Mannheimer sozialwissenschaftliche Abschlussarbeiten, Nr. 001/2007

SALIVA CORTISOL PROFILES IN FIELD RESEARCH: INTERNAL STRUCTURE, CONFOUNDING FACTORS, QUANTIFICATION, AND STABILITY

Inauguraldissertation
zur Erlangung des akademischen Grades eines
Doktors der Sozialwissenschaften
der Universität Mannheim

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Tag der Disputation: 31. Januar 2007

Schriftenreihe: Mannheimer sozialwissenschaftliche Abschlussarbeiten

Vorwort

Die Fakultät für Sozialwissenschaften der Universität Mannheim bildet in den Fächern Politikwissenschaft, Soziologie, Psychologie und Erziehungswissenschaft qualifizierten Forschungs- und Führungsnachwuchs aus. Viele Abschlussarbeiten der Studierenden zeugen von dem hohen wissenschaftlichen Niveau der Mannheimer Ausbildungsinhalte, die insbesondere gekennzeichnet sind von der empirisch-analytischen Ausrichtung unter Betonung quantitativer Methoden.

Die Ergebnisse und Inhalte vieler dieser Arbeiten sind publikationswürdig. Aus diesem Grund bietet die Fakultät für Sozialwissenschaften ihren besten Absolventen die Möglichkeit, ihre Arbeiten einem breiteren Publikum zu präsentieren und hat hierfür diese Schriftenreihe ins Leben gerufen. Diese Schriftenreihe soll dazu beitragen, die wissenschaftlichen Ergebnisse der besten Abschlussarbeiten dem Fachpublikum zugänglich zu machen. Damit sind sie für weitere Untersuchungen verfügbar und können eventuell eine Grundlage für weitere Forschungen bieten.

In dieser Reihe werden nur Abschlussarbeiten veröffentlicht, die von beiden Gutachtern mit „sehr gut“ bewertet und für veröffentlichungswürdig befunden wurden.

Prof. Dr. Josef Brüderl
Dekan der Fakultät für Sozialwissenschaften

Acknowledgements

The present study represents my doctoral thesis submitted to the faculty of social sciences (department of psychology) at the University of Mannheim, Germany.

The data of the present study were collected within the scope of the research project *Individual Combinations of Psychological and Mental Risk Factors at Work* (*Untersuchung zur individuellen Risikokonstellation psychischer und mentaler Faktoren am Arbeitsplatz*; head of project: Dr. med. Erich Marquard, BAD-Preventive Health Care and Safety Engineering, Eppelheim, Germany) funded by Hauptverband der gewerblichen Berufsgenossenschaften and Rheinischer Gemeindeunfallversicherungsverband (GUVV).

I am deeply indebted to my supervisor Prof. Dr. Rupert Hölzl who supported my work with valuable ideas and critical comments. He has encouraged the development of my research skills from the beginning of my studies when I started working with him as an undergraduate student. In addition, he has provided an excellent infrastructure and a pleasant working atmosphere.

Furthermore, I thank Dr. Erich Marquard (BAD Eppelheim, Germany) for generously yielding the data of his former research project to our disposal and Dr. Viola Weber (formerly BAD Eppelheim, Germany) for preparing and conducting the data collection.

My thanks also go to all my colleagues at the Laboratory of Clinical Psychophysiology at the Otto-Selz-Institute, University of Mannheim, in particular to Dipl.-Psych. Dagmar Baus not only for helpful discussions, but also for her encouragement and support. I would also like to particularly thank Dipl.-Psych. Judith Leinweber for her support and, last but not least, I would like to thank my student assistants Aline Bosle, Karin Alles and Johanna Möller for their assistance in literature research and preparation of data.

I gratefully acknowledge additional financial support from Berufsgenossenschaft Nahrungsmittel und Gaststätten, BGN.

Alexandra A. Bernhardt, October 2006

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Abbreviations

ACTH	adrenocorticotropic hormone
ADS	Allgemeine Depressionsskala (Hautzinger & Bailer, 1995)
ANOVA	analysis of variance
AUC	area under the curve
BAD	BAD-Preventive Health Care and Safety Engineering, Germany
BMI	body mass index: weight (kg) / height squared (m^2)
BMS	between-subject mean square (between-subject variance)
CAR	cortisol awakening rise
CBG	corticosteroid-binding globulin
CES-D	Center for Epidemiological Studies Depression Scale (Radloff, 1977)
CRH	corticotropin-releasing hormone
GBB	Giessener Beschwerdebogen (Braehler & Scheer, 1995)
HDL	high density lipoprotein
HPA	hypothalamic-pituitary-adrenal
ICC	intraclass correlation coefficient
IQR	interquartile range (25^{th} – 75^{th} percentile)
KAB	Kurzfragebogen zur aktuellen Beanspruchung (Mueller & Basler, 1993)
ln	logarithmus naturalis
MBI	Maslach Burnout Inventory (Maslach & Jackson, 1986)
MDBF	Mehrdimensionaler Befindlichkeitsfragebogen (Steyer et al., 1997)
MDD	major depressive disorder
OC	oral contraceptives
PTSD	post-traumatic stress disorder
PVN	paraventricular nucleus
Q1	first quartile; 25^{th} percentile
Q2	second quartile; median; 50^{th} percentile

Q3	third quartile; 75th percentile
SAM	sympathetic-adrenal-medullary
SCN	suprachiasmatic nucleus
SD	standard deviation
STAI-T	State-Trait Anxiety Inventory (Spielberger et al., 1980)
SWE	Generalized Self-Efficacy Scale (Schwarzer & Jerusalem, 1995)
t(+0)	sampling time: directly after awakening
t(+30)	sampling time: 30 minutes after awakening
t(08:00)	sampling time: 8 a.m.
t(11:00)	sampling time: 11 a.m.
t(15:00)	sampling time: 3 p.m.
t(16:00)	sampling time: 4 p.m.
t(20:00)	sampling time: 8 p.m.
WHR	waist-to-hip ratio: waist circumference (cm) / hip circumference (cm)
WMS	within-subject mean square (within-subject variance)

1. Introduction

Particularly the last two decades of research on hypothalamus-pituitary-adrenal (HPA) axis activity have established salivary cortisol as a prevalent biological marker of stress (Kirschbaum & Hellhammer, 1989, 1994). In numerous experimental studies the HPA-axis has shown to respond sensitively to external stimulation, i.e. acute stressors inducing a significant increase in salivary cortisol secretion. A recent meta-analysis by Dickerson and Kemeny (2004) that reviewed 208 laboratory studies employing acute psychosocial stressors provided cogent evidence that psychosocial stressors can reliably activate the HPA-axis, in particular when stressor tasks incorporate social-evaluative threats and outcome uncontrollability, as does e.g. the Trier Social Stress Test (TSST; Kirschbaum et al., 1993).

1.1 Cortisol as a biomarker of stress

The neuroendocrine system plays a prominent role in the transmission of stress signals to functional systems, such as circulation, metabolism, immune defence, respiration, and digestion, resulting from the coupling of neuronal and hormonal networks. Two major signal pathways are involved in physiologic stress responses: the *sympathetic-adrenal-medullary (SAM) axis* controls acute responses to stress, releasing the catecholamines adrenaline and noradrenaline from the adrenal medulla. The SAM system is commonly referred to as the fight or flight response; it provides a rapidly responding mechanism to mobilize the body's energy resources, thus primarily controlling activation processes (Carlson, 2004).

The endocrine axis which is mainly involved in the long-term stress response is known as the *hypothalamic-pituitary-adrenal (HPA) axis* involving a cascade of physiologic responses: on perception of stressors, parvocellular neurons in the paraventricular nucleus (PVN) of the hypothalamus are stimulated to synthesise and release corticotropin-releasing hormone (CRH) which in turn binds on CRH-receptors of corticotropic cells of the anterior pituitary triggering the secretion of adrenocorticotropic hormone (ACTH). CRH is released from neurosecretory nerve terminals at the median eminence of the hypothalamus and transported to the anterior pituitary through the hypothalamic capillaries of the portal blood vessel system of the anterior pituitary. ACTH in turn is transported via blood circula-

tion to the cortex of the adrenal gland where it stimulates the biosynthesis and secretion of glucocorticoids, the primary one being cortisol released from the zona fasciculata of the adrenal cortex. The circulating glucocorticoids exert a number of metabolic (i.e. largely catabolic) and immune-modulating effects to ensure the organism's adaptation to critical conditions. Negative feedback loops to the pituitary gland and the PVN of the hypothalamus ensure that cortisol secretion stays within certain limits. The circadian cortisol release is controlled by the suprachiasmatic nucleus (SCN) of the hypothalamus, which also provides the primary control over the timing of sleep cycles. Neuronal signals from the SCN stimulate the PVN of the hypothalamus to release pulses of CRH, resulting in HPA axis activation and, finally, in cortisol release which displays a marked circadian rhythm with pronounced activity in the morning and a marked decrease in activity throughout the remainder of the day until reaching a nadir in the middle of the night (Carlson, 2004; Ehlert & Hellhammer, 2000; Kirschbaum, 1991; Sapolsky et al., 2000; Schmidt & Thews, 2004).

Cortisol as the main glucocorticoid hormone in humans is released both spontaneously and in response to psychosocial stimuli and biochemical agents. Mineralocorticoid receptors in hippocampal neurons are reported to control the regulation of circadian rhythms of HPA-axis activity, whereas stress responses seem to be mediated by glucocorticoid receptors (de Kloet et al., 1998). While merely 5-10% of cortisol circulates unbound ("free"), roughly 90% of the glucocorticoid cortisol is bound to blood-borne carriers, i.e. corticosteroid-binding globulin (CBG) and albumin. The unbound fraction of cortisol is assumed to be the biologically active one. Due to its small size (molecular weight 362) and since cortisol is a highly lipid soluble molecule, the unbound fraction rapidly diffuses through lipid-rich cell membranes via passive intracellular diffusion. Thus, free cortisol appears in all bodily fluids and, most important for research methods, accurately reflects the amount of free cortisol in blood (Kirschbaum & Hellhammer, 1989, 1994). Hence, saliva sampling is a non-invasive method easily to employ within the scope of field studies to obtain an index of the biologically active fraction of cortisol.

Early theories by Cannon and Selye have conceptualised stress as a response to threats to the homeostasis resulting in an adaptive response (Goldstein & McEwen, 2002). However, the concept of homeostasis cannot link such responses to long-term effects of negative

health outcomes. Moreover, since body functions such as blood pressure, heart rate, endocrine output, and neural activity are in fact adaptive processes that adjust continually to environmental stimuli, homeostasis cannot explain these processes adequately. Sterling and Eyer (1988) have introduced the term allostasis to describe the operating range of these adaptive processes. McEwen and Stellar (1993) defined allostasis as the process that actively sustains homeostasis and have extended the concept incorporating temporal aspects: allostatic load denotes the cost of the body for its repeated adaptation in the long run. While the adaptive process, allostasis, has protective effects in the short run, long-term exposure to stressors and resulting increased release of stress hormones can result in allostatic load. McEwen (1998) describes four situations that can lead to allostatic load, i.e. four response patterns that describe how mediators of allostasis give way to the so-called *wear and tear* of the involved systems: a) multiple stressors over a long period of time eliciting repeated increases in stress mediators, b) lack of adaptation of hormonal stress responses to the same stressor resulting in overexposure to stress mediators, c) a prolonged stress response without the usual recovery or with delayed recovery, and d) an inadequate response that leads to compensatory hyperactivity of other mediators (McEwen, 1998). Thus, stress responses are subject to a paradox: while under critical conditions physiological stress responses indeed have a protective effect (i.e. allostasis), yet when activated chronically they can have a damaging effect in terms of disease susceptibility (i.e. allostatic load). The benefit of this new conceptualization consists in the prospect to link the progression of pathophysiology from primary mediators via secondary outcomes to actual disease. Within this framework at present, primary mediators are cortisol, adrenaline, noradrenalin and DHEA (McEwen & Seeman, 1999). Primary effects (to date not measured within this framework) are organ- and tissue-specific effects that are regulated as a part of allostasis by primary mediators. Secondary outcomes denote integrated processes that reflect the cumulative outcome of the primary effects such as abnormal metabolism and the risk for cardiovascular disease (parameters: waist-to-hip ratio, blood pressure, gylcosylated haemoglobin, cholesterol/HDL ratio; McEwen & Seeman, 1999). Tertiary outcomes are the actual diseases or disorders resulting from allostatic load. So far, cardiovascular disease, decreased physical capacity, and severe cognitive decline have been studied as outcomes in associated studies (McEwen & Seeman, 1999); however, to refine the conceptualization of allostatic load more tertiary outcomes

need to be considered along with other disease-specific primary effects and secondary outcomes.

1.2 Impact of confounding factors on salivary cortisol levels

The central issue in investigating HPA-axis functioning within the scope of field studies is to determine the relevance of the salivary cortisol response and its various quantifications as a reliable psychobiological indicator of stress. However, methodological standards regarding sampling protocols and the quantification of cortisol output are inconsistent across studies, which make it rather difficult to compare results from different studies. A recent review of literature by Hjortskov et al. (2004) revealed insufficient evidence for the association between self-reported mental stress and salivary cortisol responses in field studies which was largely due to the considerable diversity in study designs, the types and measures of mental stress, and the various saliva sampling strategies.

Given the large interindividual variation regarding cortisol levels, various variables or confounding factors doubtlessly account for these differences. A multitude of studies within the last years have endeavoured to unravel these differences. The most important confounding factors within the scope of field studies that are liable for the large variation in cortisol levels are outlined in the following sections.

Adherence to sampling protocols. One major problem in field studies involving data collection by the participants themselves is the ostensible loss of control over the participants' adherence to stipulated sampling regimes. Psychoendocrine studies employing saliva sampling within the participants' domestic setting have to rely upon the participants' voluntary adherence to the stipulated sampling protocol.

Adherence to a medication regime is a common problem to health care providers. Several measures of adherence are traditionally employed which can be classified into direct and indirect methods: direct methods involve e.g. directly observing the intake of medication or the measurement of the medication's metabolites in the blood, while indirect methods involve the patients' self-reports, pill counts and electronic medication monitors. However, indirect methods do not document whether the patient actually ingested the correct medication or the correct dose (Osterberg & Blaschke, 2005).

The same problem arises with the collection of saliva samples in field studies. One issue in this context is the participants' failure to provide samples, i.e. the amount of missing samples. However, most important in endocrine research is the correct timing of sampling, i.e. the adherence to a strictly timed sampling protocol. Particularly in the morning, i.e. in the post-awakening period, cortisol levels increase rapidly and decrease rather quickly within the first 60 minutes after awakening (Wuest et al., 2000). Thus, in order to reliably estimate the size of the cortisol response subsequent to awakening, adherence to stipulated sampling times is reported to be of primary importance: Kudielka et al. (2003) investigated ambulatory saliva collections in 47 healthy participants. Electronic monitoring devices were given to the participants; one half of the participants were informed about the monitoring, while the other half remained ignorant. A significant number of subjects failed to reliably collect saliva samples in the ambulatory setting and a clear impact of compliance was observed on salivary cortisol levels: while compliant participants displayed a robust increase in cortisol in the post-awakening period, this could not be observed in non-compliant participants, resulting in a flatter diurnal decline in cortisol. Informed participants adhered more closely to the stipulated sampling times than noninformed participants. With respect to self-reported compliance significant differences emerged between informed and noninformed participants, with significantly higher self-reported sampling accuracy than objectified compliance in the noninformed group. A subsequent associated study by Broderick et al. (2004) that compared patients and healthy volunteers – both groups randomised to aware and unaware conditions regarding objective monitoring – obtained comparable results: the aware participants' objective compliance was consistent with their self-reported compliance, yet significantly higher than the unaware participants' objective compliance, which in turn was significantly lower than their self-reported compliance. Patients are reported to be slightly more compliant than healthy volunteers. Thus, self-reported compliance in participants that were unaware of being monitored substantially overestimates the actual adherence accuracy. While compliant participants displayed the expected rise in cortisol after awakening, no such rise was observed in the non-compliant sample, resulting in a flatter diurnal decrease in cortisol.

Awakening time. Among other functions, the suprachiasmatic nucleus (SCN) controls sleep cycles and hormone secretion (Carlson, 2004). Given the interrelation of HPA-axis activity and the control of awakening, i.e. elevations in plasma ACTH and cortisol levels after awakening (Born et al., 1999), the time of awakening may affect salivary cortisol levels. Empirical evidence regarding the effect of awakening time on salivary cortisol levels is rather inconsistent: a multitude of studies failed to find significant associations between awakening time and salivary cortisol levels (e.g. Brooke-Wavell et al., 2002; Kunz-Ebrecht et al., 2004b; Pruessner et al., 1997; Wuest et al., 2000), while other studies reported significant elevations in post-awakening cortisol levels in early awakeners compared to late awakeners (e.g. Edwards et al., 2001b; Federenko et al., 2004; Kudielka & Kirschbaum, 2003; Schlotz et al., 2004). Hucklebridge et al. (2000) compared post-awakening cortisol levels after normal and nocturnal awakening (i.e. four hours prior to habitual awakening). While cortisol levels consistently increased after awakening independent of the awakening condition, lower overall cortisol levels were observed in nocturnal compared to normal awakening. Thus, awakening per se seems to be a potent stimulus to trigger cortisol release, but it remains ambiguous whether the time of awakening is significantly associated with salivary cortisol levels.

Sleep: duration and quality. The characteristic sleep-associated cortisol profile consists of very low cortisol concentrations during the first hours of sleep which is dominated by slow wave sleep and a subsequent nocturnal increase in cortisol during the second half of the night (Friess et al., 1995). Complete sleep deprivation is reported to result in a discrete increase in nocturnal plasma cortisol, i.e. an elevation of the nocturnal nadir, and a significant rise in post-awakening plasma cortisol levels compared to undisturbed sleep (Schultes & Fehm, 2004; Spaeth-Schwalbe et al., 1991). Patients with primary insomnia are reported to display blunted salivary cortisol levels subsequent to awakening (Backhaus et al., 2004). Several studies investigating salivary cortisol levels in ambulatory setting failed to find significant associations between sleep duration, i.e. total hours slept, and cortisol levels after awakening (Kirschbaum et al., 2005; Pruessner et al., 1997; Wuest et al., 2000). With regard to the quality of sleep, a pilot study by Waye et al. (2003) revealed significantly blunted salivary cortisol levels within the post-awakening period following nocturnal exposure to

low frequency noise. In addition, impaired subjective sleep quality due to exposure to traffic noise also resulted in significantly blunted cortisol levels in the post-awakening period.

Gender differences. Gender differences in basal salivary cortisol levels are hardly ever found: no gender differences in the cortisol awakening response (measured in 15-minutes intervals within the first hour of the post-awakening period) were found in a study by Kudielka and Kirschbaum (2003) who also failed to find significant differences among women with respect to the menstrual cycle phase (follicular vs. luteal phase). An earlier study by Kirschbaum et al. (1999) also failed to reveal significant gender differences in basal salivary cortisol levels regarding the cortisol awakening response as well as basal diurnal cortisol profiles. Investigating the cortisol awakening response on work days and weekends in a subsample of the Whitehall II cohort (Marmot et al., 1991), Kunz-Ebrecht et al. (2004b) revealed that cortisol levels directly after awakening were comparable among men and women, yet women displayed a considerably larger cortisol awakening rise on work days, but not on weekends. No significant gender differences with respect to the diurnal salivary cortisol pattern were found in a study by Schulz and Merck (1997) who investigated basal salivary cortisol profiles in laboratory employees; however women were reported to display slightly higher levels directly before work and lower levels at about noon, resulting in a steeper decline form morning to midday.

