, we computed three level linear mixed models with cortisol measurements (level 1) nested within timepoints (level 2), nested within participants (level 3). The packages nlme (Pinheiro et al., 2021) and MuMIn (Bartoń, 2013) were used for the analysis. We added random intercepts for both participants and timepoints as well as random slopes for minutes. The variable *timepoint* was entered as categorical variable and recoded, thus, model intercept parameters represented cortisol at the first timepoint. The CAR at the first level was modelled with the categorical variable *minutes* consisting of 0, 30 and 45 minutes after awakening. The final model contained the following fixed effects: *timepoint*, *group*, and the interactions *minutes x group*, *minutes x timepoint*, *group x timepoint*, and *minutes x timepoint x group* to test for differences between the two groups at the six timepoints (model 1). As covariates, we added the *hormonal status* (0 = women not using hormonal contraceptives, 1 = women using hormonal contraceptives and 2 = men), its interaction with *minutes* and the person mean centered variable *awakening time* and *awakening time x minutes* because these two variables were shown to have an impact on the CAR in our data (model 2).

To further investigate alterations in the SG, similar three level models only containing the SG were computed (SG.model). The predictors were added separately as main effects, in interaction with *minutes* and *timepoint* and as three-way interaction (*minutes x timepoint x predictor*) to test if the predictor had an influence on the alterations of the CAR. In total, we tested seven models, one for each of the predictors (AA stress scale over the time course, anxiety and depression symptoms, test anxiety, work overload, excessive demands from work and chronic worrying at t1). For the AA stress scale, we computed a mean value of the ten queries of the AA stress scale for the day of the CAR assessment, which was centered on the person mean. The other predictors were grand mean centered. To test for a possible influence on our findings, we also added *post-hoc* the self-report items ‘sleep duration’ and ‘sleep quality’, that were assessed on saliva sampling days as part of the AA morning questionnaire, to the SG.model.

Cortisol data was log-transformed to base 10. Seventeen cortisol values were excluded because of participants' nonadherence to the study protocol and physiologically implausible values (e.g., only one extremely high value within one CAR measurement). The residuals of the final models displayed satisfactory approximation to normal distribution.

3 Results

3.1 Demographic, university studies related and psychological variables

Demographic information of the sample is presented in Table 1. No differences between cohort A and B could be observed in the examined variables. Therefore, only results from the total sample are presented. None of the demographic variables differed significantly between the SG and the CG, except for age ($t(450) = -11.96$, $p < .001$), and none of the reported study related and psychological variables (anxiety, depression, etc., see below) differed significantly at baseline ($ps > .113$) except the subscale social tensions of the TICS ($t(450) = 1.92$, $p = .056$).

Regarding the self-report of academic study time in hours per week, significant differences between the SG and the CG over time were observed with a significant main effect for *timepoint* ($F[3.14, 1184.72] = 185.69$, $p < .001$, $\eta^2 = .33$), as well as a significant interaction *timepoint x group* ($F[3.14, 1184.72] = 164.33$, $p < .001$, $\eta^2 = .30$). For students in the SG, a rise in academic study time until t3 and a distinct decrease thereafter was found. The CG, in contrast, stayed relatively stable. In the last months prior exam, students in the SG indicated spending 49.12 ±14.90 hours per week with study related issues, while students in the CG indicated spending 34.98 ±14.19 hours per week.

Table 1. Demographic characteristics of the total sample, cohort A and cohort B.

	Total sample		Cohort A		Cohort B	
	Stress group	Control group	Stress group	Control group	Stress group	Control group
n	226	226	97	107	129	119
Age (Mean ± *SD*)	22.98*	21.04*	22.84* (±1.82)	20.95* (±1.93)	23.09* (±1.62)	21.11* (±1.59)

	(±1.71)	(±1.75)				
Women	*n* = 165 (73.0%)	*n* = 175 (77.4%)	*n* = 67 (69.1%)	*n* = 84 (78.5%)	*n* = 98 (76.0%)	*n* = 91 (76.5%)
Women using hormonal contraceptives	*n* = 105	*n* = 106	*n* = 49	*n* = 50	*n* = 56	*n* = 56
BMI (Mean ± *SD*)	22.22 (±3.10)	21.90 (±2.82)	22.37 (±2.67)	22.02 (±3.18)	22.10 (±3.39)	21.79 (±2.47)

Note. *SD* = standard deviation. Cohort A (*n* = 204) consisted mainly of law students from Regensburg; Cohort B (*n* = 248) consisted of law students from other Bavarian universities who completed a less elaborate study protocol. * marks significant differences between stress and control group.