In contrast to basal salivary cortisol levels significant gender differences can be found in stress-related HPA-axis responses to psychosocial stressors. In their comprehensive review on gender differences in HPA-axis responses to stressors Kudielka and Kirschbaum (2005) summarise results of a multitude of studies, revealing that in contrast to animal studies, which have shown consistently that glucocorticoid levels in males were lower than in females after HPA-axis stimulation, empirical evidence regarding gender differences in cortisol responses to stressors in humans is less consistent: while some studies failed to reveal gender differences in HPA-axis responses to psychosocial stressors, most studies reported higher cortisol responses in young men compared to young women subsequent to either acute real-life psychological stress or controlled laboratory stress tests. ACTH and cortisol levels were reported to be twice as high in men compared to women and anticipation of the impending psychosocial stress tasks resulted in significantly elevated cortisol levels in men

only. ACTH responses are generally higher in men than in women irrespective of menstrual cycle phase and intake of oral contraceptives. Regarding salivary cortisol responses, levels in women in the luteal phase were comparable to those of men, but significantly higher than those of women in the follicular phase or in women taking oral contraceptives. Thus, it is imperative to distinguish between the total cortisol secretion and the free, unbound cortisol in saliva (Kudielka & Kirschbaum, 2005). For this reason, the study group of Kirschbaum and co-workers reanalysed HPA-axis responses to psychosocial stress tasks of five independent studies conducted at their laboratory including only healthy, unmedicated participants (n= 102, age: 9 – 76 years; Kudielka et al., 2004). Regardless of age and gender, significant HPA-axis responses were observed in all participants. Salivary cortisol responses in elderly men were higher than in elderly women only, no gender differences could be found in younger participants. Regardless of gender, ACTH responses were reported to be higher in younger than in elder participants, yet most markedly in younger men. Thus, ACTH and plasma cortisol responses seemed to be decreasing with age in men, resulting in similar ACTH responses in elderly women and men (Kudielka & Kirschbaum, 2005).

Kirschbaum and Hellhammer (1989) concluded in their review on salivary cortisol that unstipulated, basal cortisol levels are usually unaffected by the use of oral contraceptives in women, which was replicated in several other studies (e.g. Kirschbaum et al., 1995, 1999), apart from Pruessner et al. (1999) who found overall lower levels in salivary awakening cortisol in women taking oral contraceptives in a study examining the cortisol awakening response in teachers. Experimental studies that employed psychosocial stressor tasks consistently found lower salivary cortisol responses in women using oral contraceptives (e.g. Kirschbaum et al., 1995, 1999; Kudielka & Kirschbaum, 2005), thus providing empirical evidence for blunted HPA-axis responses in women taking oral contraceptives, while basal, unstimulated salivary cortisol levels were unaffected.

Age-related differences. According to the glucocorticoid cascade hypothesis introduced by Sapolsky et al. (1986) age related changes in HPA-axis functioning result from structural hippocampal alterations. In animal studies, increasing age and exposure to chronic stressors lead to a blunted negative feedback function of the hippocampus which in turn results in heightened basal hormone levels and HPA-axis responses to stressors. Due to the resulting

diminution of hippocampal glucocorticoid receptors (receptor down-regulation) glucocorticoid feedback on the hippocampal level continues to be impaired. Deteriorating HPA-axis functioning with increasing age may be a result of this vicious circle initiated by these processes. Ferrari et al. (2001) indeed found a significant age-related increase in nocturnal serum cortisol levels in physiological aging and even more distinct in senile dementia (participants' age above 69 years) compared to young controls (19-43 years of age). This marked elevation of cortisol nadir levels was also observed by Raff et al. (1999) with respect to salivary cortisol levels in elderly men and women (above 70 years of age) and by Nicholson et al. (1997). Elevated cortisol levels in aging are reported to be associated with deteriorating memory in old age (Wolf & Kirschbaum, 2003; Wolf et al., 2005) and are presumed to be involved in the aetiology of sleep disorders in the elderly (van Cauter et al., 1996). Lupien et al. (1996), however, concluded from results of a six-year longitudinal study investigating basal serum (total and free) cortisol levels in healthy elderly that age was unrelated to cortisol levels and to the pattern of change over the years. Kudielka et al. (1999) failed to disclose age-related differences in basal salivary cortisol levels of the post-awakening period comparing post-menopausal to young women. In addition, comparing healthy elderly and young men revealed similar salivary morning cortisol profiles in the post-awakening period as well as comparable HPA-axis responses when exposed to psychosocial stressor tasks (Kudielka et al., 2000). A more recent study by Kudielka and Kirschbaum (2003), however, found a weak positive correlation between age and the cortisol awakening response.

Work-related issues. The modern work environment is presumed to contribute substantially to stress responses and negative health outcomes (e.g. Cox et al., 2004; Lundberg, 2000; Siegrist, 2005). A major problem in this context is the issue of objectifying work-related stress in terms of physiological responses. Shift work serves as a model in the attempt to objectify physiological reactions to stressors, since shift work substantially interferes with the circadian rhythms due to the mismatch between the external environment and the internal biological clock (Goh et al., 2000). A marked reversal of the circadian rhythm with higher evening than morning salivary cortisol levels was observed in nurses after their fifth night of night shifts, while undisturbed circadian cortisol rhythms were found in early shifts. Moreover, a subgroup of non-adapters, i.e. intolerant to shift work, was identified

(Hennig et al., 1998). Circadian disturbances of HPA-axis functioning due to shift work were found in several other studies as well (e.g. Goh et al., 2000; Leese et al., 1996; Motohashi, 1992; Munakata et al., 2001). However, when the pattern of light and darkness exposure was controlled, physiological rhythms could successfully be adapted to night shift work (James et al., 2004).

The Whitehall II study (Marmot et al., 1991) of British civil servants is a large-scale longitudinal study examining the influences on health circumstances at work, at home and in the wider community with particular emphasis on health inequalities linked to social positioning. The Whitehall II study has yielded a series of studies assessing psychobiological correlates of work-related stress:

Basal salivary cortisol levels in relation to work-related stress were investigated in a subsample of the Whitehall II cohort (Kunz-Ebrecht et al., 2004a). Perceived job demands and perceived job control following the demand/control model (Karasek & Theorell, 1990) were used as a measure for work-related stress. The study revealed that salivary cortisol responses to awakening were affected by job demands but not by job control, yet attenuated by higher socio-economic status. Over the remainder of the day high job demands led to significant increases in average diurnal cortisol levels in women with lower socio-economic status, while in men low job control was related to elevated cortisol levels throughout the day. Thus, men seem to be particularly vulnerable to effects of low job control, while women with lower socio-economic status are affected by high job demands.

Another study on the Whitehall II cohort (Kunz-Ebrecht et al., 2004a) revealed that the cortisol awakening response, i.e. the rise in salivary cortisol within 30 minutes after awakening from baseline levels, was greater on work days than weekends; on work days the cortisol awakening response was considerably larger in women compared to men, while gender differences were not observed on weekends. Thus, the cortisol awakening response seems to be sensitive to the influence of work-related stress and presumably its anticipation. These results are in line with other studies that investigated the association of HPA-axis functioning with work-related stress: elevated salivary cortisol levels in the morning are reported to be associated with high workload (Lundberg & Hellstroem, 2002; Schulz et al., 1998), work-

related burnout and perceived stress in teachers (Pruessner et al., 1999), and with high job strain and anger expression (Steptoe et al., 2000).

The effort-reward imbalance model (Siegrist, 1996) based on the reciprocity of work contracts was tested in yet another Whitehall II study to analyse psychobiological correlates of perceived work-related stressors (Steptoe et al., 2004). Overcommitment was significantly associated with chronic cardiovascular and neuroendocrine activation (i.e. elevated salivary cortisol levels after awakening and for the remainder of the day) in particularly in men and may thus mediate in part the impact of work-related stress on cardiovascular disease risk in men, since increased HPA-axis activation has been shown to influence cardiovascular processes in experimental studies (Steptoe et al., 2004).

Stress-related disorders. In the last years, behavioural medicine has placed particular emphasis on the impact of stress on health and illness, linking psychosocial factors to specific disease processes that are involved in the aetiology and maintenance of disorders and illnesses (e.g. Kudielka & Kirschbaum, 2001; Nater et al., 2006; Tsigos & Chrousos, 2002).

A large number of stress-related disorders are reported to be associated with blunted cortisol levels, i.e. at least in subgroups of patients, consequently raising the question whether hypocortisolism plays a decisive part in the development of stress-related pathology, in particular whether hypocortisolism is a neurobiological mediator between stress and the manifestation of physical complaints (Heim et al., 2000). Attenuated basal cortisol levels and blunted cortisol stress responses have been observed in chronic fatigue syndrome (Gur et al., 2004; Jerjes et al., 2005; Roberts et al., 2004), in pain-related syndromes, such as chronic pelvic pain (Heim et al., 1998), fibromyalgia (Griep et al., 1998; Gur et al., 2004), and rheumatoid arthritis (Catley et al., 2000; Dekkers et al., 2000a, 2000b), in post-traumatic stress disorder (Heim et al., 1998, 2000; Lauc et al., 2004; Rohleder et al., 2004; Wessa et al., 2006; Yehuda et al., 1993), irritable bowel syndrome (Boehmelt et al., 2005), allergies and asthma (Agarwal & Marshall, 2001; Buske-Kirschbaum et al., 2002, 2003), in burnout (Pruessner et al., 1999), and in metabolic syndrome and abdominal obesity (Rosmond et al., 1998). These dysregulations of HPA-axis functioning are determined by reduced biosynthesis or release of CRH from the hypothalamus, ACTH from the pituitary and cortisol from the adrenal glands, by an enhanced sensitivity to negative feedback of glucocorticoids, reduced avail-

ability of unbound cortisol, and thus decreased effects of cortisol on target tissue (Fries et al., 2005; Heim et al., 2000). A developmental model is introduced by Fries et al. (2005) based on findings by Hellhammer and Wade (1993) and the concept of allostatic load (e.g. McEwen, 1998; McEwen & Stellar, 1993): chronic exposure to stressors leads to short-term hyperactivity of the HPA-axis and excessive glucocorticoid release. In the long run, the HPA-axis adjusts in terms of a down-regulation of specific receptors on all levels of the HPA-axis (hypothalamus, pituitary, adrenal gland), resulting in a reduced biosynthesis of CRH, ACTH and cortisol and increased negative feedback sensitivity to glucocorticoids. Thus, chronic exposure is presumed to lead to hypoactive HPA-axis functioning, and it is further presumed that the resulting hypocortisolism is a protective response attenuating the chronically heightened HPA-axis activity by reducing the damaging effects of excessive glucocorticoid responses to stress, yet accepting the occurrence of symptoms such as elevated stress sensitivity, pain and fatigue (Fries et al., 2005).

With respect to depressive disorders, empirical evidence for hyperactive HPA-axis functioning is unequivocal: patients with major depressive disorder display increased cortisol levels (e.g. Burke et al., 2005b; Holsboer et al., 2001; Plotsky et al. 1998), yet their HPA-axis responses to negative daily events and social stressors are reported to be blunted (Peeters et al., 2003; Young et al., 2000). However, Gould and Chrousos (2002) postulate that studies of depression should allow for the heterogeneity of the illness, i.e. the diagnostic subtypes of melancholic and atypical depression, since apart from their discriminative phenomenology of symptoms (i.e. although both types are associated with dysphoria and anhedonia, melancholic depression is characterised by intense anxiety, feelings of worthlessness and guilt, insomnia, and severity of depressed mood that is greatest in the morning, while atypical depression is characterised by fatigue, lethargy, excessive sleepiness, and severity of depressed mood that is greatest later in the day) distinct differences in the neurobiology of both types can be observed. Gold and Chrousos (2002) describe melancholic depression as a prolonged and intensified stress response with marked hypercortisolism and blunted ACTH responses resulting from chronically overstimulated and hyperresponsive adrenal glands, while atypical depression is characterised by hypoactivity of the stress system resulting in significantly reduced levels of cortisol and catecholamines.

Smoking status. Apart from well-known negative effects of tobacco smoking on health, i.e. among others, carcinogenic processes and inflammatory effects, pronounced effects on the endocrine function are observed (two recent comprehensive reviews: Rohleder & Kirschbaum, 2006; Steptoe & Ussher, 2006). Tobacco smoking leads acutely to elevated cortisol levels (Kirschbaum et al., 1992b; Mendelson et al., 2005), while psychosocial stressors elicit only blunted HPA-axis responses in habitual smokers (Kirschbaum et al., 1992b, 1993b). With respect to basal salivary cortisol levels, empirical evidence is equivocal: some studies failed to disclose significant differences in basal salivary cortisol levels between habitual smokers and non-smokers (Edwards et al., 2001a; Hansen et al., 2001; Kirschbaum et al., 1994; Pruessner et al., 1997), while in several other studies significantly elevated cortisol levels were observed in habitual smokers (Kirschbaum et al., 1992b; Olff et al., 2006; Steptoe & Ussher, 2006; Wuest et al., 2000). Thus, HPA-axis reactivity to psychosocial stress seems to be impaired in habitual smokers; however, it remains unclear whether basal cortisol levels are affected as well and if so, whether there are subgroups of habitual smokers with normal and altered basal salivary cortisol levels depending on exposure-related issues, such as quantity of cigarettes smoked habitually per day or number of years of habitual smoking.

1.3 Quantification of salivary cortisol levels

Functioning under basal conditions, diurnal salivary cortisol profiles in healthy adults are characterized by peak values in the post-awakening period and a consecutive decline thereafter (Kirschbaum & Hellhammer, 1989, 1994). Thus, diurnal salivary cortisol profiles are usually subdivided into the cortisol awakening response (cortisol samples directly after awakening and within up to 60 minutes after awakening) and into the actual diurnal profile (remaining samples over the day). In the recent literature a whole range of composite cortisol measures referring to both components of the diurnal cycle are presented: the diurnal cortisol pattern is commonly quantified as the overall level of cortisol output over the waking day, the amplitude of the increase in cortisol levels subsequent to awakening, and the decrease in cortisol levels over the course of the day.

The cortisol awakening response covering the period from immediately after awakening to up to 60 minutes thereafter usually measured in 15-minutes intervals is most frequently computed as the total output, i.e. the area under the response curve (e.g. Edwards et al.,

2001a, 2001b, 2003; Federenko et al., 2004; Hucklebridge et al., 2000; Kudielka & Kirschbaum, 2003; Roberts et al., 2004; Wuest et al., 2000) or as the average cortisol output after awakening (e.g. Schlotz et al., 2004). The change in cortisol levels from baseline within the post-awakening period is usually computed as the mean increase (e.g. Edwards et al., 2001b, 2003; Federenko et al., 2004; Pruessner et al., 1997; Wuest et al., 2000) or as the absolute increase from baseline, i.e. the difference between cortisol levels directly after awakening and 30 minutes later (e.g. Kudielka et al., 2003; Kunz-Ebrecht et al., 2004a; Polk et al., 2005; Pruessner et al., 1997). Diurnal cortisol profiles are generally quantified as the overall output over the day, i.e. either the average diurnal level (e.g. den Hartog et al, 2003; Edwards et al., 2001a, 2001b; Kunz-Ebrecht et al., 2004a; Steptoe et al., 2004a, 2004b; Vedhara et al., 2003) or the area under the curve (e.g. Boehmelt et al., 2005; Bower et al., 2005; Dekkers et al., 2000a, 2000b; Edwards et al., 2003; Polk et al., 2005). Furthermore, diurnal profiles are quantified as the decrease in cortisol levels over the day by either computing difference scores between morning and evening samples (e.g. Broderick et al., 2004; den Hartog et al., 2003; Edwards et al., 2001a; Kudielka et al., 2003; Steptoe et al., 2000, 2005) or linear slopes of the diurnal change, i.e. regressing cortisol levels on time of day (e.g. Bower et al., 2005; Edwards et al., 2003; Sephton et al., 2000; Smyth et al., 1997; Stone et al., 2001).

Basically, the variety of published measures quantifying basal salivary cortisol profiles can be classified into *level* and *dynamic parameters* reflecting both state and trait aspects (Table 1). Level parameters describe the overall or average levels of cortisol output over a specified period of time, while dynamic parameters represent changes of cortisol levels within a specified period of time. State parameters are cortisol parameters of one single day, while trait parameters refer to parameters computed from cortisol values aggregated over several sampling days.

Table 1: Categories of composite salivary cortisol measures

Specification	Level measures	Dynamic measures
State	*Total morning cortisol release*: average cortisol output in the post-awakening period	*Cortisol awakening rise*: increase in cortisol within the post-awakening period
	Total diurnal cortisol release: a) average diurnal cortisol output b) AUC of diurnal cortisol output	*Diurnal cortisol decline*: a) decrease in cortisol output from morning to evening b) linear slope of the diurnal change in cortisol levels
Trait	*Total morning cortisol release* aggregated over several sampling days	*Cortisol awakening rise* aggregated over several sampling days
	Total diurnal cortisol release aggregated over several sampling days	*Diurnal cortisol decline* aggregated over several sampling days

1.4 Stability and normal values of salivary cortisol levels

So far, the stability of basal salivary cortisol values over several sampling days has not been investigated systematically. Usually stability coefficients, i.e. in most cases correlation coefficients in terms of retest reliability, are reported in addition to the actual research questions and results: Edwards et al. (2001b) reported low to moderate stability for dynamic measures of the post-awakening period (r= .34 - .50; covering the first 45 minutes subsequent to awakening), the diurnal decline in cortisol levels (r= .45 - .55), and for the diurnal mean (r= .45) based on two sampling days in 40 healthy adults. Federenko et al. (2004) investigated cortisol levels of the post-awakening period within the first 60 minutes in shift working nurses and students with a regular sleep-wake cycle on two sampling days, revealing low to high stability for single samples (nurses: r= .38 - .74; students: r= .53 - .81), and moderate to high stability for dynamic measures of the cortisol awakening response (nurses: r= .56 - .65; students: r= .50 - .84). Pruessner et al. (1997) examined cortisol levels in the post-awakening

period covering 30 to 60 minutes after awakening on two and three sampling days in three independent studies, obtaining low to moderate stability (r= . 39 - .67) of the cortisol increase within this period. Wuest et al. (2000) pooled salivary cortisol levels measured within the first 60 minutes after awakening of 509 healthy adults from four independent studies and found low to moderate stability of single samples (r= .37 - .66; with the lowest stability for cortisol levels directly after awakening) and moderate stability for the increase in cortisol levels within this period (r= .47 - .63). Thus, studies assessing the stability of basal salivary cortisol profiles apply correlation analyses, i.e. test-retest reliability, using either parametric Pearson product-moment coefficients or non-parametric Spearman rank coefficients (e.g. Edwards et al., 2001b; Federenko et al., 2004; Pruessner et al., 1997; Wuest et al., 2000). However, correlation coefficients are measures of association, i.e. measuring the strength of linear association; they are not measures of agreement (Altman & Bland, 1983; Bland & Altman, 1986). Thus, intraclass correlation coefficients are recommended to assess the degree of agreement (Rankin & Stokes, 1998), i.e. assessing the ratio of within-subject variance to total variance (Shrout & Fleiss, 1979).

Normal values of basal salivary cortisol levels in healthy adults have been published by Clow et al. (2004), Kirschbaum and Hellhammer (1989) and Wuest et al. (2000). It is of concern that these published normal values vary to large extent across studies which implies that even in healthy participants basal cortisol levels are sensitive to a range of confounding factors. Due to the lack of normative values regarding cortisol levels the diagnostic appropriateness of cortisol profiles is challenged: it remains unclear what constitutes too high or too low a cortisol awakening response or too steep or too flat a diurnal decline in cortisol levels. This issue also raises the question of the relevance or validity of cortisol levels as indicators of HPA-axis dysregulation or as an indicator of disease: most studies compare patient groups with specified disorders to healthy controls to evaluate HPA-axis dysfunctioning, but since normal values of healthy adults are liable to such a large variation (e.g. Smyth et al., 1997; Stone et al., 2001), it remains doubtful whether the observed dysregulation in HPA-axis functioning results solely from the disease process. Moreover, it remains unclear within the scope of such cross-sectional study designs whether the observed HPA-axis dys-

regulation reflects acute effects or chronic dysregulation persisting over time and whether the dysregulation constitutes the cause or sequelae of the disease process.

2. Aim and research questions

Based on these findings the goal of the present study was to investigate basal diurnal salivary cortisol levels in two independent samples of employees working in the human service sector within their natural environment. Preliminary analyses of both samples have already been provided by Weber (2005), yet the present study was designed to gain an in-depth insight in the internal structure of basal salivary cortisol levels, in the effectiveness of empirically proved confounding factors to which HPA-axis functioning is sensitive, and in the stability of cortisol profiles and their compound measures within the scope of field research.

Diurnal salivary cortisol profiles were quantified as composite measures reflecting the average level of cortisol output and those reflecting dynamic aspects of the change in cortisol over the day (Table 1).