We found a significant main effect for *timepoint* and a significant interaction *timepoint x group* for the variables anxiety and depression symptoms and sleep disturbances. In contrast to the CG, we observed a distinct and statistically significant increase in anxiety and depression symptoms as well as in sleep disturbances until t3 in the SG. All variables decreased after the exam to similar levels measured at t1 and in the CG (test statistics can be found in Table 2).

At baseline, 17.0% of the SG and 19.0% of the CG participants already exceeded the clinically relevant score of 11 for anxiety symptoms, which is consistent with previous findings in student cohorts (Bunevicius et al., 2008; Moreira de Sousa et al., 2018). At t3, this proportion reached 47.7% in the SG and decreased thereafter to the initial level. The same pattern was found for depression symptoms (cut-off ≥ 11) and sleep disturbances (cut-off ≥ 13). At t3, 19.2% exceeded the cut-off for depression symptoms (t1 = 3.0%) and 5.2% for sleep disturbances (t1 = 0.4%).

Regarding the different facets of chronic stress measured with the TICS, the scales work overload, work discontent, excessive demands from work, lack of social recognition, social tensions, social isolation and chronic worrying showed an increase in the SG compared to the CG (test statistics can be found in Table 2; figures can be found on https://doi.org/10.5283/epub.51920).

Table 2. Test statistics for repeated measures ANOVAs for stress related questionnaire variables.

		F	*p*	η^2
HADS				
Anxiety symptoms	timepoint	44.37	<.001	.10

	timepoint x group	33.06	<.001	.08
Depression symptoms	timepoint	44.67	<.001	.10
	timepoint x group	27.53	<.001	.07
RIS				
Sleeping problems	timepoint	20.23	<.001	.05
	timepoint x group	18.72	<.001	.05
TICS				
Work overload	timepoint	0.13	.875	.00
	timepoint x group	18.35	<.001	.04
Social overload	timepoint	6.93	.001	.02
	timepoint x group	0.82	.436	.00
Pressure to perform	timepoint	8.18	<.001	.02
	timepoint x group	2.50	.085	.01
Work discontent	timepoint	0.46	.620	.00
	timepoint x group	6.93	.001	.02
Excessive demands from work	timepoint	4.36	.014	.01
	timepoint x group	19.58	<.001	.05
Lack of social recognition	timepoint	0.44	.631	.00
	timepoint x group	3.37	.038	.01
Social tensions	timepoint	0.04	.947	.00
	timepoint x group	4.68	.011	.01
Social isolation	timepoint	5.48	.005	.01
	timepoint x group	4.22	.016	.01
Chronic worrying	timepoint	0.46	.624	.00
	timepoint x group	8.24	<.001	.02

3.2 Stress induced alterations in the AA stress scale and the cortisol awakening response

The most important self-report instrument of the present study was the AA stress scale. In cohort A, it was assessed in an extensive AA design with 100 queries per participant, while in cohort B we applied a less extensive design comprising only 12 queries for each participant. In the following, only the results for cohort A (*n* = 204) are presented.

On average, participants who completed at least the first timepoint, responded to 91.35 (±11.19) out of 100 queries. The model containing a cubic trajectory represented the best fit for the data (compared to the preceding model: linear model ΔAIC = 3751.34; quadratic model ΔAIC = 1209.52;

cubic model ΔAIC = 268.22). The trajectory of the perceived stress levels differed significantly between the CG and the SG (*timepoint x SG b* = .39, $p < .001$; *timepoint*^{2} *x SG b* = -.19, $p < .001$; *timepoint*^{3} *x SG b* = .02, $p < .001$). In the SG, mean perceived stress increased until the exam and showed a decline thereafter. The stress levels in the CG stayed relatively stable with just a slight linear increase (*timepoint b* = .05, $p < .001$) (see Figure 2 & supplementary Table S2). There was no significant difference between the two groups at t1 (*SG b* = .09, $p = .059$). Since the covariate *sex* showed no significant effect on perceived stress, the parameter was excluded from the final model. The overall explained variance of the final model was 65.1% and the variance explained by the fixed effects was 8.9%. It should be noted that perceived stress levels in cohort B were higher in both the stress and the control group over the entire study period, but the overall trajectories in SG and CG were very similar to those shown in Figure 2 (see supplementary Table S3 and S4).