Given the exploratory nature of this study, we first analysed the internal structure of salivary cortisol samples with respect to:

- the pattern of missing values, i.e. the distribution of missing cortisol samples and missing indication of sampling times;
- the identification of extreme cortisol values and their distribution among sampling times and days as well as their association with potential confounding factors;
- the adherence to the stipulated sampling protocol regarding:
  a) the extent of adherence to the sampling protocol, i.e. the number of non-compliant measures and the deviation in minutes from the stipulated sampling protocol comparing objectified (i.e. electronically verified) and self-reported compliance,
  b) the variation in the length of the sampling time interval between awakening cortisol samples due to non-adherence and its effect on cortisol levels of the post-awakening period,

c) the effect of adherence and non-adherence to scheduled sampling times (clock time and relative time) on corresponding cortisol samples;

- the effect of awakening cortisol levels on subsequent cortisol samples and
- the predictive power of single cortisol samples in representing mean diurnal cortisol levels.

In a next step, the effect of potential confounding factors on basal salivary cortisol levels of single samples and composite measures was examined regarding:

- the time of awakening;
- sleep duration and quality of sleep;
- gender differences and intake of oral contraceptives;
- age;
- occupational groups;
- shift work;
- the somatic health status with respect to self-reported disorders and diseases, somatic complaints, smoking status, and cardiovascular risk factors;
- the mental health status with respect to depressive symptomatology, trait anxiety, work-related burnout and self-efficacy, and
- diurnal mood and subjective well-being.

Taking into account those confounding factors that proved to be significantly associated with basal diurnal cortisol levels in our samples in the preceding analyses described above, the stability of basal salivary cortisol levels was investigated with respect to:

- the stability of the average diurnal pattern, i.e. diurnal salivary cortisol profiles;
- the stability of single diurnal cortisol samples;
- the stability of composite measures of the diurnal cortisol pattern reflecting average levels of cortisol output (total morning cortisol release and total diurnal release) and those reflecting dynamic aspects of the change in cortisol output over the day (cortisol awakening rise and diurnal decline in cortisol) and
- the stability of diurnal salivary cortisol levels regarding the replication of both independent samples, i.e. comparing cortisol levels of equally timed and varying sampling

occasions as well as comparing composite measures of basal salivary cortisol profiles between both study samples to obtain normal values of basal salivary cortisol levels in a large sample of employees of the human service sector.

The stability of basal salivary cortisol levels was investigated in order to evaluate whether the aggregation of cortisol values, i.e. averaging cortisol levels over sampling days, and whether the use of aggregated cortisol values as trait measures can be empirically justified.

3. Methods

3.1 Participants

In two separate studies, a total of 440 participants (132 men, 308 women) were investigated for diurnal cortisol secretory activity. Participants covered four occupational groups in study 1 (49 nurses, 22 teachers, 9 hotel employees, 23 social service assistants) and five occupational groups in study 2 (85 nurses, 110, teachers, 17 hotel employees, 90 social service assistants, 35 mixed group). The mean age of participants was 40.5 ± 11.3 years in study 1 and 42.4 ± 11.0 years in study 2. Participants in both studies were recruited through company physicians from German BAD centres (BAD-Preventive Health Care and Safety Engineering, Germany). Study 1 was conducted from May 2002 to January 2003, study 2 from February 2003 to May 2004 within the scope of the BAD research project *Individual Combinations of Psychological and Mental Risk Factors at Work*. Participating companies were informed in written form in advance about the purpose and procedures of the studies. Participants did not receive monetary incentives for participation. Participation was voluntary and participants gave written informed consent in accordance with the Helsinki declaration. In both studies, participants underwent a general medical examination and were given psychometric questionnaires (specified in the following chapters). Table 2 summarizes the characteristics of both samples.

Table 2: Sample characteristics

	Study 1	Study 2	Chi²	F
N	103	337		
Age (in years), mean ± SD	40.5 ± 11.3	42.4 ± 11.0	--	2.26
Gender, N (%)				
Male : Female	23 : 80 (22.3 % : 77.7 %)	109 : 228 (32.3 % : 67.7%)	3.76	--
Oral contraceptive users, N (%)	17 (21.2 %)	49 (21.5 %)	0.42	--
Occupational groups, N (%)			23.91**	--
Nurses	49 (47.6 %)	85 (25.2 %)		
Teachers	22 (21.4 %)	110 (32.7 %)		
Hotel staff	9 (8.7 %)	17 (5.0 %)		
Social service assistants	23 (22.3 %)	90 (26.7 %)		
Mixed group	--	35 (10.4 %)		
Shift work, N (%)	45 (44 %)	89 (26.4 %)	6.48*	--
Smokers, N (%)	38 (36.8)	96 (28.5 %)	7.21**	--
Body mass index[1], mean ± SD	24.2 ± 3.8	24.6 ± 4.2	--	0.42
Waist-to-hip ratio[2], mean ± SD	--	0.9 ± 0.1	--	--
Blood pressure(mmHg), mean ± SD				
Systolic blood pressure	129.2 ± 18.5	121.9 ± 15.7	--	5.24*
Diastolic blood pressure	79.7 ± 10.4	78.6 ± 9.7	--	1.00
Somatic complaints[3], mean ± SD	49.2 ± 27.1	57.8 ± 26.4	--	7.56**
Depressive symptoms[4], mean ± SD	56.4 ± 33.2	49.9 ± 27.8	--	3.65
Trait anxiety[5], mean ± SD	64.1 ± 29.2	61.8 ± 27.4	--	0.51
Burnout[6], mean ± SD				
Emotional exhaustion	50.3 ± 11.8	50.4 ± 10.7	--	0.01
Depersonalization	51.5 ± 13.4	51.5 ± 11.7	--	0.00
Personal accomplishment	52.1 ± 11.9	53.0 ± 11.1	--	0.46
Self-efficacy[7], mean ± SD	44.7 ± 11.7	48.7 ± 8.3	--	14.33**

[1]Body mass index: weight (kg) / height squared (m²); [2]waist-to-hip ratio: waist circumference (cm) / hip circumference (cm); [3]somatic complaints: overall somatic distress, percentile rank (GBBB; Braehler & Scheer, 1995); [4]depressive symptoms: percentile rank (ADS, Hautzinger & Bailer, 1995); [5]trait anxiety: percentile rank (STAI-T; Laux et al., 1981); [6]burnout: T-value (MBI, Enzmann & Kleiber, 1989); [7]self-efficacy: T-value (SWE; Schwarzer & Jerusalem, 1995); *p< 0.05; **p< 0.01.

3.2 Saliva sampling procedure

Saliva samples were obtained using cotton swabs which were stored in standard centrifugation tubes ("Salivette"; Sarstedt Inc., Rommelsdorf, Germany) labelled individually for each day and measurement. Participants were instructed to collect samples immediately after awakening (t(+0)), 30 minutes later (t(+30)), and then at 08:00 h, 11:00 h, 15:00 h, 20:00 h on two consecutive working days (study 1) and 16:00 h and 20:00 h on three consecutive working days (study 2). In case of shift work, participants were asked to collect samples on working days with dayshift, to ensure conformance with the stipulated sampling protocol of

scheduled clock-time sampling occasions. They were told not to brush their teeth as to avoid microvascular contamination and to refrain from smoking, consuming food or drinking other than water at least 30 minutes prior to sampling. Participants were briefed in detail in saliva sampling and were given written instructions as well as easy-to-administer saliva sampling devices. In study 1, 69 participants were informed that adherence to the sampling protocol was controlled using electronic drug exposure monitors (MEMS ® V TrackCap, Aardex, CH-Untermuehli); all other participants were not controlled, but were requested to adhere strictly to the sampling protocol. Participants were instructed to record precise sampling times (clock-time) in a stress diary (see 3.3.4 Stress diary). Participants were instructed additionally to keep saliva samples frozen until returning them to the investigators personally. Saliva samples were then kept frozen at the investigators' laboratory at -25° C until analyses. Cortisol was analysed using a biotin-streptavidin fluorescence immunoassay as described in detail elsewhere (Dressendoerfer et al., 1992). Intraassay and interassay coefficients of variation were less than 10% and 12%, respectively.

3.3 Measures

3.3.1 Quantification of diurnal cortisol secretory activity

The diurnal cortisol release was quantified in terms of level and dynamic measures as presented in Table 1.

Level state measures of cortisol release were quantified as

(1) the *total morning cortisol release* of each sampling day

a) mean of awakening samples

study 1/2: $[C_{t(+0)} + C_{t(+30)}] / 2$ [1]

(2) the *total diurnal cortisol release* of each sampling day

a) diurnal mean including t(+30)

study 1: $[C_{t(+0)} + C_{t(+30)} + C_{t(08:00)} + C_{t(11:00)} + C_{t(15:00)} + C_{t(20:00)}] / 6$

study 2: $[C_{t(+0)} + C_{t(+30)} + C_{t(16:00)} + C_{t(20:00)}] / 4$

[1] $C_{t(xx)}$ denotes cortisol measurements at the specified time point (+0 = directly after awakening; +30 = 30 minutes after awakening; 08:00 = 08:00 am; 11:00 = 11:00 am; 15:00 = 03:00 pm; 16:00 = 04:00 pm; 20:00 = 08:00 pm).

b) diurnal mean excluding t(+30)

study 1: $[C_{t(+0)} + C_{t(08:00)} + C_{t(11:00)} + C_{t(15:00)} + C_{t(20:00)}] / 5$

study 2: $[C_{t(+0)} + C_{t(16:00)} + C_{t(20:00)}] / 3$

c) AUC including t(+30) [2]

study 1: $[(C_{t(+30)}+C_{t(+0)}) \times t_1] / 2 + [(C_{t(08:00)}+C_{t(+30)}) \times t_2] / 2 + [(C_{t(11:00)}+C_{t(08:00)}) \times t_3] / 2 + [(C_{t(15:00)}+C_{t(11:00)}) \times t_4] / 2 + [(C_{t(20:00)}+C_{t(15:00)}) \times t_5] / 2$ [3]

study 2: $[(C_{t(+30)}+C_{t(+0)}) \times t_1] / 2 + [(C_{t(16:00)}+C_{t(+30)}) \times t_2] / 2 + [(C_{t(20:00)}+C_{t(16:00)}) \times t_3] / 2$

d) AUC excluding t(+30)

study 1: $[(C_{t(08:00)}+C_{t(+0)}) \times t_1] / 2 + [(C_{t(11:00)}+C_{t(08:00)}) \times t_2] / 2 + [(C_{t(15:00)}+C_{t(11:00)}) \times t_3] / 2 + [(C_{t(20:00)}+C_{t(15:00)}) \times t_4] / 2$

study 2: $[(C_{t(16:00)}+C_{t(+0)}) \times t_1] / 2 + [(C_{t(20:00)}+C_{t(16:00)}) \times t_2] / 2$

Dynamic state measures of cortisol output were quantified as

(3) the *cortisol awakening rise* of each sampling day

a) absolute difference between awakening cortisol samples

study 1/2: $C_{t(+30)} - C_{t(+0)}$

(4) the *diurnal cortisol decline* of each sampling day

a) linear slope of the diurnal change in cortisol release excluding t(+0) [4]

study 1: *regressing cortisol values on sampling time (beta estimates):* $C_{t(+30)}$, $C_{t(08:00)}$, $C_{t(11:00)}$, $C_{t(15:00)}$, $C_{t(20:00)}$

study 2: *regressing cortisol values on sampling time (beta estimates):* $C_{t(+30)}$, $C_{t(16:00)}$, $C_{t(20:00)}$

b) linear slope of the diurnal change in cortisol release excluding t(+30)

study 1: *regressing cortisol values on sampling time (beta estimates):* $C_{t(+0)}$, $C_{t(08:00)}$, $C_{t(11:00)}$, $C_{t(15:00)}$, $C_{t(20:00)}$

study 2: *regressing cortisol values on sampling time (beta estimates):* $C_{t(+0)}$, $C_{t(16:00)}$, $C_{t(20:00)}$

[2] AUC: area under the curve with respect to zero according to Pruessner et al. (2003b) assessing the overall diurnal cortisol release.

[3] t_i denotes the time interval between measurements

[4] Interpretation of beta estimates: smaller betas denote a rapid decrease in cortisol throughout the day, while larger betas denote a flatter diurnal rhythm.

c) diurnal decline from t(+30) to t(20:00), difference score [5]

study 1/2: $C_{t(+30)} - C_{t(20:00)}$

d) diurnal decline from t(+0) to t(20:00), difference score

study 1/2: $C_{t(+0)} - C_{t(20:00)}$

Level and dynamic trait measures were calculated analogously using mean cortisol values over sampling days instead of single day values.

3.3.2 Mental health status

Depressive symptoms were assessed with the German adaptation (ADS; Hautzinger & Bailer, 1995) of the Center for Epidemiological Studies Depression Scale (CES-D; Radloff, 1977), a 20-item, self-report depression scale inferring to the frequency of symptoms during the past week. The four-point frequency scale consists of items addressing four factors: depressive affect, somatic symptoms, positive affect, and interpersonal relations (*rarely or none of the time / some or little of the time / occasionally or a moderate amount of time / most or all of the time*). Percentile ranks of German normative data (Hautzinger & Bailer, 1995) were used for analyses.

Trait anxiety was measured with the German version of the State-Trait Anxiety Inventory (STAI-T; Laux et al., 1981; Spielberger, 1980) which consists of 20 items assessing anxiety as a general trait, i.e. the general tendency to respond with anxiety to perceived threats in the environment, on a four-point frequency scale (*almost never / sometimes / often / almost always*). Percentile ranks of German normative data (Laux et al., 1981) were used for analyses.

Burnout was assessed with the German version (Enzmann & Kleiber, 1989) of the Maslach Burnout Inventory (MBI; Maslach & Jackson, 1986) measuring three components of burnout: emotional exhaustion, depersonalization, and reduced personal accomplishment using 22 items in form of statements about personal feelings and attitudes on a six-point scale (intensity and frequency of experience: 1=*very mild* to 6=*very strong*). T-values of German normative data (Schwarzer & Jerusalem, 1999) were used for analyses.

[5] Interpretation of difference scores: smaller deltas denote a flatter diurnal rhythm, while larger deltas denote a rapid decline of cortisol from morning to evening.

Self-efficacy, i.e. optimistic self-beliefs in the ability to cope with a variety of difficult demands in life, was assessed with the Generalized Self-Efficacy Scale (Schwarzer & Jerusalem, 1995). The scale consists of ten items assessing a general sense of self-efficacy (e.g. *I can always manage to solve difficult problems if I try hard enough*) on a four-point Likert-type scale (*not at all true / hardly true / moderately true / exactly true*). Cronbach's alphas derived from samples from 23 nations ranged from .76 to .90; T-values from German normative data (Schwarzer & Jerusalem, 1999) were used for analyses.

3.3.3 Somatic health status

Somatic symptoms were assessed by the Giessen Subjective Complaints List (GBB; Braehler & Scheer, 1995) which consists of 24 somatic symptoms representing the scales exhaustion, cardiovascular, musculoskeletal, and gastrointestinal complaints. Participants were asked to estimate the degree of distress caused by each somatic complaint on a five-point frequency scale (*never / rarely / sometimes / often / always*). The sum of the four scales makes up the fifth scale score, overall distress or indulgence to complaining. Percentile ranks from German normative data (Braehler & Scheer, 1995) were used for analyses.

A ***general medical examination*** performed by company physicians from German BAD centres (BAD-Preventive Health Care and Safety Engineering, Germany) recorded sociodemographic data (age, gender, occupation, shift work) as well as smoking status (current smoker vs. non-smoker), intake of oral contraceptives in women, systolic and diastolic blood pressure (mmHg), weight and height to determine the body mass index (BMI = weight in kg / height in m^2), waist and hip circumference to determine waist-to-hip ratio (waist circumference in cm / hip circumference in cm; in study 2 only) and self-reported disorders and diseases (endocrine, psychiatric, neurological, and sleep disorders).

3.3.4 Stress diary

A stress diary was kept during sampling days (study 1: two days; study 2: three days). Participants were equipped with a pocket-sized booklet to be completed in parallel with the saliva sampling schedule assessing psychological strain (study 1) and self-rated mood and subjective well-being (study 2). In study 1, current psychological strain was assessed with the Short Questionnaire of Current Psychological Strain (KAB; Mueller & Basler, 1993). The

KAB consists of six bipolar items rated on a six-point intensity scale (e.g. *At the moment I feel tense … extremely / moderately / mildly / mildly / moderately / extremely … composed*). In study 2, current mood and subjective well-being were assessed using an adaptation of the three principal bipolar dimensions (*good vs. bad mood*; *alertness vs. tiredness*; *relaxation vs. agitation*) of the Multidimensional Well-being Questionnaire (MDBF; Steyer et al., 1997) on a ten-point numeric rating scale ranging from 0 (*very bad mood / very tired / very agitated*) to 10 (*very good mood / very alert / very relaxed*). In addition, participants filled in a short sleep protocol (study 1: self-reported sleep deprivation, i.e. lack of sufficient sleep; study 2: length of sleep as well as frequency and duration of sleep disruption episodes).

3.4 Statistical analyses

3.4.1 Initial treatment of cortisol data

As raw cortisol data were positively skewed, they were log-transformed to obtain symmetrically distributed data. Normal probability plots of raw and transformed data (log- and square-root-transformations) were compared; while not perfectly symmetric, log-transformed data (natural logarithm of raw values: ln(x+1)) provided the best approximation to a normal distribution (Figure 1). Composite cortisol measures were log-transformed subsequent to their computation using raw values. All statistical analyses of cortisol data were performed with log-transformed values; to facilitate interpretation, however, raw values are presented in tables and figures.

3.4.2 Statistical analyses

a) Examination of participant adherence

Participant adherence to the sampling protocol was controlled in two ways: for 69 participants in study 1 the participants' self-reported collection times were compared with those indicated by the drug exposure monitors. All other remaining participants' self-reported sampling times were compared with the scheduled times. A 10 minute time window for samples collected 30 minutes after awakening and a 60 minute time window for all subsequent samples was allowed. Non-compliant measures were compared to compliant ones as to avoid artefacts in results, i.e. inconsistencies in cortisol responses could justifiably be at-

tributed to genuine physiological characteristics as opposed to non-adherence to the regime (Clow et al., 2004; Kudielka et al., 2003).