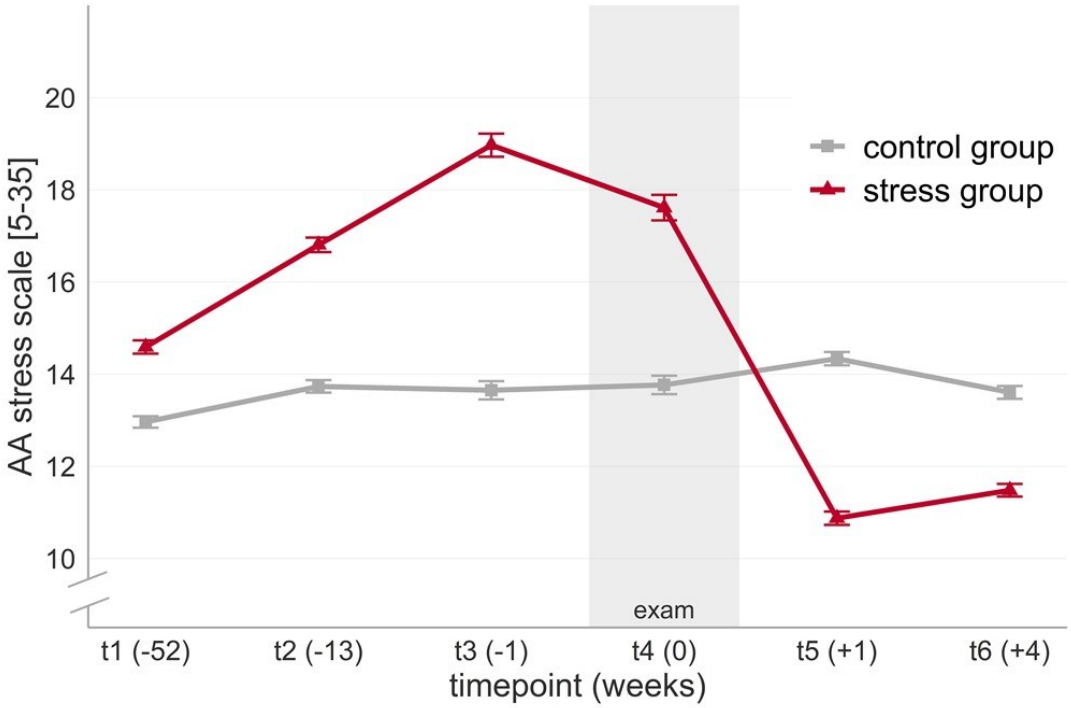

Figure 2. Time course of mean perceived stress levels (± SEM) in the stress group (SG) and the control group (CG) over the study period (cohort A).

The compliance rate regarding saliva sampling was rather high. Among participants who completed all timepoints, an average of 20.78 (±0.88) out of 21 saliva samples have been successfully collected resulting in 4009 observations.

A key hypothesis of the present study was the assumption of a decreased mean CAR over the 13-months period in the SG compared to the CG due to exam preparation. Our findings are consistent with this hypothesis. Due to a significant model improvement, the covariates *awakening time*, *hormonal status*, and their interaction with *minutes* (model 1 - model2: ΔAIC = 158.67) were included in the final model. We found a significant increase of cortisol after awakening (*0 min b* = .80, $p < .001$; *30 min b* = .35, $p < .001$; *45 min b* = .36, $p < .001$) with no difference between SG and CG at the first timepoint (*SG x min ps* ≥ .292). Compared to the CG, the SG showed significantly lower mean cortisol values 30 and 45 minutes after awakening during the exam (*SG x t4 x 30 min b* = -.07, $p = .041$; *SG x t4 x 45 min b* = -.10, $p = .004$) (see Figure 3 & Table 3). The overall explained variance of the model was 86.0%; 25.2% thereof could be explained by the fixed effects.

The models for further analysis within the SG comprised 97 students, 86 of whom completed the whole study protocol. Compared to the baseline measure at t1, lower cortisol concentrations during the exam at t4 (*t4 x 30 min b* = -.12, $p < .001$; *t4 x 45 min b* = -.16, $p < .001$) could be observed. At t2 at awakening and at t3, a trend for lower cortisol concentrations became visible (*t2 x 0 min b* = -.05, $p = .075$; *t3 x 30 min b* = -.05, $p = .073$; *t3 x 45 min b* = -.06, $p = .075$). The full output of the model can be found in Table S5 in the supplements. The overall explained variance for the full model was 84.0% and the variance explained by the fixed effects was 24.6%. The *post-hoc* tested variables *sleep duration* and *sleep quality* and their interaction with *minutes* and *timepoint* did not lead to an improvement of the model, so the significant CAR effect was not explained by concomitant changes in reported sleep behavior (*duration*: ΔAIC = -12.72; *quality*: ΔAIC = -19.68).

Table 3. Parameter estimates for overall effects for the final group model (model 2).

Fixed Effects	Estimate	Std. Error	*p*
Intercept	.80	0.03	**<.001**
30 min	.35	0.03	**<.001**

45 min	.36	0.03	**<.001**
SG	.02	0.04	.517
SG x 30 min	-.02	0.03	.415
SG x 45 min	-.04	0.04	.292
T2	-.01	0.03	.753
T3	-.01	0.03	.801
T4	-.01	0.03	.648
T5	-.07	0.03	**.023**
T6	-.10	0.03	**.001**
T2 x 30 min	-.01	0.03	.616
T2 x 45 min	-.04	0.03	.157
T3 x 30 min	.00	0.03	.918
T3 x 45 min	-.02	0.03	.480
T4 x 30 min	-.03	0.03	.250
T4 x 45 min	-.04	0.03	.188
T5 x 30 min	-.01	0.03	.638
T5 x 45 min	-.02	0.03	.594
T6 x 30 min	.02	0.03	.538
T6 x 45 min	.03	0.03	.432
SG x t2 x 0 min	-.04	0.04	.392
SG x t2 x 30 min	-.02	0.03	.625
SG x t2 x 45 min	.03	0.03	.450
SG x t3 x 0 min	.02	0.04	.703
SG x t3 x 30 min	-.04	0.03	.252
SG x t3 x 45 min	-.03	0.03	.385
SG x t4 x 0 min	.00	0.04	.960
SG x t4 x 30 min	-.07	0.03	**.041**
SG x t4 x 45 min	-.10	0.03	**.004**
SG x t5 x 0 min	.03	0.04	.498
SG x t5 x 30 min	.06	0.03	.085
SG x t5 x 45 min	.05	0.03	.106
SG x t6 x 0 min	.07	0.04	.109
SG x t6 x 30 min	.06	0.03	.060
SG x t6 x 45 min	.06	0.03	.092
Covariates			
Women using HC	.02	0.03	.541
Women using HC x 30 min	-.11	0.02	**<.001**
Women using HC x 45 min	-.10	0.03	**<.001**
Men	-.03	0.04	.465
Men x 30 min	-.07	0.03	**.007**
Men x 45 min	-.10	0.03	**.001**
Awakening time	.15	0.01	**<.001**
Awakening time x 30 min	-.12	0.02	**<.001**
Awakening time x 45 min	-.16	0.02	**<.001**

Random Effects	*SD*	Correlation	
		(Intercept)	30 min
Subject (Intercept)	0.17		
30 min	0.10	-.71	

45 min	0.13	-.76	1
Timepoint (Intercept)	0.20		
30 min	0.15	-.70	
45 min	0.19	-.74	1
Residual	0.10		

Note. Std.Error: Standard error; *SD*: Standard deviation; Min: Minutes after awakening; SG: Stress group; T: Timepoint; HC: Hormonal contraception.