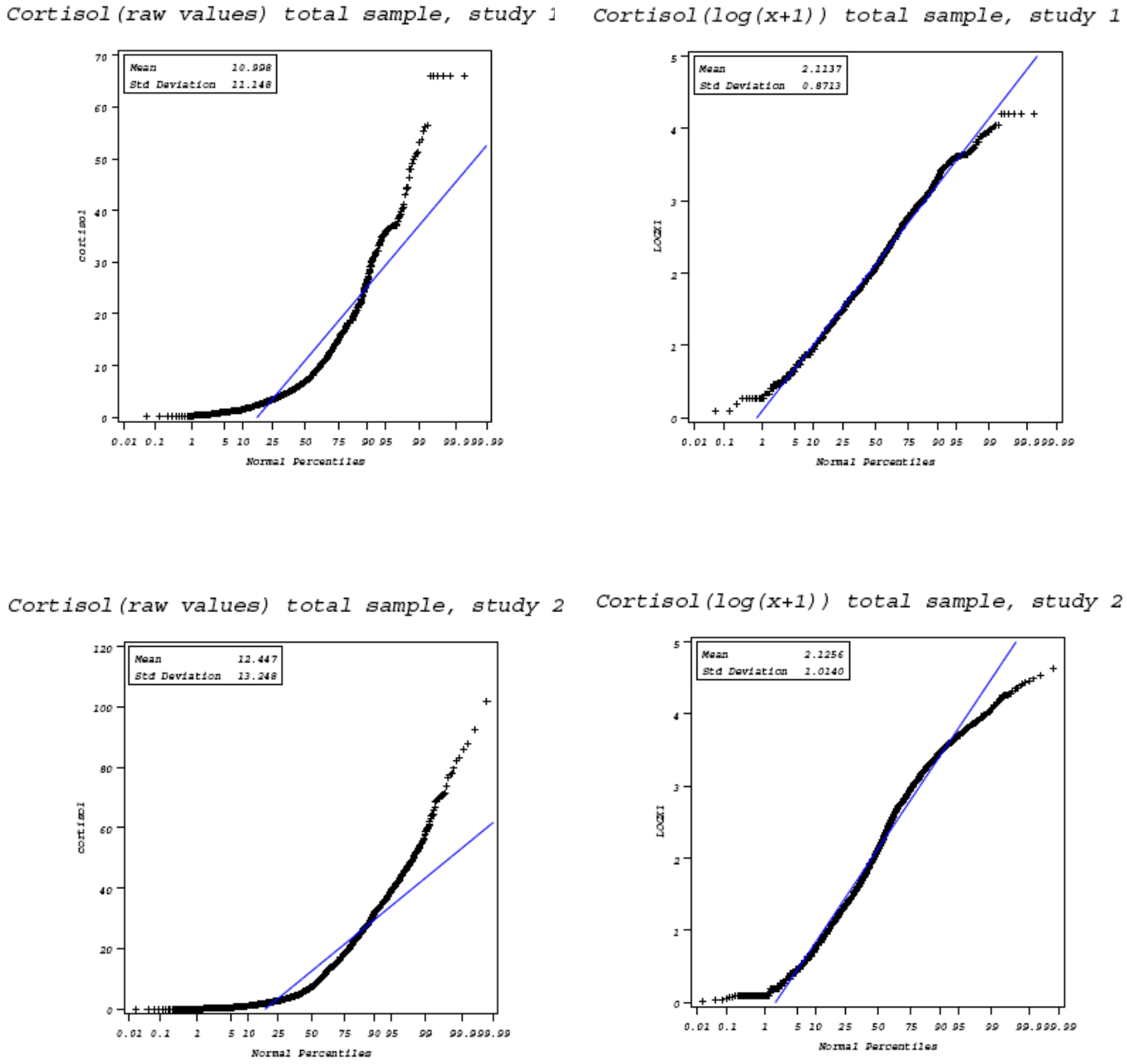

Figure 1*:* Normal probability plots of salivary cortisol data; on the left: raw values (nmol/l); on the right: transformed values (ln(x+1); above: study 1; below: study 2).

b) Statistical analyses

Statistical analyses were performed with the SAS® System for Windows®, Release 9.1. Chi-square tests (PROC FREQ) were applied to analyse categorical variables. Prior to analyses of compliance rates, an arcsine (angular) transformation was performed on percentage/proportion data, as proportions usually have a binomial distribution, thus the appropriate transformation would be the arcsine transformation (i.e. finding an angle whose sine is the square-root of the proportion) to obtain an approximation to the normal distribution of data (Sokal & Rohlf, 1981).

One-way ANOVA (PROC GLM) analyses were used to test differences among groups. Mixed model analyses for repeated measures (PROC MIXED, REPEATED statement) were performed to test group differences involving repeated measures (i.e. cortisol data, current mood/well-being). Significant interaction effects in mixed model analyses were decomposed in two ways: a) effect slices were examined to obtain simple main effects, i.e. each level of between-subjects factor was compared with each level of the within-subjects factor, and b) differences of least square means were examined to obtain contrasts between levels of the variables in the interaction (Khattree & Naik, 1999). Pearson product-moment correlation coefficients were calculated (PROC CORR) as a measure of the strength and direction of the linear association between continuous variables.

With regard to stability of cortisol single samples and composite measures across sampling days intraclass correlation coefficients (ICC) were calculated according to Shrout and Fleiss (1979) as a measure of homogeneity (ICC = ratio of within-subject variance to total variance). In their classic article on intraclass correlation coefficients as a measure of reliability (Shrout & Fleiss, 1979), six intraclass correlations are discussed. According to their notation ICC (1,1) is used when each target is rated by multiple raters, raters are assumed to be randomly assigned to targets, and all targets have the same number of raters (one-way ANOVA design); ICC(2,1) is used when all targets are rated by the same raters who are assumed to be a random sample of all possible raters (two-way ANOVA design); ICC(3,1) is used when all targets are rated by the same raters who are assumed to be the entire population of raters (two-way ANOVA design); ICC(1,k), ICC(2,k), and ICC(3,k) apply analogously assessing the reliability for the mean of k ratings. Here, the ICC(1,1) was chosen, as for each

target (i.e. cortisol sample, composite measure) k different raters from the pool of raters (i.e. sampling days) were randomly assigned (i.e. one-way ANOVA in which the cortisol sample or composite measure is a random effect, and sampling days are viewed as measurement error). ICC(1,1) was calculated as

$$[BMS - WMS] / [BMS + (k\text{-}1) \times WMS]$$

where BMS denotes the between-subject mean square (between-subject variance), WMS the within-subject mean square (within-subject variance), and k the number of raters, i.e. sampling days (Bartko, 1966, 1976; Shrout & Fleiss, 1979). High ICC values denote small within-subjects variance. According to Bartko (1966) the ICC(1,1) can be interpreted as a correlation coefficient, thus ICC values were interpreted according to Guilford's (1954) interpretation of the correlation coefficient ($r \leq .20$: slight / almost negligible relationship; .20 - .40: low / definite, but small relationship; .40 - .70: moderate / substantial relationship; .70 - .90: high / marked relationship; .90 - 1.0: very high / very dependable relationship).

4. Results

4.1 Internal structure of cortisol samples

4.1.1 Pattern of missing data

Three types of missing values were identified: (a) no cortisol sample provided, (b) no sampling time reported (by self-report or verified by MEMS TrackCaps), and (c) the combination of both. Participants provided a total of 1158 (93.7%) cortisol samples and 1182 (95.6%) reported sampling times of the 1236 expected sampling occasions in study 1 (103 participants × 6 measurements per day × 2 sampling days) and a total of 3954 (97.8%) cortisol samples and 3881 (96.0%) reported sampling times of the 4044 expected sampling occasions in study 2 (337 participants × 4 measurements per day × 3 sampling days), respectively (Appendix 1, Table 1).

In both studies, missing values of cortisol samples and/or reported sampling times were evenly distributed across sampling days (Appendix 1, Table 2). Cortisol missing values were also evenly distributed across sampling times, whereas missing values for reported sampling

times and the combination of both were more likely to occur at later time points (Appendix 1, Table 3; Chi-square: $p<0.05$ and $p<0.01$, respectively). In study 1, men had more missing values than women (Appendix 1, Table 4; Chi-square: $p<0.01$ and $p<0.05$), while in study 2 men and women did not differ significantly (Appendix 1, Table 4; Chi-square: n.s.). In both studies, the occupational group "hotel staff" accounted for differences in the distribution of missing values (Appendix 1, Table 5): In study 1, missing values accumulated in the group "hotel staff" (Chi-square: $p<0.01$), while in study 2 the group "hotel staff" accounted for the fewest missing values (Chi-square: $p<0.01$).

Subsequent to missing analyses, cortisol samples were set to missing when a corresponding sampling time (reported and/or verified by MEMS TrackCaps) was not specified, since these cortisol samples could not be assigned reliably to a specified point in time. In study 1, one participant failed to report sampling times for all cortisol samples and was thus excluded from further analyses, resulting in 102 participants in study 1. In study 2, five participants failed to report sampling times for all cortisol samples and were therefore excluded from further analyses. In addition, one participant in study 2 was excluded from subsequent analyses due to substantial deviations from the stipulated sampling regime on all three sampling days (t(+0) at 13:00, thus, t(16:00) at 22:00, and t(20:00) at 02:00), resulting in 331 participants in study 2.

4.1.2 Identification of extreme cortisol values

Extreme cortisol values were defined in three different ways:

(1) Mean + (4 * SD)

(2) Q3 + (3* IQR)

(3) Q2 + 4*(Q3 – Q2)

Extreme cortisol values were assessed for each timed sample and sampling day separately (see Appendix 1, Table 6 for descriptive statistics of cortisol values and their corresponding outlier fences).

First, cortisol values ranging more than 4 standard deviations above the sample mean were defined as outliers (Broderick et al., 2004; Catley et al., 2000). In study 1, two extreme

values were observed in two participants. In study 2, a total of 31 extreme values were observed in 26 participants.

Second, cortisol values ranging more than 3 interquartile ranges above the 75th percentile were defined as outliers. In study 1, 12 extreme values were observed in 10 participants. In study 2, 58 extreme values were observed in 44 participants.

Third, cortisol values ranging [4*(Q3 – Q2)] above the sample median were defined as outliers (Rousseeuw et al., 1999). This method generated the largest amount of outliers: In study 1, 34 extreme values were observed in 25 participants. In study 2, 151 extreme values were observed in 92 participants.

Extreme values were evenly distributed across sampling times and days, gender, and occupational groups. There was no systematic bias in extreme cortisol values regarding depression and anxiety scores, and somatic complaints or disorders; consequently, extreme values were not eliminated from data sets (see Appendix 1, Table 7-8 for a detailed description of cortisol outliers and Appendix 2 for the illustration of single cortisol profiles of each participant with outlier fences and the corresponding extreme cortisol values).

4.1.3 Adherence to sampling protocol: compliance rates and deviation in minutes

Adherence to the stipulated sampling protocol (i.e. compliance with saliva sampling) was examined in both studies (computation see chapter 3.4.2). The number of compliant and non-compliant samples for each sampling occasion on each sampling day is given in Appendix 1, Table 9 (study 1) and Appendix 1, Table 10 (study 2). Analogously, the deviation in minutes from the stipulated sampling protocol for each sampling occasion on each sampling day is given in Appendix 1, Table 11 (study 1) and Appendix 1, Table 10 (study 2). Mean compliance rates and mean deviation in minutes were calculated across sampling occasions, sampling days and participants (Table 3).

In study 1, 69 participants used drug exposure monitors to record sampling times and additionally provided self-reported sampling times. The remaining 33 participants in study 1 as well as the total sample of study 2 provided self-reported sampling times, only.

Table 3: Average rates of compliance and average deviation in minutes across participants

Compliance	Electronically monitored			Not electronically monitored		
	N	Compliance rate*	Deviation in minutes#	N	Compliance rate*	Deviation in minutes#
Study 1						
Verified	69	89 % ± 0.19 (20 – 100 %)	0:28 ± 0:51 (0:02 – 5:06)	--	--	--
Self-reported	69	93 % ± 0.16 (20 – 100 %)	0:19 ± 0:36 (0:00 – 3:27)	33	94 % ± 0.11 (60 – 100 %)	0:19 ± 0:18 (0:00 – 1:13)
Study 2						
Self-reported	--	--	--	331	96 % ± 0.08 (55 – 100 %)	0:09 ± 0:10 (0:00 – 1:20)

*Values are proportions: mean ± SD and range across participants and sampling times; #values are hh:mm: mean ± SD and range across participants and sampling times.

In study 1, mixed model analyses for repeated measures revealed a significant difference in mean compliance rates between verified (sampling times indicated by drug exposure monitors) and self-reported sampling times in participants using drug exposure monitors ($F= 11.89$, $p< 0.01$; Table 3): Verified compliance rates in participants using drug exposure monitors were significantly lower compared to their additionally self-reported compliance rates. The same effect could be observed with regard to deviation in minutes from the sampling protocol: deviation in minutes from the stipulated sampling times was much stronger in verified sampling times compared to self-reported sampling times in participants using drug exposure monitors (mixed model analyses for repeated measures: $F= 4.39$, $p< 0.05$; Table 3). However, there was no difference in self-reported compliance rates (one-way ANOVA: $F= 0.03$, $p> 0.8$) or self-reported deviation in minutes (one-way ANOVA: $F= 0.02$, $p> 0.8$) between participants using drug exposure monitors and those with self-reported data only (Table 3).

In study 2, participants provided self-reported sampling times only. Compliance rates of self-reported sampling times of participants in study 2 were comparable to those of participants in study 1 with or without drug exposure monitors (one-way ANOVA: $F= 0.03$, $p> 0.7$; Table 3). However, self-reported deviation in minutes of participants in study 2 was considerably lower compared to self-reported deviation of participants in study 1 with or without

drug exposure monitors (one-way ANOVA: F= 14.15, p< 0.01; post-hoc Scheffé's tests; Table 3).

Most studies examining the effect of adherence to saliva sampling protocols on cortisol profiles (e.g. Kudielka et al., 2003) classify participants non-compliant in case of one or more non-compliant single sample. We think that this procedure results in unfavourable confounding of individual cortisol profiles with a mixture of compliant and non-compliant measures. Thus, the next two sections investigate the effect of adherence to stipulated sampling times on each single cortisol sample separately.

4.1.4 Adherence to sampling protocol: sampling time interval of awakening cortisol

In a next step, the variation in the length of the sampling time interval between early morning cortisol samples at t(+0) and t(+30) due to non-adherence to the sampling protocol and the effect on the magnitude of the *cortisol awakening rise* and *total morning cortisol release* were examined. According to the stipulated sampling protocol, the first diurnal cortisol sample was scheduled directly after awakening, the second sample 30 minutes thereafter.

Mixed model analyses for repeated measures failed to reveal significant differences in the length of sampling time intervals across sampling days in both studies (study 1: F= 0.98, p= 0.32; study 2: F= 1.89, p= 0.15; Appendix 1, Table 12).

In both studies, *cortisol awakening rise* as well as *total morning cortisol release* and length of sampling time intervals between early morning cortisol samples were uncorrelated (Table 4, Figure 2), i.e. there was no linear relationship between the length of the time interval and the size of the *cortisol awakening response* or *morning cortisol release*.

Table 4: Correlation of sampling time interval and post-awakening cortisol levels

Time interval t(+0) to t(+30)	N	Cortisol awakening rise	Morning cortisol release
Study 1	192	0.07	0.05
Day 1	95	0.10	0.12
Day 2	97	0.03	-0.02
Study 2	915	-0.04	0.02
Day 1	305	-0.04	-0.06
Day 2	307	-0.04	0.09
Day 3	303	-0.02	0.00

Pearson correlation coefficients

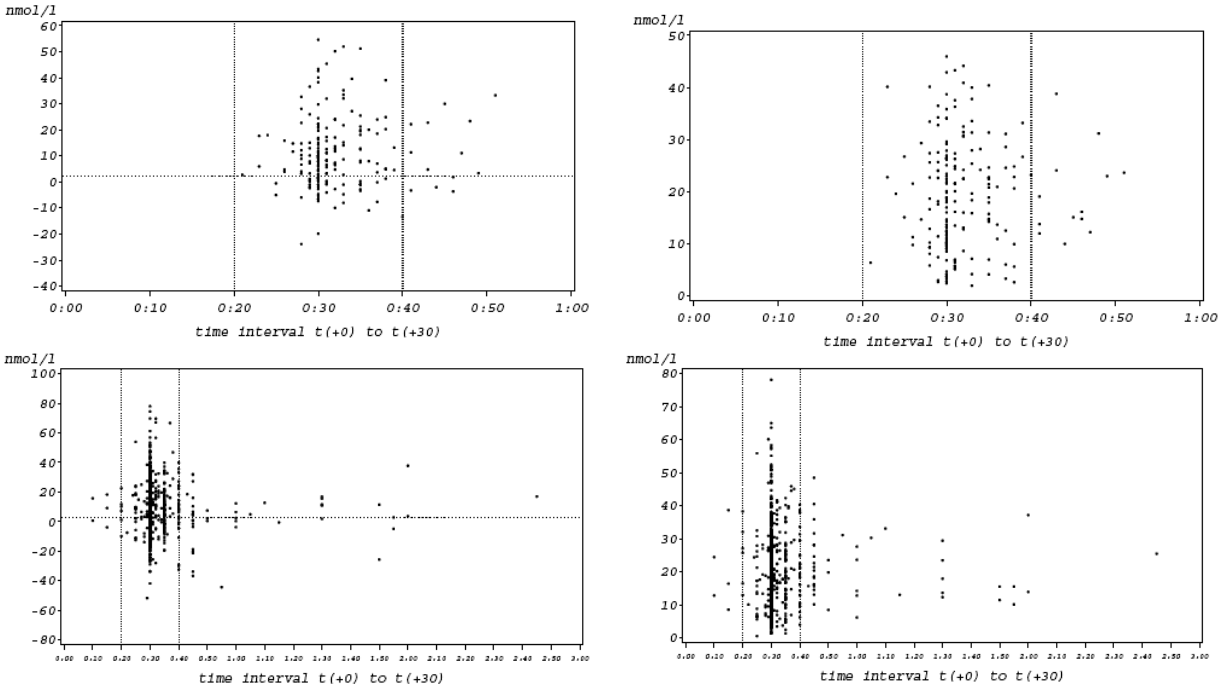

Figure 2: Correlation of cortisol levels and length of time interval between t(+0) and t(+30). On the left: cortisol awakening rise, the horizontal reference line divides cortisol awakening response measures into responders (CAR > 2.5 nmol/l) and non-responders (CAR < 2.5 nmol/l; Wuest et al., 2000); the vertical lines specify the categories of the time interval between t(+0) and t(+30): 0-19, 20-40, 41-60, > 60 minutes (see text); on the right: total morning cortisol release; above: study 1; below: study 2.

According to studies on compliance with saliva sampling protocols (Broderick et al., 2004; Kudielka et al., 2003; Kunz-Ebrecht et al., 2004b) that define compliance with cortisol sampling on awakening and 30 minutes thereafter by a time window of ± 10 minutes, the sampling time interval was subdivided into four categories: *0 to 19 minutes*, *20 to 40 minutes*, *41 to 60 minutes*, and *more than 60 minutes*. Additionally, the *cortisol awakening rise* was classified in responders and non-responders according to the criterion introduced by Wuest et al. (2000): an increase of at least 2.5 nmol/l above the individual baseline was defined as a cortisol response to awakening.

In study 1, 179 of 204 samples (102 participants × 2 sampling days) were collected within 20-40 minutes after awakening (with 129 responders and 50 non-responders), 13 of 204 within 41-60 minutes after awakening (with 9 responders and 4 non-responders), and none within 0-19 and > 60 minutes after awakening. Mixed model analyses for repeated measures for the total sample (Appendix 1, Table 13; Figure 3; due to the small sample size of non-

responders in these categories significance testing was not appropriate) revealed no significant main effect of sampling time interval categories with regard to cortisol values at t(+0) (F=0.52, p>0.4), cortisol values at t(+30) (F=0.0, p> 0.50), *cortisol awakening rise* (F= 0.14, p>0.70), and *total morning cortisol release* (F=1.59, p> 0.20).

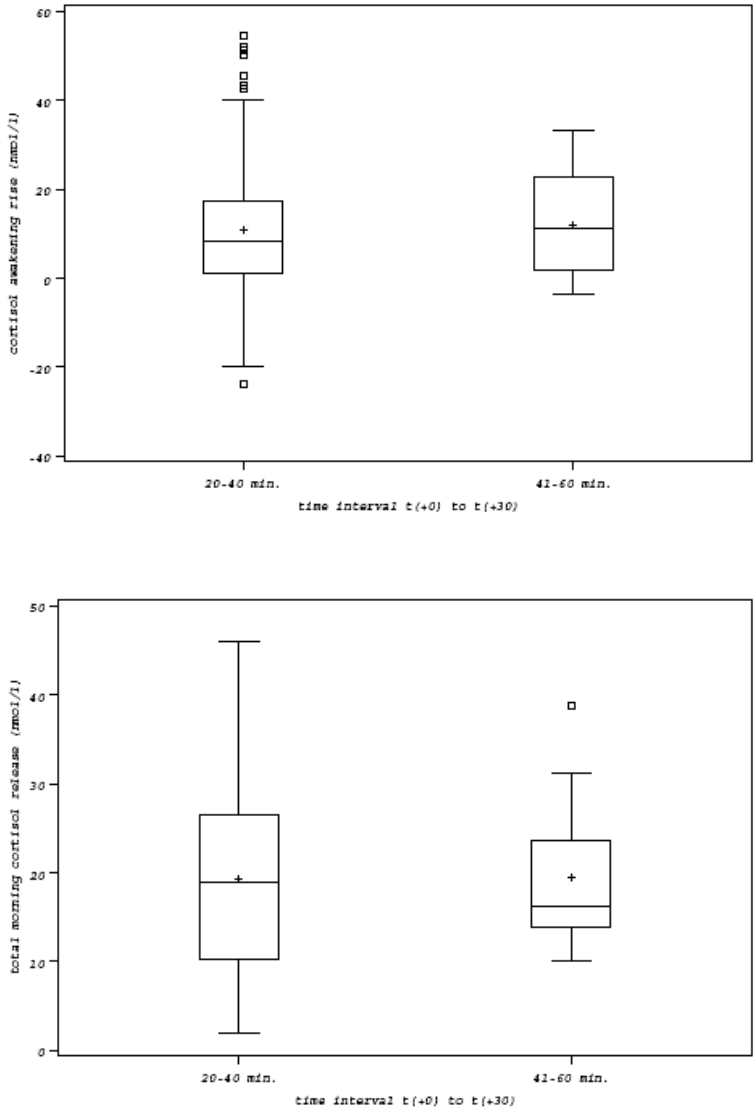

Figure 3: Cortisol levels and length of time interval between t(+0) and t(+30). Above: cortisol awakening rise; below: total morning cortisol release (study 1).