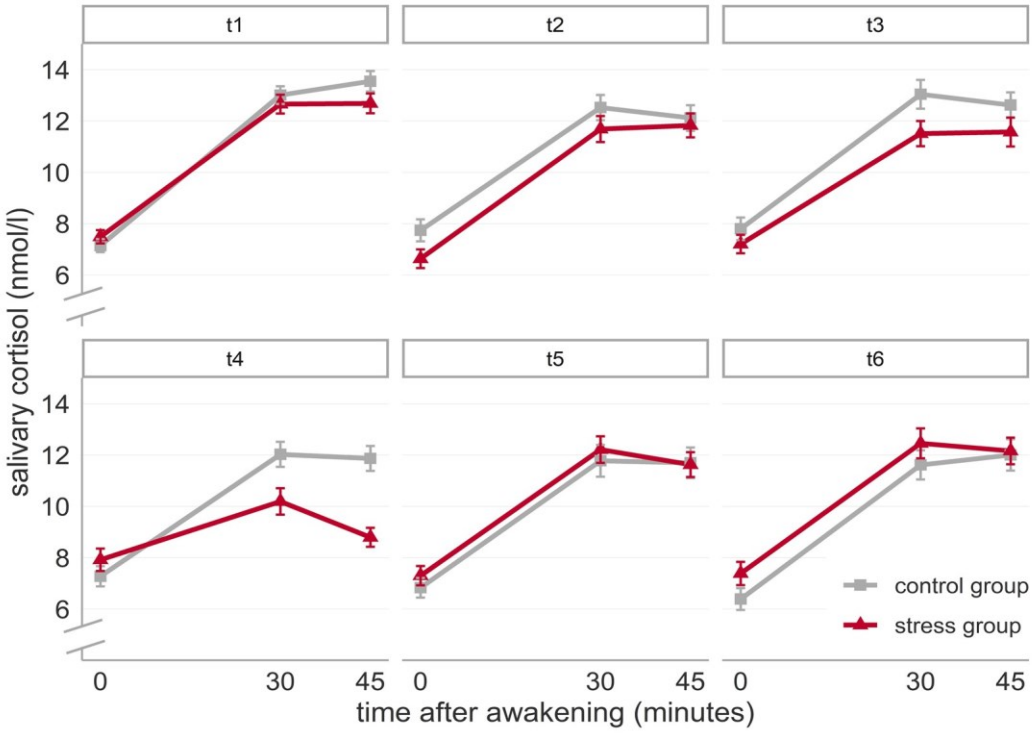

Figure 3. Mean cortisol values (± SEM) for the stress group (SG) and control group (CG) over the study period. *Note.* Timepoints: t1 (1 year before the exam), t2 (-3 months), t3 (-1 week), t4 (during exam in the stress group), t5 (+1 week), t6 (+1month).

3.3 Predictors of the alterations in the cortisol awakening response

Adding the stress scale to the SG.model on level 2 did not lead to a significant improvement (ΔAIC = -12.14). Thus, we failed to find an association between the cortisol awakening response and perceived stress. Furthermore, we could not detect significant associations between any of the predictors measured at t1, namely anxiety and depression symptoms, test anxiety as well as chronic stress (work overload, excessive demands at work and chronic worrying) and the alterations of the CAR (all ΔAICs < -9.65; for fit indices of the models see supplementary Table S6).

4 Discussion

In this report, we present the first results from the controlled prospective-longitudinal LawSTRESS project. Here, we examined the effects of the long-term preparation for and the exposure to the first state examination for German law students on perceived stress and the cortisol awakening response. The combination of a longitudinal design with a baseline measurement about one year prior exam offered the opportunity for a detailed analysis of the trajectories of different stress related variables including the CAR and their interrelations.