In study 2, 5 of 993 samples (331 participants × 3 sampling days) were collected within 0-19 minutes after awakening (3 responders, 2 non-responders), 867 samples within 20-40 minutes (598 responders, 269 non-responders), 28 samples within 41-60 minutes (13 responders, 15 non-responders), and 15 samples after more than 60 minutes following awak-

ening (11 responders, 4 non-responders). Mixed model analyses for repeated measures for the total sample (Appendix 1, Table 13; Figure 4; due to the small sample size of non-responders in these categories significance testing was not appropriate) revealed no significant main effect of sampling time interval categories with regard to cortisol values at t(+0) ($F=0.85$, $p>0.4$), cortisol values at t(+30) ($F=0.20$, $p> 0.80$; responders), and *total morning cortisol release* ($F=0.37$, $p> 0.70$). A significant main effect of sampling time interval categories regarding the *cortisol awakening rise* was observed for the total sample ($F= 3.85$, $p= 0.02$), subsequent separate analyses for responders and non-responders, however, were inappropriate due to the small sample size of responders and non-responders (Appendix 1, Table 13).

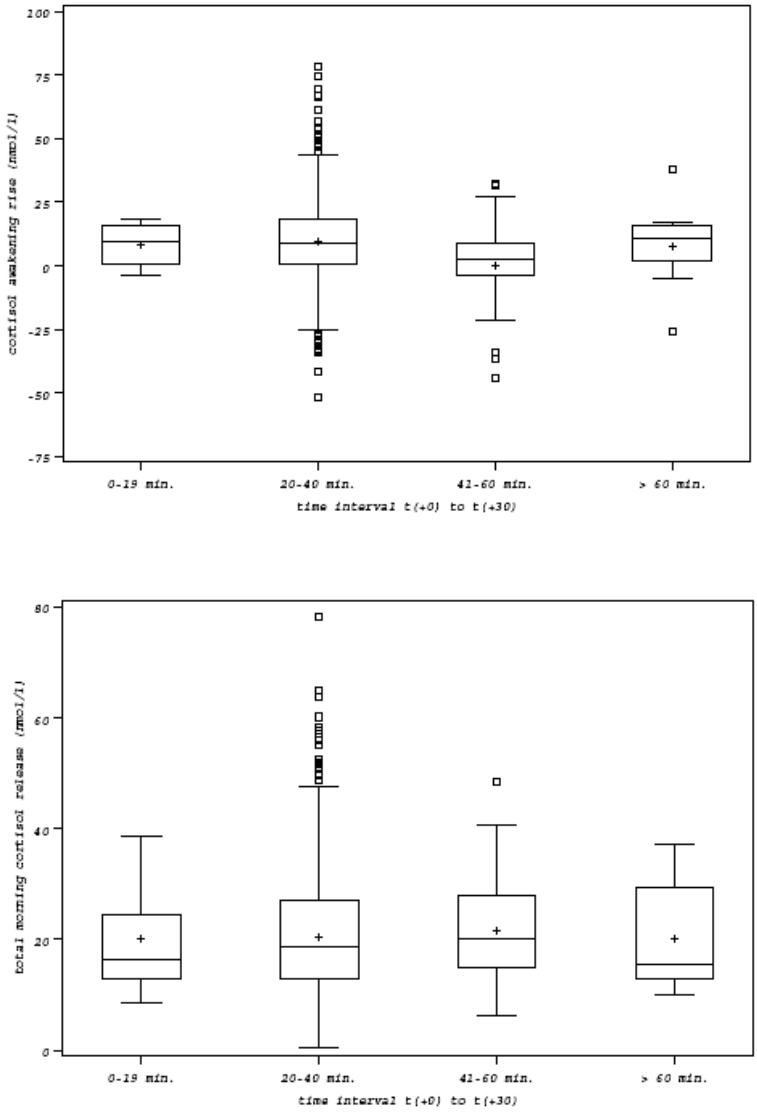

Figure 4: Cortisol levels and length of time interval between t(+0) and t(+30). Above: cortisol awakening rise; below: total morning cortisol release (study 2).

4.1.5 Adherence to sampling protocol: sampling times and corresponding cortisol levels

a) Clock time

In a next step, the impact of adherence to scheduled sampling times (clock time) subsequent to morning cortisol collection on the corresponding cortisol values was examined (study 1: sampling times at 08:00, 11:00, 15:00, 20:00; study 2: sampling times at 16:00 and 20:00).

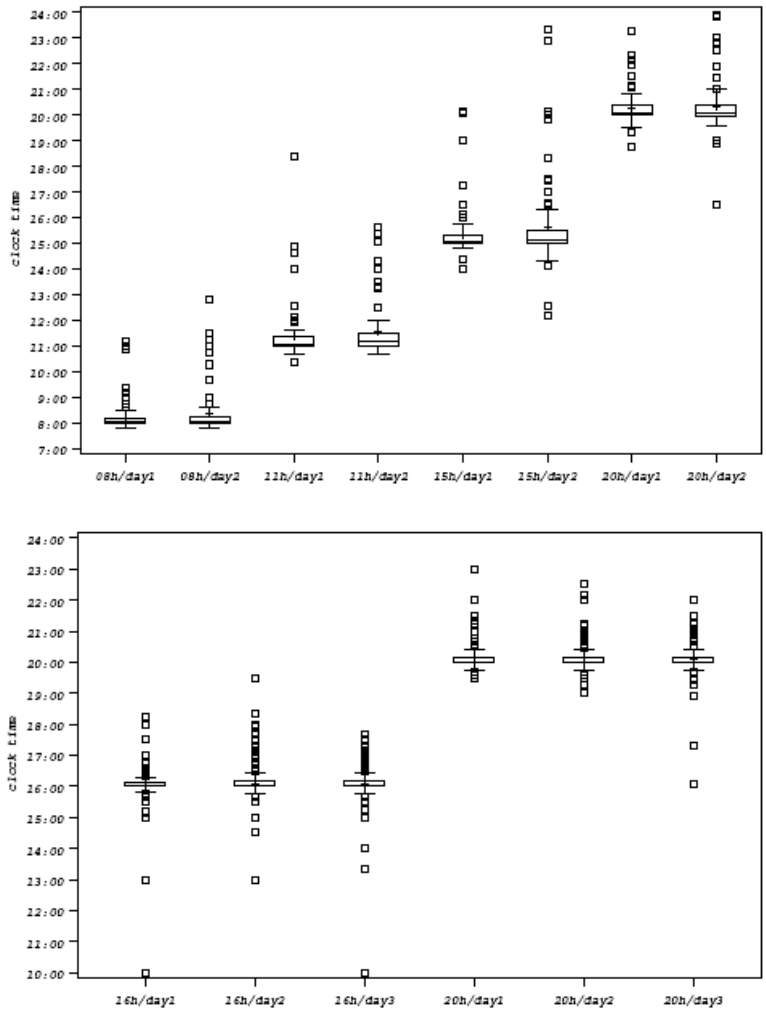

Figure 5: Mean sampling times (clock time). Above: study 1; below: study 2

The mean sampling times are displayed in Figure 5. In study 1, mixed model analyses for repeated measures revealed a significant main effect for sampling day ($F= 14.26$, $p< 0.001$; descriptive statistics: Appendix 1, Table 14), but no interaction effect of sampling day × sampling time ($F= 0.79$, $p> 0.4$). Subsequent contrast analyses revealed significant differences

between sampling days for sampling times scheduled at 15:00 (F= 9.94, p< 0.01). In study 2, however, sampling times did not differ across sampling days (descriptive statistics: Appendix 1, Table 14, mixed model analyses for repeated measures: t(16:00): F= 0.94, p>0.3; t(20:00): F= 0.78, p>0.4).

In study 1, correlation coefficients of cortisol secretion and time of sampling were rather small and unsystematic (Table 5): cortisol secretion and time of sampling were negatively correlated at t(08:00) across sampling days (Pearson's r= -.19, p< 0.01) and on sampling day 2 (r= -.24, p< 0.05), but not on sampling day 1 (r= -.13, p> 0.1), negatively correlated at t(15:00) across sampling days (Pearson's r= -.29, p< 0.001) and on both sampling days separately (day 1: r= -.26, p<0.05; day 2: -.33, p<0.01), and uncorrelated for samples at t(11:00) and t(20:00) (Table 5). Significant correlation coefficients ranged from -.19 to -.33 with maximum coefficients of determination of .11, i.e. 11% of the variance in cortisol output at most could be attributed to the variance in time of sampling. Consistent with correlation analyses, mixed model analyses for repeated measures revealed a significant main effect of sampling time at t(08:00) (F=15.44, p< 0.01) and t(15:00) (F=5.19, p< 0.05), but not for t(11:00) (F= 3.13, p> 0.08) and t(20:00) (F=3.01, p> 0.09).

Table 5: Correlation of sampling time (clock time) and corresponding cortisol samples

	Time of measurement (hh:mm)							
	Day 1+2		**Day 1**		**Day 2**		**Day 3**	
	N	**r⁺**	**N**	**r**	**N**	**r**	**N**	**r**
Study 1								
t(08:00)	184	**-.19****	91	-.13	93	**-.24***	--	--
t(11:00)	186	-.11	93	-.17	93	-.04	--	--
t(15:00)	186	**-.29****	95	**-.26***	91	**-.33****	--	--
t(20:00)	178	-.13	91	-.17	87	-.12	--	--
	Day 1+2+3		**Day 1**		**Day 2**		**Day 3**	
	N	**r⁺**	**N**	**r**	**N**	**r**	**N**	**r**
Study 2								
t(16:00)	957	-.05	318	-.02	324	-.08	315	-.04
t(20:00)	925	-.04	309	-.04	305	-.03	311	-.05

⁺Pearson correlation coefficients; *p< 0.05; **p< 0.01

In study 2, cortisol output and time of sampling were uncorrelated across sampling days and for each sampling day separately (Table 5); this lack of association was confirmed by

subsequent mixed model analyses for repeated measures (t(16:00): F= 1.47, p> 0.2; t(20:00): F= 0.30, p> 0.5). Appendix 3.1 illustrates the correlation between cortisol secretion and time of sampling for both studies.

According to studies on compliance with saliva sampling protocols (Broderick et al., 2004; Kudielka et al., 2003; Kunz-Ebrecht et al., 2004b), that define adherence to the sampling protocol for diurnal cortisol samples by a time window of ± 60 minutes, sampling times were partitioned into three categories: *more than 60 minutes before scheduled time*, *± 60 minutes within scheduled time*, and *more than 60 minutes after scheduled time*. The number of samples outside and within the compliance time windows for each scheduled sampling time as well as the matching mean cortisol output, for both studies across sampling days are given in Appendix 1, Table 15. Due to the small sample size of samples outside the compliance time windows significance testing of differences in cortisol secretion of compliant vs. non-compliant samples was inappropriate.

b) Sampling time synchronised to awakening

Additionally, sampling times synchronised to awakening were calculated for each scheduled sampling time (study 1: t(08:00), t(11:00), t(15:00), t(20:00); study 2: t(16:00), t(20:00)). The mean (relative) sampling times synchronised to awakening are displayed in Figure 6. In both studies, sampling times synchronised to awakening did not differ across sampling days (Appendix 1, Table 16).

In study 1, cortisol secretion and sampling time synchronised to awakening were negatively correlated at t(08:00) on sampling day 2 (Pearson's correlation coefficient r= -.26, p< 0.05) and at t(15:00) across sampling days (r= -.17, p< 0.05) and on sampling day 2 (r= -.25, p< 0.05). All other sampling times were uncorrelated to cortisol output (Table 6). Again, significant correlation coefficients were small and unsystematic with maximum coefficients of determination of .07, i.e. 7% of variance in cortisol output at most could be attributed to variations in sampling times. In consistence with these unsystematic associations, mixed models for repeated measures failed to reveal significant main effects for sampling times (t(11:00): F= 1.12, p> 0.2; t(15:00): F= 2.98, p> 0.08; t(20:00): F= 0.46, p> 0.4), apart from t(08:00) (F= 4.42, p< 0.05).

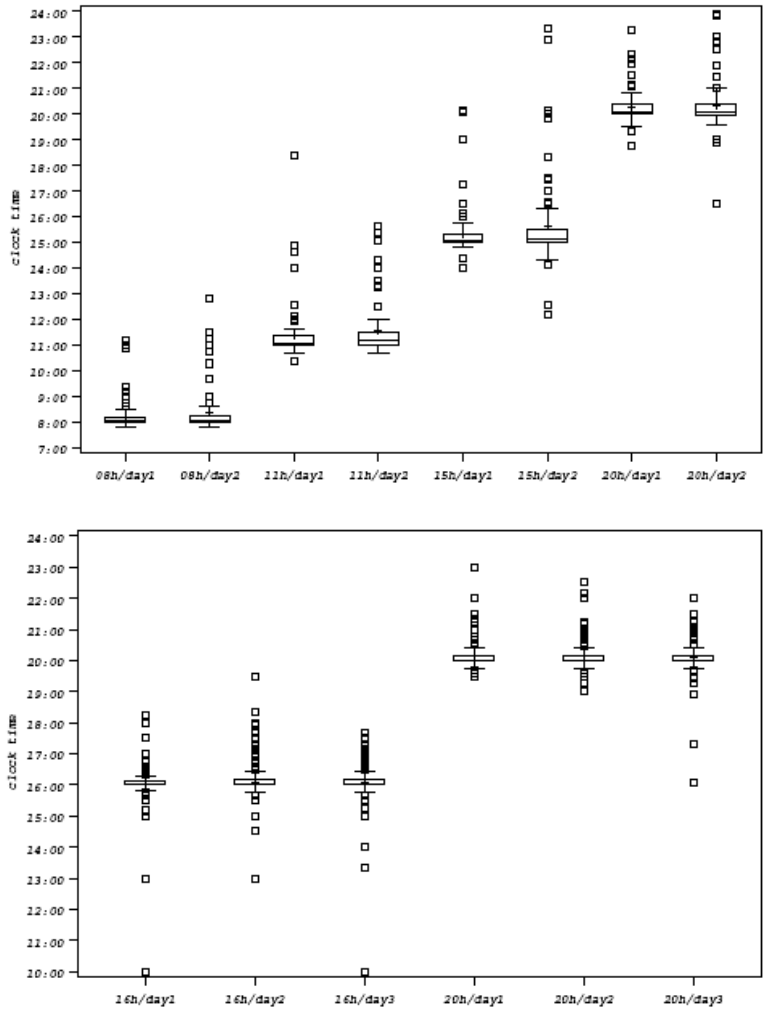

Figure 6: Mean sampling times (relative time). Above: study 1; below: study 2

Table 6: Correlation of relative sampling time and corresponding cortisol samples

	Time of measurement (hh:mm)							
	Day 1+2		**Day 1**		**Day 2**		**Day 3**	
	N	**r⁺**	**N**	**r**	**N**	**r**	**N**	**r**
Study 1								
t(08:00)	181	-.13	90	.01	91	**-.26***	--	--
t(11:00)	182	-.04	91	-.08	91	-.01	--	--
t(15:00)	181	**-.17***	92	-.04	89	**-.25***	--	--
t(20:00)	174	-.06	89	-.06	85	-.06	--	--
	Day 1+2+3		**Day 1**		**Day 2**		**Day 3**	
	N	**r⁺**	**N**	**r**	**N**	**r**	**N**	**r**
Study 2								
t(16:00)	925	-.02	305	-.08	314	.03	306	.00
t(20:00)	896	-.05	298	-.05	296	-.05	302	-.05

⁺Pearson correlation coefficients; *p< 0.05; **p< 0.01

In study 2, cortisol secretion and sampling time synchronised to awakening were uncorrelated across sampling days and for each sampling day separately (Table 6). Likewise, mixed models for repeated measures failed to yield significant effects of sampling time on cortisol output (t(16:00): F= 0.36, p> 0.5; t(20:00): F= 0.58, p> 0.4).

Due to their large range (Appendix 1, Table 16), sampling times synchronised to awakening were subdivided into three categories: sampling times *below the interquartile range* (the first quartile Q1), *within the interquartile range* (IQR= Q3 – Q1), and *above the interquartile range* (the third quartile Q3). Appendix 1, Table 17 shows mean cortisol output across these categories. In both studies, however, cortisol secretion did not differ significantly between sampling time categories, which explains the lack of systematic association between cortisol output and sampling times derived from preceding correlation analyses. Appendix 3.1 illustrates the correlation between cortisol secretion and time of sampling synchronised to awakening for both studies.

4.1.6 Effect of awakening cortisol levels on diurnal cortisol levels

In study 1, there was a significant positive relationship between cortisol secretion at awakening (t+0) and 30 minutes later across sampling days (t(+30); Pearson's correlation coefficient r= .59, p< 0.01; Table 7), i.e. participants with higher levels of cortisol at the time of awakening had higher levels of cortisol 30 minutes thereafter. However, levels of cortisol at awakening were not associated with *cortisol awakening rise* (r= .07, p>0.2; Table 8), although they were significantly correlated with the *diurnal mean* (r= .36, p< 0.01; mean of samples at t(08:00), t(11:00), t(15:00), t(20:00)). Thus participants with high levels of cortisol at awakening did not exhibit a larger *cortisol awakening rise*, but had higher levels of diurnal cortisol. Correlations between cortisol levels at awakening and single diurnal samples were small to medium sized (Table 7). Both, *cortisol awakening rise* and *total morning cortisol release* (mean of samples at t(+0) and t(+30)) were positively correlated with the *diurnal mean* (mean of samples at t(08:00), t(11:00), t(15:00), t(20:00)) across sampling days (*cortisol awakening rise*: r= .36, p< 0.01; *total morning cortisol release*: r= .52, p< 0.01; Table 9). Thus participants with higher cortisol reactivity in the morning exhibited higher diurnal cortisol levels.

These findings, however, could not be replicated in study 2: The association between cortisol levels at awakening and 30 minutes later was by far weaker (r= .28, p< 0.01; Table 7) and correlations between cortisol levels at awakening and single diurnal samples were insignificant (Table 7). In contrast to study 1, cortisol levels at awakening were negatively correlated with *cortisol awakening rise* (r= -.29, p< 0.01; Table 8) and there was no association between cortisol levels at awakening and the *diurnal mean* (r= .05, p> 0.10; Table 8). There was also no association between the *diurnal mean* and both *cortisol awakening rise* (r= 0.02, p> 0.50; Table 9) or *total morning cortisol release* (r= .10, p< 0.01; Table 9). Thus participants with higher levels of cortisol at awakening exhibited smaller sized cortisol awakening rise levels and neither cortisol levels at awakening, nor the *cortisol awakening no rise, or total morning cortisol release* were indicative of the *diurnal mean*. Appendix 3.2 illustrates the reported correlation analyses for both studies.