In the stress group, we found significant increases in self-reported anxiety and depression symptoms, sleep disturbances as well as regarding several facets of perceived chronic stress until the exam. Furthermore, perceived stress in everyday life – measured at high frequency with the AA stress scale – increased significantly until the examination period, whereas non-exam students stayed relatively stable. At closer inspection, a considerable number of participants could be identified who temporally clearly exceeded the cut-off levels for anxiety and depression. We also found clear evidence for a fast recovery. Mean anxiety, depression and stress levels as well as reported sleep disturbances returned to initial levels four weeks after the exam. These results confirm and expand previous findings and they highlight the impact of academic stress on students' health and well-being (Gonzalez-Cabrera et al., 2014; Koudela-Hamila et al., 2020).

Regarding cortisol regulation, a blunted CAR during the examination days (t4) in the SG compared to the baseline measure and to the CG could be observed. This effect is driven by lower cortisol concentrations 30 and 45 minutes after awakening and not by a higher awakening value as observed in other studies (Koudela-Hamila et al., 2020; Weik and Deinzer, 2010). It has to be noted that t4 data were assessed at the weekend between exam days and that a lower CAR on regular weekend days compared to regular weekdays has been previously reported (Schlotz et al., 2004). However, based on a pilot study (self-report in *n* = 197 law students from Regensburg), we concluded that in the final phase of the exam preparation and during the actual exam block a typical weekend-weekday rhythm

does not exist. Our finding that momentary stress levels at t4 were slightly lower than at t3 but still very high, supports this view (see Figure 2). Furthermore, our statistical model controlled for awakening time. Moreover, the significant effect at t4 was preceded by a trend for a reduced CAR at t2 and t3, suggesting a plausible development over time, peaking during the examination days. In our view, this apparent temporal trajectory provides further support for the assumption that the observed blunted CAR at t4 indeed is a valid finding. To date, studies investigating the influence of stress due to academic examinations on the CAR have not yielded consistent results. In the context of examination stress, enhanced morning cortisol responses (e.g., Hewig et al., 2008; Weik and Deinzer, 2010) as well as dampened cortisol levels after awakening (e.g., Duan et al., 2013; Koudela-Hamila et al., 2020) or even no change in the CAR (e.g., O'Flynn et al., 2018) have been reported. However, this partly contradictory results pattern can probably be explained by methodological differences, e.g., heterogenous samples or varying durations and intensities of the exam period. Law and Clow (2020) recently concluded that studies with convincing designs and reliable methods relatively consistently reported a decreased CAR to be linked to chronic stress. In their cross-sectional study in male students, Duan et al. (2013) observed a blunted CAR in timely proximity to an examination period compared to a control group. This effect was more pronounced in students with higher perceived stress levels. The CAR was assessed twice in a longitudinal study by Koudela-Hamila et al. (2020), once at the beginning of the semester and once at the end, one week prior the examination period. Heightened cortisol levels at awakening as well as reduced subsequent increases were found. Based on a longitudinal design, a real baseline measurement, a control group and a long stress period, our study could confirm these findings. Moreover, we also had the opportunity to collect saliva samples at two timepoints after the exam and, on average, we found a distinct and quick recovery of the CAR already one week after the exam. On the one hand, this trajectory is perfectly in line with those of our measurements of anxiety, depression and perceived stress. On the other hand, the velocity of this change that could be interpreted as indicator of a fast regeneration of cortisol regulation back to normal, is somewhat unexpected.

The finding of a blunted CAR in males and females shows indications for a down-regulation of the HPA axis and hypocortisolism due to chronic examination stress. Interestingly, we could not find a preceding hyperactivity of the HPA axis, as often proposed in the context of a developing hypocortisolism due to ongoing stress (Fries et al., 2005; Miller et al., 2007). However, evidence for this plausible model is scarce. Miller et al. (2007) conducted a meta-analysis mainly based on cross-sectional data and they showed an inverse association between cortisol and the time since stressor onset. Nevertheless, they highlighted the need for longitudinal studies and that the impact of chronic stress on the HPA axis activity seems to depend not only on the timing of the stressor but also on several different features of the stressor and characteristics of the person experiencing it (Boggero et al., 2017; Miller et al., 2007). We assume that, at least in our cohort, long-term examination stress triggered a temporary hypocortisolism. Apparently, HPA axis activity on average seemed to quickly return to baseline levels after the exam. Nevertheless, we suggest that a temporal hypocortisolism in a critical period might be of great psychobiological relevance, considering the numerous effects of cortisol on energy metabolism, mood, and immune function (Sapolsky et al., 2000). First results of a longitudinal study by McGregor et al. (2016) implicated an association between a flattened CAR due to university studies and a decrease in CD+19 lymphocytes. Further research is needed to examine possible effects of this short-term reduction in morning cortisol. In summary, we found that chronic examination stress in young and healthy students was related to a temporary reduction of the CAR, followed, on average, by a rapid recovery. Interestingly, this mean course of the CAR appears consistent with the mean trajectories of the measured psychometric variables.