Table 7: Correlation of awakening cortisol levels to subsequent diurnal samples

	t(+30)		**t(08:00)**		**t(11:00)**		**t(15:00)**		**t(20:00)**	
	N	r	N	r	N	r	N	r	N	r
Study 1										
t(+0), Day 1-2	192	**.59****	181	**.34****	182	**.22****	181	**.34****	175	.13
Day 1	95	**.56****	90	**.26***	91	.17	92	**.28****	90	.03
Day2	97	**.63****	91	**.45****	91	**.27****	89	**.42****	85	**.25****
	t(+30)						**t(16:00)**		**t(20:00)**	
	N	r					N	r	N	r
Study 2										
t(+0), Day 1-3	924	**.28****	--	--	--	--	925	.04	896	**.07***
Day1	307	**.29****	--	--	--	--	305	-.03	298	-.03
Day 2	310	**.27****	--	--	--	--	314	.07	296	.07
Day 3	307	**.29****	--	--	--	--	306	.09	302	**.14***

Pearson correlation coefficients; *p< 0.05; **p< 0.01

Table 8: Correlation of awakening cortisol levels to cortisol awakening rise / diurnal mean

	Cortisol awakening rise		Diurnal mean	
	N	r	N	r
Study 1				
t(+0), Day 1-2	192	-.07	194	**.36****
Day 1	95	-.08	97	**.27****
Day2	97	-.07	97	**.48****
Study 2				
t(+0), Day 1-3	924	**-.29****	948	.05
Day1	307	**-.25****	315	-.05
Day 2	310	**-.36****	318	.08
Day 3	307	**-.26****	315	.11

Pearson correlation coefficients; *p< 0.05; **p< 0.01

Table 9: Correlation of post-awakening cortisol levels to the diurnal mean

	Cortisol awakening rise		Total morning cortisol release	
	N	r	N	r
Study 1				
Diurnal mean				
Day 1-2	190	**.36****	196	**.52****
Day 1	95	**.31****	97	**.44****
Day2	95	**.41****	99	**.60****
Study 2				
Diurnal mean				
Day 1-3	918	.02	948	**.10****
Day1	306	.04	315	.00
Day 2	308	.03	318	**.13***
Day 3	304	-.01	315	**.17****

Pearson correlation coefficients; *p< 0.05; **p< 0.01

To check whether the lack of association between cortisol levels at awakening / *cortisol awakening rise* / *total morning cortisol release* and diurnal levels of cortisol in study 2 was due to a different sampling protocol, correlation analyses in study 1 were rerun with mean diurnal cortisol levels calculated analogue to study 2 (diurnal mean – short profile in study 1: mean of samples at t(15:00) and t(20:00) instead of long profile: mean of samples at t(08:00), t(11:00), t(15:00), t(20:00)). Although to a lesser extent, the positive relationship

between diurnal levels of cortisol and cortisol levels at awakening (t(+0); r= .29, p< 0.01) / *cortisol awakening rise* (r= .23, p< 0.01) / *total morning cortisol release* (r= .40, p< 0.01) remained unaffected.

4.1.7 Relationship between single samples and diurnal mean

In consistency with a study by Edwards and co-workers (2001a) investigating the predictive power of single samples in representing mean diurnal basal cortisol levels, we examined whether single cortisol samples were indicative of basal cortisol secretion throughout the day. In study 1, each single sample was positively correlated with diurnal cortisol levels across sampling days and on both sampling days separately (Table 10), whereas in study 2 these associations could not be replicated (Table 10).

Table 10: Correlation of cortisol single samples with diurnal mean

	Day 1-2		Day 1		Day 2			
	N	r	N	r	N	r		
Study 1								
t(+0) with mean of t(08:00), t(11:00), t(15:00), t(20:00)	194	**.36****	97	**.26****	97	**.48****		
t(08:00) with mean of t(+0), t(11:00), t(15:00), t(20:00)	184	**.55****	91	**.53****	93	**.59****		
t(11:00) with mean of t(+0), t(08:00), t(15:00), t(20:00)	186	**.50****	93	**.51****	93	**.48****		
t(15:00) with mean of t(+0), t(08:00), t(11:00), t(20:00)	186	**.59****	95	**.61****	91	**.57****		
t(20:00) with mean of t(+0), t(08:00), t(11:00), t(15:00)	179	**.32****	92	**.27****	87	**.37****		
	Day 1-3		**Day 1**		**Day 2**		**Day 3**	
	N	**r**	**N**	**r**	**N**	**r**	**N**	**r**
Study 2								
t(+0) with mean of t(16:00), t(20:00)	948	.05	315	-.05	318	.08	315	.11
t(16:00) with mean of t(+0), t(20:00)	952	**.12****	315	.04	323	**.18****	314	**.14***
t(20:00) with mean of t(+0), t(16:00)	923	**.19****	308	.10	305	**.20****	310	**.26****

Pearson correlation coefficients; *p< 0.05; **p< 0.01

Again, to check whether this lack of association in study 2 was due to the shorter sampling protocol, correlation analyses in study 1 were repeated with diurnal cortisol levels calculated analogue to study 2 (diurnal levels: mean of samples at t(15:00), t(20:00)): al-

though diminished in size, the positive correlation of single samples with diurnal cortisol levels persisted (cortisol levels at awakening – t(+0): r= .29, p< 0.01; cortisol levels at t(15:00): r= .34, p< 0.01; cortisol levels at t(20:00): r= .24, p< 0.1).

4.2 Confounding factors

4.2.1 Awakening time

The mean awakening times are displayed in Figure 7. In both studies, awakening times did not differ significantly across sampling days (Appendix 1, Table 18; study 1: F=3.31, p=0.07; study 2: F=2.19, p=0.11).

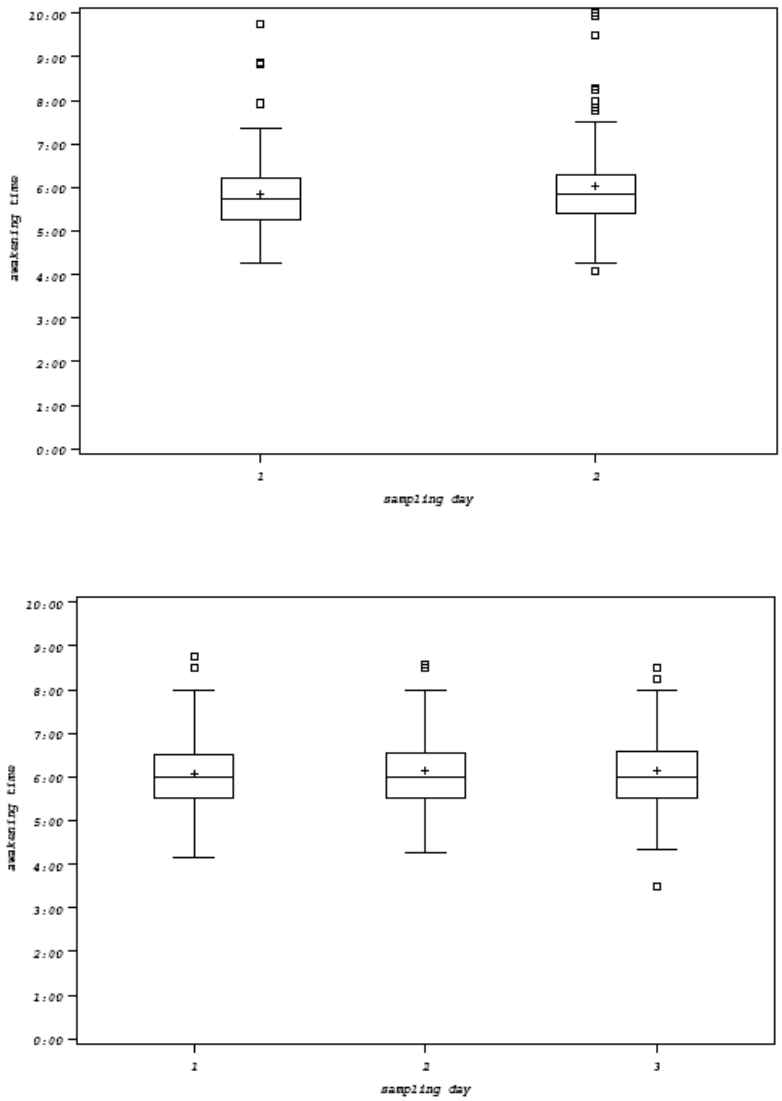

Figure 7: Mean awakening times. Above: study 1; below: study 2.

In study 1, awakening time was negatively correlated (Pearson's correlation coefficient r) with the *AUC including t(+30)* as well as *AUC excluding t(+30)*, see Table 11. All other cortisol measures, however, were uncorrelated with awakening time and the significant correlation coefficients were relatively small and unsystematic: significant correlation coefficients ranged from -.26 to .15 with maximum coefficients of determination of .07, i.e. 7 % of the variance of cortisol values or parameters at most could be attributed to the variance of awakening time.

In study 2, awakening time was negatively correlated with the *cortisol awakening rise*, the *AUC including t(+30)*, and the *slope of change excluding t(+30)*, and positively correlated with cortisol secretion at t(+0), see Table 11. Comparable to study 1, the significant correlation coefficients were not substantial and unsystematic: significant correlation coefficients ranged from -.15 to .14 with maximum coefficients of determination of .02, i.e. 2 % of the variance of cortisol values or parameters at most could be attributed to the variance of awakening time.

Table 11: Association of awakening time and cortisol values and parameters

	Time of awakening (hh:mm)							
	Day 1+2		**Day 1**		**Day 2**		**Day 3**	
	N	**r⁺**	**N**	**r**	**N**	**r**	**N**	**r**
Study 1								
t(+0)	196	.09	97	**.26****	99	-.07	--	--
t(+30)	192	.03	95	.06	97	.01	--	--
t(08:00)	181	-.07	90	-.11	91	-.01	--	--
t(11:00)	182	-.10	91	-.12	91	-.07	--	--
t(15:00)	181	**-.23****	92	**-.26***	89	-.20	--	--
t(20:00)	175	-.06	90	-.08	85	-.04	--	--
Cortisol awakening rise	192	.01	95	-.08	97	.10	--	--
Total morning cortisol release	196	.05	97	.13	99	-.02	--	--
Total diurnal cortisol release								
Mean [with t(+30)]	182	-.02	92	-.01	90	-.03	--	--
Mean [without t(+30)]	182	-.02	92	.00	90	-.03	--	--
AUC [with t(+30)]	182	**-.26****	92	**-.26***	90	**-.25***	--	--
AUC [without t(+30)]	182	**-.23****	92	**-.22***	90	**-.24***	--	--
Diurnal cortisol decline								
Slope [without t(+0)]	182	.00	92	.06	90	-.06	--	--
Slope [without t(+30)]	182	-.12	92	-.11	90	-.13	--	--
Delta [t(+30) – t(20:00)]	172	.02	88	-.02	84	.07	--	--
Delta [t(+0) – t(20:00)]	175	**.15***	90	.17	85	.13	--	--
	Day 1+2+3		**Day 1**		**Day 2**		**Day 3**	
	N	**r⁺**	**N**	**r**	**N**	**r**	**N**	**r**
Study 2								
t(+0)	956	**.14****	316	**.18****	321	.06	319	**.17****
t(+30)	915	**-.08***	305	-.06	307	-.04	303	**-.14***
t(16:00)	925	.00	302	.08	314	-.07	306	-.02
t(20:00)	896	.04	298	.04	296	.04	302	.02
Cortisol awakening rise	924	**-.15****	307	**-.15****	310	-.07	307	**-.21****
Total morning cortisol release	956	-.01	316	.03	321	-.03	319	-.02
Total diurnal cortisol release								
Mean [with t(+30)]	943	-.02	313	.04	315	-.02	315	-.06
Mean [without t(+30)]	943	**.11****	313	**.17****	315	.05	315	.09
AUC [with t(+30)]	943	**-.12****	313	-.02	315	**-.17****	315	**-.17****
AUC [without t(+30)]	922	-.02	304	.07	312	-.09	306	-.04
Diurnal cortisol decline								
Slope [without t(+0)]	943	.03	313	.01	315	-.05	315	**.12***
Slope [without t(+30)]	943	**-.14****	313	**-.17****	315	**-.11***	315	**-.15****
Delta [t(+30) – t(20:00)]	869	**-.11****	290	-.10	288	-.06	291	-.15
Delta [t(+0) – t(20:00)]	869	**.09****	298	**.12***	296	.05	302	.09

⁺Pearson correlation coefficients; * p< 0.05; ** p< 0.01.

4.2.2 Sleep duration and quality of sleep

a) Sleep duration

Sleep duration was not recorded in study 1, thus results are reported for study 2 only. Mean sleep duration for each night before sampling days is displayed in Figure 8. Mixed models for repeated measures revealed a significant main effect of sampling day (Appendix 1, Table 19; F= 6.02, p< 0.0001) indicating varying awakening times of day 1 and day 2.

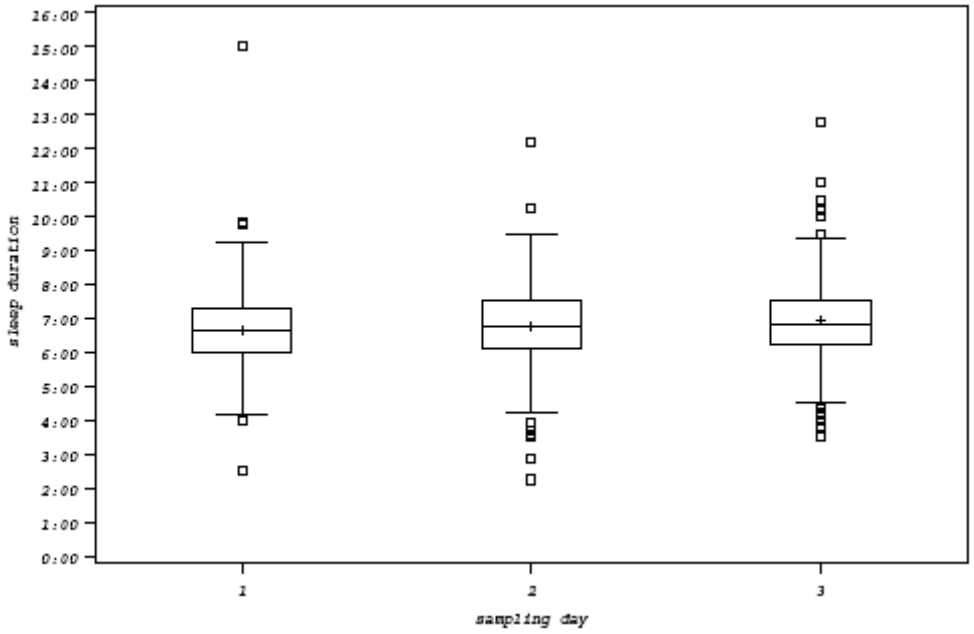

Figure 8: Sleep duration (study 2).

Sleep duration was positively correlated with cortisol levels at awakening (t(+0), with the *diurnal mean excluding t(+30)*, and *decline from t(+0) to t(20:00)* and negatively correlated with *cortisol awakening rise* and *slope of change excluding t(+30)* across nights, see Table 12. All other cortisol measures, however, were uncorrelated with sleep duration (Table 12) and significant correlation coefficients were small und unsystematic: significant correlation coefficients ranged from -.14 to .18 with maximum coefficients of determination of .03, i.e. 3 % of the variance of cortisol values or parameters could be attributed to the variance of sleep duration.

Table 12: Association of sleep duration and cortisol values and parameters

	Sleep duration (hh:mm)							
	Night 1+2+3		**Night 1**		**Night 2**		**Night 3**	
	N	**r**[+]	**N**	**r**	**N**	**r**	**N**	**r**
Study 2								
t(+0)	932	**.18****	310	**.21****	314	**.13***	308	**.18****
t(+30)	922	.00	311	.01	309	.04	302	-.04
t(16:00)	931	-.06	312	-.08	316	-.02	303	-.07
t(20:00)	900	.03	303	.06	298	-.08	299	.07
Cortisol awakening rise	900	**-.08***	301	-.07	303	-.01	296	**-.14***
Total morning cortisol release	932	**.08***	310	**.12***	314	.05	308	.06
Total diurnal cortisol release								
Mean [with t(+30)]	937	.06	314	.09	313	.04	310	.04
Mean [without t(+30)]	937	**.12****	314	**.14***	313	.07	310	**.13***
AUC [with t(+30)]	937	-.03	314	-.03	313	.02	310	-.06
AUC [without t(+30)]	917	.06	305	.08	310	.04	302	.04
Diurnal cortisol decline								
Slope [without t(+0)]	937	-.03	314	-.06	313	-.10	310	.05
Slope [without t(+30)]	937	**-.14****	314	**-.14***	313	**-.14***	310	**-.12***
Delta [t(+30) – t(20:00)]	865	.00	291	.01	287	.08	287	-.06
Delta [t(+0) – t(20:00)]	874	**.12****	292	**.15****	290	**.12***	292	.09

[+]Pearson correlation coefficients; * $p < 0.05$; ** $p < 0.01$.

b) Sleep quality

In study 1, self-reported sleep deprivation (lack of sufficient sleep) during each night before sampling days was used as a proxy for sleep quality. On sampling day 1, 40 participants reported insufficient sleep compared to 59 participants with sufficient sleep (3 missing values); on sampling day 2, 34 participants reported lack of sleep compared to 62 participants with sufficient sleep (6 missing values). Frequency of sleep deprivation did not differ across sampling days (two-sided Fisher's exact test; $p > 0.5$). There was no impact of self-reported sleep deprivation on single cortisol samples or cortisol parameters: mixed model analyses for repeated measures did not reveal significant main effects for night before day of sampling or sleep deprivation (Appendix 1, Table 20).

In study 2, the frequency and duration of self-reported sleep disruption episodes was used as a proxy for sleep quality. A significant majority of the sample in study 2 reported episodes of sleep disruption at nights before sampling days: 225 participants reported episodes of sleep disruption at night 1 (the night before sampling day 1), 189 at night 2, and 171

at night 3 (compared to 59 participants without sleep disruption at night 1, 83 at night 2, and 94 at night 3; Chi-square: $p < 0.001$). The mean frequency of sleep disruption was 2.3 at night 1 (min: 1, max: 10), 1.9 at night 2 (min: 1, max: 5), and 1.9 at night 3 (min: 1, max: 5). Appendix 1, Table 21 shows the mean duration of sleep disruption per night. There was no impact of frequency of sleep disruption episodes on cortisol levels of single samples or parameters (Table 13): mixed models for repeated measures failed to yield significant main effects of frequency of sleep disruption episodes on cortisol profiles and composite measures.

However, the duration of sleep disruption episodes affected cortisol parameters, but not cortisol levels of single samples (Table 14): the *cortisol awakening rise* (mixed models for repeated measures: $F = 4.95$, $p < 0.05$), *diurnal mean including t(+30)* ($F=4.22$, $p < 0.05$), the *AUC including t(+30)* ($F = 6.42$, $p<0.05$), and *slope of change excluding (t+0)* ($F=4.34$, $p < 0.05$). Subsequent correlation analyses of cortisol levels of single samples and parameters and duration of sleep disruption episodes (Appendix 1, Table 22) clarified these results: Although the same parameters were significantly correlated with duration of sleep disruption, the corresponding correlation coefficients were small and unsystematic: significant correlation coefficients for *cortisol awakening rise*, *AUC including t(+30*), and *slope of change excluding (t+0)* could only be obtained across days (single days were uncorrelated) and ranged from -.10 to .08 with maximum coefficients of determination of .01, i.e. 1 % of variance in cortisol levels at most could be attributed to the variance of the duration of sleep disruption episodes. Additionally, the *diurnal mean including t(+30)* was significantly correlated with duration of sleep disruption on day 3 only (Appendix 1, Table 22).