While both, perceived stress assessed in everyday life as well as the CAR showed the *a priori* postulated changes over the measurement timepoints, they were not significantly associated. In general, a lack of consistent correlations between subjective stress experience and markers of cortisol regulation is a well-known phenomenon. Moreover, previous studies on the association between CAR measurements and self-reported perceived stress on the same day yielded inconsistent results (Pruessner et al., 2003; Weekes et al., 2008). Nevertheless, we hypothesized that a significant

association between perceived stress and the CAR, theoretically representing indicators of the same construct 'stress', might become visible in the present study as several features of our design presumably enhanced the validity of our measurements (extensive AA) and facilitated the emergence of within- as well as between-subject variability. The fact that we still failed to confirm this hypothesis is in line with a recent review by Schlotz (2019) concluding that the probability for the detection of a significant association between momentary stress and cortisol measures collected over the day increases when both variables are measured simultaneously or with only a short time delay to the stressor or daily hassle. Unfortunately, such an approach was not feasible in the present study. Consistent with the absence of a significant association between the AA stress scale and the CAR, stress related psychological dimensions assessed at baseline, namely anxiety and depression symptoms, test anxiety and perceived chronic stress, did not significantly predict the ascertained CAR effect.

In our view, our study has several strengths, but it surely also has some limitations that need to be considered. First, our participants were young, healthy students with presumably above-average intelligence and socioeconomic status compared to the general population. Therefore, while our cohort was suitable to specifically study academic stress, a generalization of our findings to the general population may be less valid. Secondly, we cannot rule out a certain selection bias as we found that compared to the Bavarian average, our sample achieved better grades in the state examination. Also, the failure rate was higher in Bavaria (24% - 30% in 2019 and 2020) than in our sample (13.1%). It thus appears possible, that particularly less excellent students tended to expect a very stressful exam preparation period and consequently did not accept the extra burden related to participation in our study. Therefore, our findings may underestimate the general stress load related to the first state examination for German law students to a certain extent. However, 32% of the participants did not disclose the exam grade they had received a few months after the last measurement timepoint. Therefore, we certainly cannot rule out that this subgroup on average got lower grades, which, in part, could also explain the difference between the Bavarian average and our sample. Thirdly, our control

group was very conservatively chosen. Although the participants were not preparing for the first state examination, they did experience 'usual' university studies related strain, including minor exams. Finally, to increase the CAR assessment quality, we applied several methods (electronic monitoring devices, random codes, encouragement to report non-compliance). Unfortunately, a reliable technique to verify the exact awakening time was not available in the present study and we cannot rule out that this limitation had a confounding effect to a certain extent. However, at least a group-specific effect of this potential confounder appears unlikely as a delay between awakening and collecting the first sample should result in erroneously high cortisol levels at awakening. This was not observed in our study (see Figure 3).

In conclusion, we were able to assess psychological stress trajectories over 13 months in law students preparing for a major exam and in a control group. A significant increase of perceived stress, anxiety and depression symptoms could be documented and the number of participants showing temporally anxiety and depression scores well-above the clinically relevant cut-off scores appears alarming. These stress related psychological changes were paralleled by the stepwise development of a blunted CAR, although within participants psychological stress and the CAR were not significantly associated. Fortunately, mean psychological stress levels as well as mean cortisol awakening responses normalized briefly after the exam, suggesting a quick and distinct recovery. It appears conceivable that successfully undergoing this demanding period may improve the individual stress coping strategy and capacity. On the other hand, we certainly cannot rule out that the experience of this exceptionally long stress period may also have a sensitizing effect on psychobiological responses to future stress exposures in vulnerable individuals.

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