Table 13: Effect of frequency of sleep disruption episodes on cortisol levels

Dependent variable	Effect	NDF	DDF	F	p
Study 2					
Single samples:	DAY	2	511	1.85	
t(+0), t(+30), t(16:00), t(20:00)	TIME	3	909	667.51	**
	FREQ	1	2811	0.57	
	DAY × FREQ	2	2811	1.24	
	TIME × FREQ	3	2811	0.71	
Cortisol awakening rise	DAY	2	461	0.27	
	FREQ	1	461	1.44	
	DAY × FREQ	2	461	0.21	
Total morning cortisol release	DAY	2	485	1.29	
	FREQ	1	485	0.23	
	DAY × FREQ	2	485	2.02	
Total diurnal cortisol release					
Mean [with t(+30)]	DAY	2	488	1.38	
	FREQ	1	488	0.28	
	DAY × FREQ	2	488	1.18	
Mean [without t(+30)]	DAY	2	488	0.79	
	FREQ	1	488	0.08	
	DAY × FREQ	2	488	0.45	
AUC [with t(+30)]	DAY	2	488	0.25	
	FREQ	1	488	2.42	
	DAY × FREQ	2	488	1.22	
AUC [without t(+30)]	DAY	2	474	0.86	
	FREQ	1	474	0.05	
	DAY × FREQ	2	474	0.30	
Diurnal cortisol decline					
Slope [without t(+0)]	DAY	2	488	0.13	
	FREQ	1	488	0.17	
	DAY × FREQ	2	488	0.97	
Slope [without t(+30)]	DAY	2	488	0.97	
	FREQ	1	488	3.09	
	DAY × FREQ	2	488	0.73	
Delta [t(+30) – t(20:00)]	DAY	2	433	0.12	
	FREQ	1	433	0.28	
	DAY × FREQ	2	433	0.95	
Delta [t(+0) – t(20:00)]	DAY	2	438	0.38	
	FREQ	1	438	2.87	
	DAY × FREQ	2	438	1.24	

NDF= numerator degrees of freedom; DDF= denominator degrees of freedom; DAY= sampling day; FREQ= frequency of sleep disruption episodes; TIME= sampling time (t(+0) to t(20:00)); mixed models for repeated measures: *p<0.05; ** p<0.01

Table 14: Effect of duration of sleep disruption episodes on cortisol levels

Dependent variable	Effect	NDF	DDF	F	p
Study 2					
Single samples:	DAY	2	488	3.67	*
t(+0), t(+30), t(16:00), t(20:00)	TIME	3	893	1165.07	**
	LENGTH	1	2708	0.96	
	DAY × LENGTH	2	2708	4.92	**
	TIME × LENGTH	3	2708	2.89	*
Cortisol awakening rise	DAY	2	436	1.05	
	LENGTH	1	436	4.95	*
	DAY × LENGTH	2	436	0.20	
Total morning cortisol release	DAY	2	460	1.59	
	LENGTH	1	460	1.71	
	DAY × LENGTH	2	460	4.43	*
Total diurnal cortisol release					
Mean [with t(+30)]	DAY	2	466	1.73	
	LENGTH	1	466	4.22	*
	DAY × LENGTH	2	466	3.63	*
Mean [without t(+30)]	DAY	2	466	3.47	*
	LENGTH	1	466	0.32	
	DAY × LENGTH	2	466	3.80	*
AUC [with t(+30)]	DAY	2	466	0.90	
	LENGTH	1	466	6.42	*
	DAY × LENGTH	2	466	2.03	
AUC [without t(+30)]	DAY	2	453	2.04	
	LENGTH	1	453	0.54	
	DAY × LENGTH	2	453	3.38	*
Diurnal cortisol decline					
Slope [without t(+0)]	DAY	2	466	0.57	
	LENGTH	1	466	4.34	*
	DAY × LENGTH	2	466	0.38	
Slope [without t(+30)]	DAY	2	466	1.12	
	LENGTH	1	466	0.01	
	DAY × LENGTH	2	466	2.42	
Delta [t(+30) – t(20:00)]	DAY	2	414	0.41	
	LENGTH	1	414	2.27	
	DAY × LENGTH	2	414	0.52	
Delta [t(+0) – t(20:00)]	DAY	2	418	0.40	
	LENGTH	1	418	0.01	
	DAY × LENGTH	2	418	3.00	

NDF= numerator degrees of freedom; DDF= denominator degrees of freedom; DAY= sampling day; LENGTH= duration of sleep disruption episodes; TIME= sampling time (t(+0) to t(20:00)); mixed models for repeated measures: *$p<0.05$; ** $p<0.01$

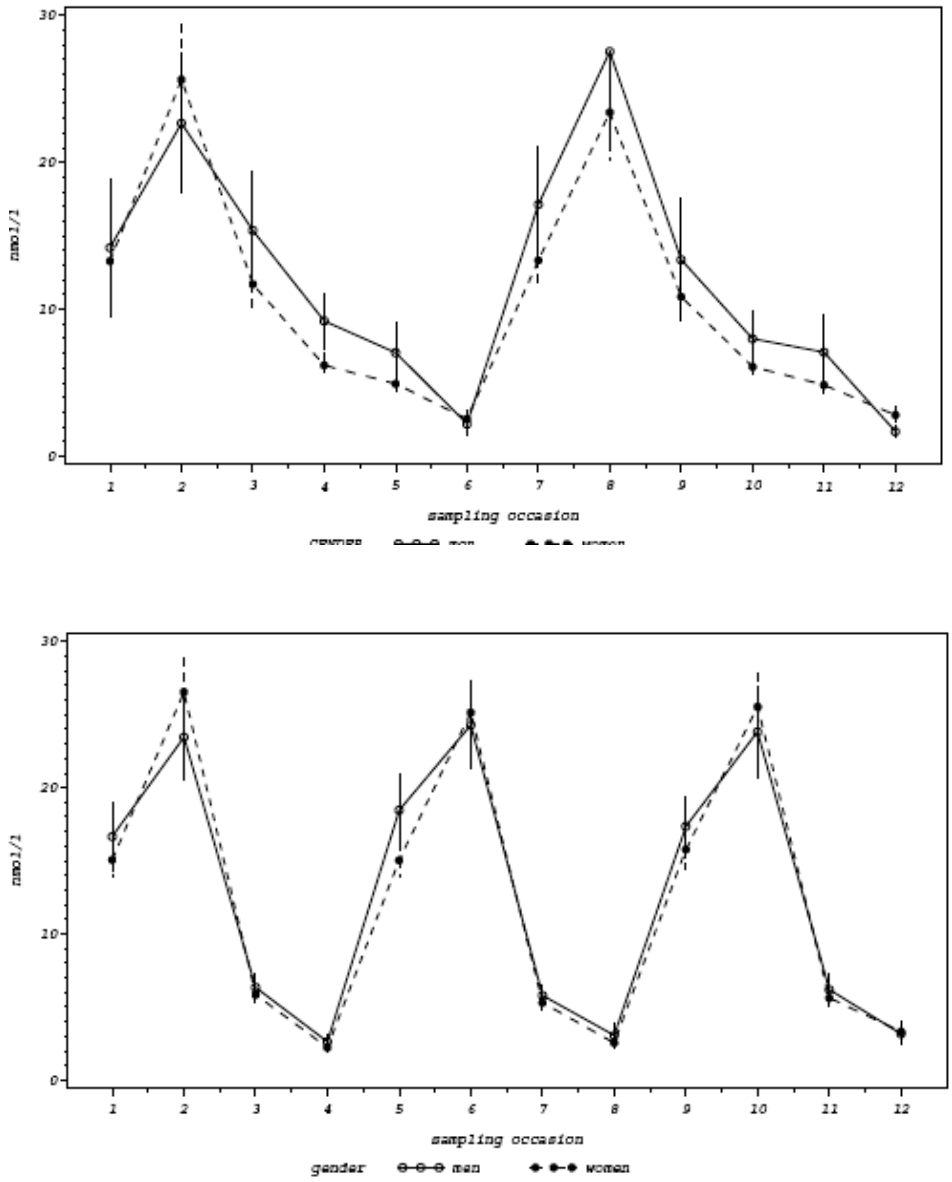

Figure 9: Diurnal salivary cortisol profiles in men and women. Above: study 1, below: study 2.

4.2.3 Gender and intake of oral contraceptives

a) Gender

In study 1, cortisol levels were slightly higher in men compared to women (Figure 9; see Appendix 1, Table 23 and 24 for descriptive statistics). With regard to diurnal cortisol profiles, mixed model analyses for repeated measures did not yield a significant main effect of gender, but a significant interaction effect of sampling time × gender ($F= 3.74$, $p< 0.01$; Table

15). Decomposing the significant interaction effect by examining the effect slices, significant differences in cortisol levels between men and women emerged for samples collected at t(08:00) (F= 4.43, p< 0.05), at t(11:00) (F= 7.32, p< 0.01), and at t(15:00) (F= 5.72, p< 0.05). Apart from the *diurnal mean excluding t(+30)*, (F= 5.22, p< 0.05) and the *AUC excluding t(+30)* (F= 6.74, p< 0.05), mixed model analyses for repeated measures did not reveal significant differences in cortisol levels between men and women (Table 15). Thus, although men and women did not differ in cortisol levels subsequent to awakening and 30 minutes thereafter (neither in single samples nor in composite measures, i.e. *cortisol awakening rise* and *total morning cortisol release*), cortisol levels reflecting diurnal cortisol output (*diurnal mean excluding t(+30), AUC excluding t(+30)*) were higher in men.

In study 2, these effects were partly confirmed: Compared to women men showed slightly higher cortisol levels (Figure 9; see Appendix 1, Table 23 and 24 for descriptive statistics). Although there was no significant main effect of gender alone, mixed model analyses for repeated measures revealed a significant interaction effect of sampling time × gender (F= 4.60, p< 0.01; Table 16). Decomposing the significant interaction effect by examining the effect slices, significant differences in cortisol levels between men and women emerged for samples collected at t(+0) (F= 4.31, p< 0.05) and t(16:00) (F= 4.32, p< 0.05). Apart from the *cortisol awakening rise* (F=8.94, p< 0.01), the *diurnal mean excluding t(+30)*, (F= 4.07, p< 0.05) and the *slope of change excluding (t+0)* (F= 4.17, p< 0.05), mixed model analyses for repeated measures did not reveal significant differences in cortisol levels between men and women (Table 16): Although cortisol levels directly after awakening were higher in men, women displayed a larger *cortisol awakening rise*. Cortisol levels reflecting the diurnal output (*diurnal mean excluding t(+30)*) were higher in men and the diurnal decline of cortisol throughout the day (*slope of change excluding (t+0)*) was steeper in men compared to women.

Table 15: Effect of gender on cortisol levels in study 1

Dependent variable	Effect	NDF	DDF	F	p
Study 1					
Single samples: t(+0), t(+30), t(08:00), t(11:00), t(15:00), t(20:00)	DAY	1	97	0.01	
	TIME	5	485	264.60	**
	SEX	1	100	3.22	
	DAY × SEX	1	97	0.06	
	TIME × SEX	5	485	3.74	**
Cortisol awakening rise	DAY	1	88	0.35	
	SEX	1	100	0.34	
	DAY × SEX	1	88	0.60	
Total morning cortisol release	DAY	1	94	1.34	
	SEX	1	100	0.65	
	DAY × SEX	1	94	1.24	
Total diurnal cortisol release					
Mean [with t(+30)]	DAY	1	84	0.16	
	SEX	1	96	2.79	
	DAY × SEX	1	84	0.31	
Mean [without t(+30)]	DAY	1	84	0.00	
	SEX	1	96	5.22	*
	DAY × SEX	1	84	0.02	
AUC [with t(+30)]	DAY	1	84	0.00	
	SEX	1	96	3.78	
	DAY × SEX	1	84	0.31	
AUC [without t(+30)]	DAY	1	84	0.06	
	SEX	1	96	6.74	*
	DAY × SEX	1	84	0.23	
Diurnal cortisol decline					
Slope [without t(+0)]	DAY	1	84	0.45	
	SEX	1	96	0.01	
	DAY × SEX	1	84	0.74	
Slope [without t(+30)]	DAY	1	84	0.71	
	SEX	1	96	1.41	
	DAY × SEX	1	84	0.71	
Delta [t(+30) – t(20:00)]	DAY	1	76	1.33	
	SEX	1	94	0.84	
	DAY × SEX	1	76	1.79	
Delta [t(+0) – t(20:00)]	DAY	1	76	2.63	
	SEX	1	95	3.28	
	DAY × SEX	1	76	2.18	

NDF= numerator degrees of freedom; DDF= denominator degrees of freedom; DAY= sampling day; SEX= gender; TIME= sampling time (t(+0) to t(20:00)); mixed models for repeated measures: *$p<0.05$; ** $p<0.01$

Table 16: Effect of gender on cortisol levels in study 2

Dependent variable	Effect	NDF	DDF	F	p
Study 2					
Single samples:	DAY	2	655	1.02	
t(+0), t(+30), t(16:00), t(20:00)	TIME	3	982	1597.13	**
	SEX	1	329	1.82	
	DAY × SEX	2	655	0.96	
	TIME × SEX	3	982	4.60	**
Cortisol awakening rise	DAY	2	593	0.07	
	SEX	1	325	8.94	**
	DAY × SEX	2	593	0.10	
Total morning cortisol release	DAY	2	622	1.13	
	SEX	1	328	0.12	
	DAY × SEX	2	622	0.91	
Total diurnal cortisol release					
Mean [with t(+30)]	DAY	2	629	0.13	
	SEX	1	327	0.00	
	DAY × SEX	2	629	0.11	
Mean [without t(+30)]	DAY	2	629	0.34	
	SEX	1	327	4.07	*
	DAY × SEX	2	629	0.55	
AUC [with t(+30)]	DAY	2	629	1.46	
	SEX	1	327	0.37	
	DAY × SEX	2	629	2.01	
AUC [without t(+30)]	DAY	2	609	0.03	
	SEX	1	326	2.77	
	DAY × SEX	2	609	0.06	
Diurnal cortisol decline					
Slope [without t(+0)]	DAY	2	629	0.30	
	SEX	1	327	1.15	
	DAY × SEX	2	629	0.58	
Slope [without t(+30)]	DAY	2	629	0.36	
	SEX	1	327	4.17	*
	DAY × SEX	2	629	0.25	
Delta [t(+30) – t(20:00)]	DAY	2	558	0.13	
	SEX	1	324	2.14	
	DAY × SEX	2	558	0.44	
Delta [t(+0) – t(20:00)]	DAY	2	566	0.45	
	SEX	1	324	3.28	
	DAY × SEX	2	566	0.45	

NDF= numerator degrees of freedom; DDF= denominator degrees of freedom; DAY= sampling day; SEX= gender; TIME= sampling time (t(+0) to t(20:00)); mixed models for repeated measures: *$p<0.05$; ** $p<0.01$

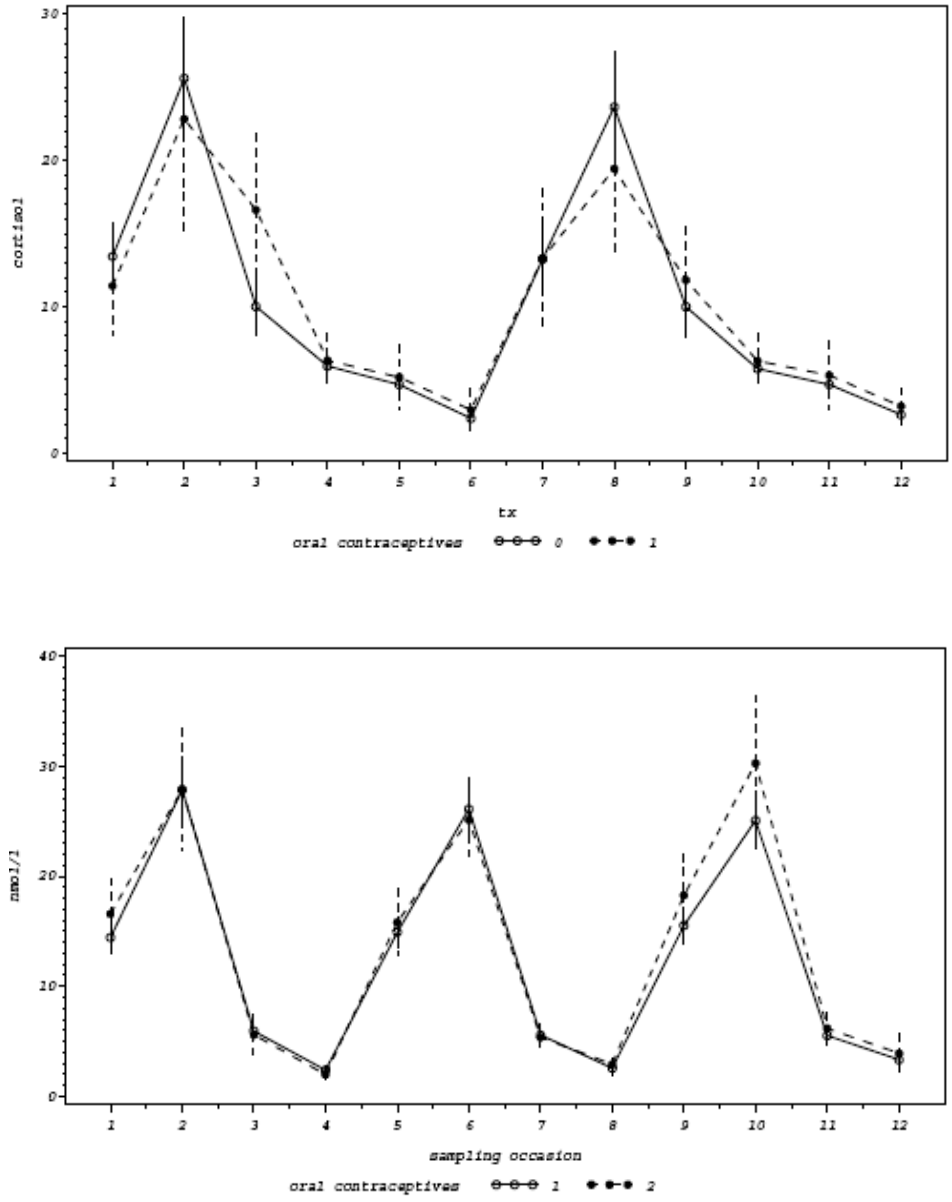

Figure 10: Diurnal salivary cortisol profiles in women with or without oral contraceptives. Above: study 1, below: study 2.

b) Oral contraceptives

In the subsample of women in study 1 (n= 80), 17 (22 %) reported to take oral contraceptives (OC). To check whether OC intake affected cortisol levels of single samples and parameters (see Appendix 1, Table 25 and 26 for descriptive statistics) mixed model analyses for repeated measures were applied: With regard to diurnal cortisol profiles there was no significant main effect of OC intake on single cortisol samples (Figure 10), apart from a sig-

nificant interaction effect of sampling time × OC intake (F= 2.32, p< 0.05). Decomposing the significant interaction effect by examining the effect slices, however, did not yield significant differences in cortisol levels at different sampling times throughout the day between women with or without OC intake (Appendix 1, Table 27). In addition, cortisol levels of composite measures did not differ significantly between women reporting to take OC and those reporting not to (Appendix 1, Table 27).

In study 2, 48 women (21 %) reported to take oral contraceptives (subsample of women in study 2: n= 225; 35 women failed to specify OC intake). There was no impact of OC intake on single cortisol samples (Figure 10) or parameters (see Appendix 1, Table 25 and 26 for descriptive statistics): mixed model analyses for repeated measures did neither reveal significant main effects of OC intake nor significant interaction effects (Appendix 1, Table 28).

4.2.4 Age

In study 1, there was a significant effect of age on morning cortisol levels (Table 17): older participants tended to display higher levels of *total morning cortisol release* across sampling days (Pearson's correlation coefficient; across sampling days: r= .25, p< 0.01; day 1: r= .31, p< 0.01; day 2: r= .20, p< 0.05). In addition, the diurnal decrease in cortisol throughout the day varied with the participants' age: older participants tended to have a steeper decline of diurnal cortisol which in all probability was due to their larger cortisol levels subsequent to awakening (*slope of change excluding t(+0)*: r= -.31, p< 0.01; *slope of change excluding t(+30)*: r= -.20, p< 0.01; *decline from t(+30) to t(20:00)*: r= .32, p< 0.01; *decline from t(+0) to t(20:00)*: r= .17, p< 0.05; Table 17). However, the significant correlation coefficients were rather small with maximum coefficients of determination of 0.10, i.e. a maximum of 10 % of variance in cortisol levels could be attributed to the variance of participants' age. Mixed model analyses for repeated measures provided the same results obtained from the preceding correlation analyses (Table 18).

Table 17: Association between age and cortisol levels

	Age (in years)							
	Day 1+2		Day 1		Day 2		Day 3	
	N	r+	N	r	N	r	N	r
Study 1								
t(+0)	196	**.19****	97	**.22***	99	.16	--	--
t(+30)	194	**.24****	95	**.30****	99	.19	--	--
t(08:00)	184	.10	91	.04	93	.16	--	--
t(11:00)	186	.06	93	-.06	93	.17	--	--
t(15:00)	186	.12	95	.06	91	.17	--	--
t(20:00)	179	-.05	92	.00	87	-.10	--	--
Cortisol awakening rise	192	**.18***	95	.18	97	.18	--	--
Total morning cortisol release	198	**.25****	97	**.31****	101	**.20***	--	--
Total diurnal cortisol release								
Mean [with t(+30)]	184	**.19***	92	**.21***	92	.16	--	--
Mean [without t(+30)]	184	.11	92	.11	92	.12	--	--
AUC [with t(+30)]	184	.07	92	.03	92	.12	--	--
AUC [without t(+30)]	184	.05	92	.00	92	.12	--	--
Diurnal cortisol decline								
Slope [without t(+0)]	184	**-.31****	92	**-.32****	92	**-.29****	--	--
Slope [without t(+30)]	184	**-.20****	92	**-.24***	92	-.17	--	--
Delta [t(+30) – t(20:00)]	174	**.32****	88	**.34****	86	**.29****	--	--
Delta [t(+0) – t(20:00)]	175	**.17***	90	**.23***	85	.11	--	--
	Day 1+2+3		**Day 1**		**Day 2**		**Day 3**	
	N	**r+**	**N**	**r**	**N**	**r**	**N**	**r**
Study 2								
t(+0)	947	.03	313	.08	318	.07	316	-.07
t(+30)	938	-.01	314	.07	313	-.03	311	-.09
t(16:00)	948	.00	315	.04	321	-.03	312	-.03
t(20:00)	916	.00	306	.04	302	.02	308	-.04
Cortisol awakening rise	915	-.05	304	-.01	307	-.08	304	-.06
Total morning cortisol release	947	.01	313	.09	318	.01	316	-.08
Total diurnal cortisol release								
Mean [with t(+30)]	953	.01	317	.09	317	.03	319	-.08
Mean [without t(+30)]	953	.02	317	.10	317	.05	319	-.08
AUC [with t(+30)]	953	-.02	317	.02	317	-.03	319	-.04
AUC [without t(+30)]	932	.01	308	.05	314	.03	310	-.05
Diurnal cortisol decline								
Slope [without t(+0)]	953	.00	317	-.03	317	-.01	319	.05
Slope [without t(+30)]	953	-.03	317	-.06	317	-.07	319	.03
Delta [t(+30) – t(20:00)]	879	.00	294	.07	290	.01	295	-.06
Delta [t(+0) – t(20:00)]	887	.05	295	.09	293	.10	299	-.03

+Pearson correlation coefficients; * p< 0.05; ** p< 0.01.

Table 18: Effect of age on cortisol levels in study 1

Dependent variable	Effect	NDF	DDF	F	p
Study 1					
Single samples:	DAY	1	98	0.33	
t(+0), t(+30), t(08:00), t(11:00),	TIME	5	490	12.06	**
t(15:00), t(20:00)	AGE	1	100	3.07	
	DAY × AGE	1	1001	0.31	
	TIME × AGE	5	1001	3.56	**
Cortisol awakening rise	DAY	1	88	0.07	
	AGE	1	100	4.77	*
	DAY × AGE	1	88	0.00	
Total morning cortisol release	DAY	1	94	1.27	
	AGE	1	100	7.15	**
	DAY × AGE	1	94	1.16	
Total diurnal cortisol release					
Mean [with t(+30)]	DAY	1	84	0.00	
	AGE	1	96	3.77	*
	DAY × AGE	1	84	0.02	
Mean [without t(+30)]	DAY	1	84	0.34	
	AGE	1	96	1.37	
	DAY × AGE	1	84	0.20	
AUC [with t(+30)]	DAY	1	84	1.24	
	AGE	1	96	0.46	
	DAY × AGE	1	84	0.17	
AUC [without t(+30)]	DAY	1	84	1.19	
	AGE	1	96	0.27	
	DAY × AGE	1	84	0.73	
Diurnal cortisol decline					
Slope [without t(+0)]	DAY	1	84	0.05	
	AGE	1	96	12.65	**
	DAY × AGE	1	84	0.00	
Slope [without t(+30)]	DAY	1	84	0.26	
	AGE	1	96	5.71	*
	DAY × AGE	1	84	0.26	
Delta [t(+30) – t(20:00)]	DAY	1	76	0.33	
	AGE	1	94	12.57	**
	DAY × AGE	1	76	0.16	
Delta [t(+0) – t(20:00)]	DAY	1	76	0.24	
	AGE	1	95	4.18	*
	DAY × AGE	1	76	0.09	

NDF= numerator degrees of freedom; DDF= denominator degrees of freedom; DAY= sampling day; AGE= age (in years); TIME= sampling time (t(+0) to t(20:00)); mixed models for repeated measures: *p<0.05; ** p<0.01

Table 19: Effect of age on cortisol levels in study 2

Dependent variable	Effect	NDF	DDF	F	p
Study 2					
Single samples:	DAY	2	651	6.81	**
t(+0), t(+30), t(16:00), t(20:00)	TIME	3	976	120.18	**
	AGE	1	326	0.00	
	DAY × AGE	2	3399	5.70	**
	TIME × AGE	3	3399	0.32	
Cortisol awakening rise	DAY	2	587	0.32	
	AGE	1	322	1.51	
	DAY × AGE	2	587	0.66	
Total morning cortisol release	DAY	2	616	5.34	**
	AGE	1	325	0.01	
	DAY × AGE	2	616	5.59	**
Total diurnal cortisol release					
Mean [with t(+30)]	DAY	2	623	4.29	*
	AGE	1	324	0.05	
	DAY × AGE	2	623	4.50	*
Mean [without t(+30)]	DAY	2	623	5.20	**
	AGE	1	324	0.19	
	DAY × AGE	2	623	4.54	*
AUC [with t(+30)]	DAY	2	623	0.35	
	AGE	1	324	0.20	
	DAY × AGE	2	623	0.49	
AUC [without t(+30)]	DAY	2	603	1.38	
	AGE	1	323	0.00	
	DAY × AGE	2	603	1.26	
Diurnal cortisol decline					
Slope [without t(+0)]	DAY	2	623	0.58	
	AGE	1	324	0.01	
	DAY × AGE	2	623	0.92	
Slope [without t(+30)]	DAY	2	623	1.22	
	AGE	1	324	0.51	
	DAY × AGE	2	623	1.15	
Delta [t(+30) – t(20:00)]	DAY	2	552	1.37	
	AGE	1	321	0.02	
	DAY × AGE	2	552	2.26	
Delta [t(+0) – t(20:00)]	DAY	2	560	2.21	
	AGE	1	321	1.19	
	DAY × AGE	2	560	2.60	

NDF= numerator degrees of freedom; DDF= denominator degrees of freedom; DAY= sampling day; AGE= age in years; TIME= sampling time (t(+0) to t(20:00)); mixed models for repeated measures: *p<0.05; ** p<0.01

In study 2, however, there was no significant relationship between age and cortisol levels: single samples and parameters were uncorrelated with age (Table 17) and mixed models for repeated measures did not reveal a significant main effect of age on cortisol levels of single samples and parameters (Table 19). Mixed model analyses for repeated measures, however, yielded a significant effect of sampling day and significant interaction effects of sampling day × age on single cortisol samples, *total morning cortisol release*, and diurnal means (Table 19), which indicates unstable cortisol levels across sampling days interacting with age.

Apart from investigating the exclusive effect of age on cortisol levels, additional mixed model analyses for repeated measures were applied to examine the interaction of age and gender on cortisol levels. However, in neither study a significant interaction of age and gender with regard to cortisol levels of single samples and parameters could be observed (Appendix 1, Table 29).

4.2.5 Occupational groups

In study 1, participants covered four occupational groups (nurses: n= 49; teachers: n= 21; hotel staff: n= 9; social service assistants: n= 23). Descriptive statistics of cortisol levels of single values and parameters in each occupational group are given in Appendix 1, Table 30-33.

Mixed model analyses for repeated measures revealed a significant main effect of occupational group on cortisol levels of single values ($F= 14.04$, $p< 0.0001$) as well as a significant interaction effect of occupational group × time of sampling ($F= 5.92$, $p< 0.0001$; Table 20). Decomposing the significant interaction effect by examining the effect slices, significant differences emerged between occupational groups at each sampling occasion (t(+0): $F= 20.17$, $p< 0.0001$; t(+30): $F= 21.70$, $p< 0.0001$; t(08:00): $F= 7.75$, $p< 0.0001$; t(11:00): $F= 3.15$, $p< 0.05$; t(15:00): $F= 8.33$, $p< 0.0001$; t(20:00): $F= 2.82$, $p< 0.05$). To check which occupational groups differed from each other in cortisol levels at each sampling occasion, single contrasts between levels of interaction variables (differences of least square means) were examined (Appendix 1, Table 35, Figure 11). At most sampling occasions nurses had the lowest cortisol levels (except for the afternoon and evening samples): at t(+0) nurses differed significantly from all other groups; at t(+30) nurses differed significantly from teachers and

social service, but not from hotel staff, who differed significantly from social service; at t(08:00) nurses differed significantly from teachers and social service, but not from hotel staff, who differed significantly from social service; at t(11:00) nurses had slightly lower cortisol levels differing significantly from teachers only; at t(15:00) lowest cortisol levels could be observed in hotel staff differing significantly from teachers and social service, whereas nurses differed significantly from both teachers and social service; at t(20:00) lowest cortisol levels were observed in hotel staff differing significantly from social service, who also differed significantly from nurses.

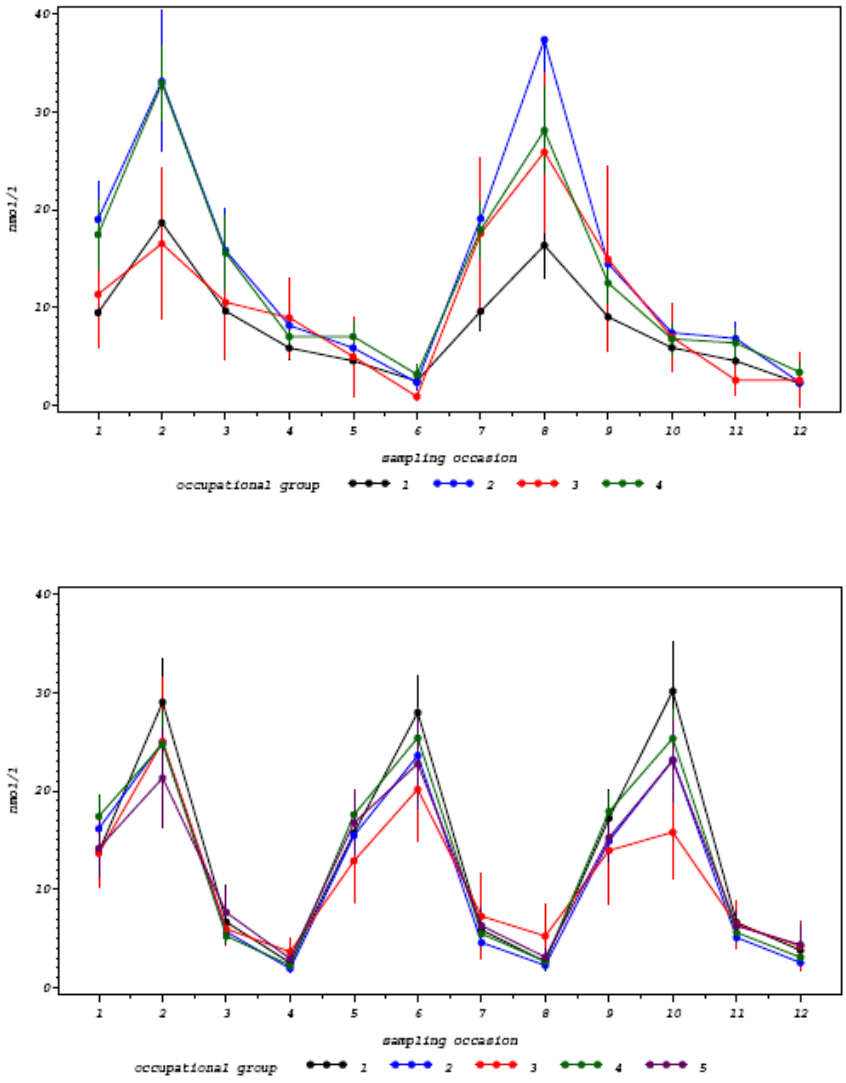

Figure 11: Diurnal salivary cortisol profiles in occupational groups (1= nurses; 2= teachers; 3= hotel staff; 4= social service assistant; 5= mixed group, study 2 only; above: study 1, below: study 2).

Table 20: Effect of occupational group on cortisol levels in study 1

Dependent variable	Effect	NDF	DDF	F	p
Study 1					
Single samples: t(+0), t(+30), t(08:00), t(11:00), t(15:00), t(20:00)	DAY	1	95	1.08	
	TIME	5	475	313.49	**
	OCC	3	98	14.04	**
	DAY × OCC	3	95	1.44	
	TIME × OCC	15	475	5.92	**
Cortisol awakening rise	DAY	1	86	0.01	
	OCC	3	98	2.97	*
	DAY × OCC	3	86	1.79	
Total morning cortisol release	DAY	1	92	3.83	
	OCC	3	98	20.93	**
	DAY × OCC	3	92	3.64	*
Total diurnal cortisol release					
Mean [with t(+30)]	DAY	1	82	3.05	
	OCC	3	94	18.37	**
	DAY × OCC	3	82	7.29	**
Mean [without t(+30)]	DAY	1	82	1.97	
	OCC	3	94	13.41	**
	DAY × OCC	3	82	3.60	*
AUC [with t(+30)]	DAY	1	82	0.28	
	OCC	3	94	8.49	**
	DAY × OCC	3	82	4.16	**
AUC [without t(+30)]	DAY	1	82	0.82	
	OCC	3	94	8.44	**
	DAY × OCC	3	82	2.95	*
Diurnal cortisol decline					
Slope [without t(+0)]	DAY	1	82	0.37	
	OCC	3	94	12.48	**
	DAY × OCC	3	82	4.87	**
Slope [without t(+30)]	DAY	1	82	2.63	
	OCC	3	94	10.17	**
	DAY × OCC	3	82	2.75	*
Delta [t(+30) – t(20:00)]	DAY	1	74	0.05	
	OCC	3	92	16.74	**
	DAY × OCC	3	74	4.38	**
Delta [t(+0) – t(20:00)]	DAY	1	74	1.69	
	OCC	3	93	12.73	**
	DAY × OCC	3	74	0.47	

NDF= numerator degrees of freedom; DDF= denominator degrees of freedom; DAY= sampling day; OCC= occupational group (nurses; teachers; hotel staff; social service; mixed group – study 2 only); TIME= sampling time (t(+0) to t(20:00)); mixed models for repeated measures: *p<0.05; ** p<0.01

Mixed model analyses for repeated measures revealed a significant main effect of occupational group on each composite cortisol measure (Table 20). To check which occupational groups differed from each other in each composite cortisol measure, mixed model analyses for repeated measures were rerun to obtain contrasts between levels of the between subject factor occupational group (Appendix 1, Table 36). Due to their lowest diurnal cortisol profiles, levels of composite cortisol measures were also lowest in nurses: with regard to the *cortisol awakening rise* nurses differed significantly from teachers only; significant differences in *total morning cortisol release* could be observed between nurses and teachers, nurses and social service as well as between teachers and hotel staff; in both, *diurnal mean including t(+30)* and *diurnal mean excluding t(+30)*, nurses differed significantly from all other groups; in both, the *AUC including t(+30)* and *AUC excluding t(+30)*, nurses differed significantly from teachers and social service, but not from hotel staff; in both *slope of change excluding t(+0)* and *slope of change excluding t(+30)*, nurses differed significantly from teachers and social service; with regard to *decline from t(+30) to t(20:00)* nurses differed significantly from teachers and social service, but not from hotel staff who differed significantly from teachers; in *decline from t(+0) to t(20:00)* nurses differed significantly from all other groups.

In study 2, participants covered five occupational groups (nurses: n= 82; teachers: n= 108; hotel staff: n= 17; social service assistants: n= 89; mixed group: n= 35). Descriptive statistics of cortisol levels of single values and parameters in each occupational group are given in Appendix 1, Table 30-34.

Mixed model analyses for repeated measures revealed a marginally significant main effect of occupational group on cortisol levels of single values ($F= 2.23$, $p= 0.06$) and a significant interaction effect of occupational group × sampling occasion ($F= 4.81$, $p< 0.0001$; Table 21). Decomposing the significant interaction effect by examining the effect slices, significant differences emerged between occupational groups at most sampling occasions (t(+0): $F= 2.82$, $p< 0.05$; t(+30): $F= 2.53$, $p< 0.05$; t(16:00): $F= 2.27$, $p= 0.06$; t(20:00): $F= 6.16$, $p< 0.0001$). Consequently, to check which occupational groups differed from each other at t(+0), t(+30), and t(20:00), single contrasts between levels of interaction variables (differences of least square means) were examined (Appendix 1, Table 37, Figure 11). In contrast to study 1, ho-

tel staff had the lowest cortisol levels subsequent to awakening and 30 minutes thereafter: at t(+0) hotel staff differed significantly from social service, who – with highest cortisol levels – differed significantly from nurses and teachers; at t(+30) nurses (with highest cortisol levels) differed significantly from hotel staff, teachers, and mixed group. At t(20:00) highest cortisol levels could be observed in hotel staff differing significantly from nurses, teachers, and social service, whereas teachers had the lowest cortisol levels differing significantly from nurses, hotel staff, and mixed group.

Mixed model analyses for repeated measures revealed a significant main effect of occupational group on *cortisol awakening rise*, *AUC including t(+30)*, *decline from t(+30) to t(20:00)*, and *decline from t(+0) to t(20:00)* (Table 21). Thus, to check which occupational groups differed from each other in these parameters, mixed model analyses for repeated measures were rerun to obtain contrasts between levels of the between subject factor occupational group (Appendix 1, Table 38). In contrast to study 1, nurses had the highest and hotel staff the lowest cortisol levels with regard to the *cortisol awakening rise*, *AUC including t(+30)*, *decline from t(+30) to t(20:00)*, and *decline from t(+0) to t(20:00)*. Nurses differed significantly from all other groups in *cortisol awakening rise*, from teachers in *AUC including t(+30)*, and from teachers, hotel staff, and mixed group in *decline from t(+30) to t(20:00)*. With regard to *decline from t(+0) to t(20:00)* hotel staff with lowest cortisol levels differed significantly from teachers and social service.

Table 21: Effect of occupational group on cortisol levels in study 2

Dependent variable	Effect	NDF	DDF	F	p
Study 2					
Single samples:	DAY	2	649	0.44	
t(+0), t(+30), t(16:00), t(20:00)	TIME	3	973	1081.43	**
	OCC	4	326	2.23	*
	DAY × OCC	8	649	1.28	
	TIME × OCC	12	973	4.81	**
Cortisol awakening rise	DAY	2	587	1.79	
	OCC	4	322	3.63	**
	DAY × OCC	8	587	0.64	
Total morning cortisol release	DAY	2	616	0.36	
	OCC	4	325	2.02	
	DAY × OCC	8	616	1.59	
Total diurnal cortisol release					
Mean [with t(+30)]	DAY	2	623	0.23	
	OCC	4	324	1.25	
	DAY × OCC	8	623	1.07	
Mean [without t(+30)]	DAY	2	623	0.29	
	OCC	4	324	1.94	
	DAY × OCC	8	623	1.24	
AUC [with t(+30)]	DAY	2	623	1.48	
	OCC	4	324	2.85	*
	DAY × OCC	8	623	0.46	
AUC [without t(+30)]	DAY	2	603	0.00	
	OCC	4	323	1.73	
	DAY × OCC	8	603	1.27	
Diurnal cortisol decline					
Slope [without t(+0)]	DAY	2	623	1.80	
	OCC	4	324	2.35	
	DAY × OCC	8	623	0.91	
Slope [without t(+30)]	DAY	2	623	0.16	
	OCC	4	324	2.27	
	DAY × OCC	8	623	0.96	
Delta [t(+30) – t(20:00)]	DAY	2	552	2.17	
	OCC	4	321	3.41	**
	DAY × OCC	8	552	0.78	
Delta [t(+0) – t(20:00)]	DAY	2	560	0.08	
	OCC	4	321	2.62	*
	DAY × OCC	8	560	1.12	

NDF= numerator degrees of freedom; DDF= denominator degrees of freedom; DAY= sampling day; OCC= occupational group (nurses; teachers; hotel staff; social service; mixed group – study 2 only); TIME= sampling time (t(+0) to t(20:00)); mixed models for repeated measures: *p<0.05; ** p<0.01

4.2.6 Shift work

In study 1, 45 participants (51 %; nurses: n= 11; teachers: n= 15; hotel staff: n= 5; social service assistants: n= 14; 14 participants failed to specify scheduled working hours) reported to work shifts. Descriptive statistics of cortisol levels of single values and parameters in participants working shifts and those working normal hours are given in Appendix 1, Table 39 and 40.

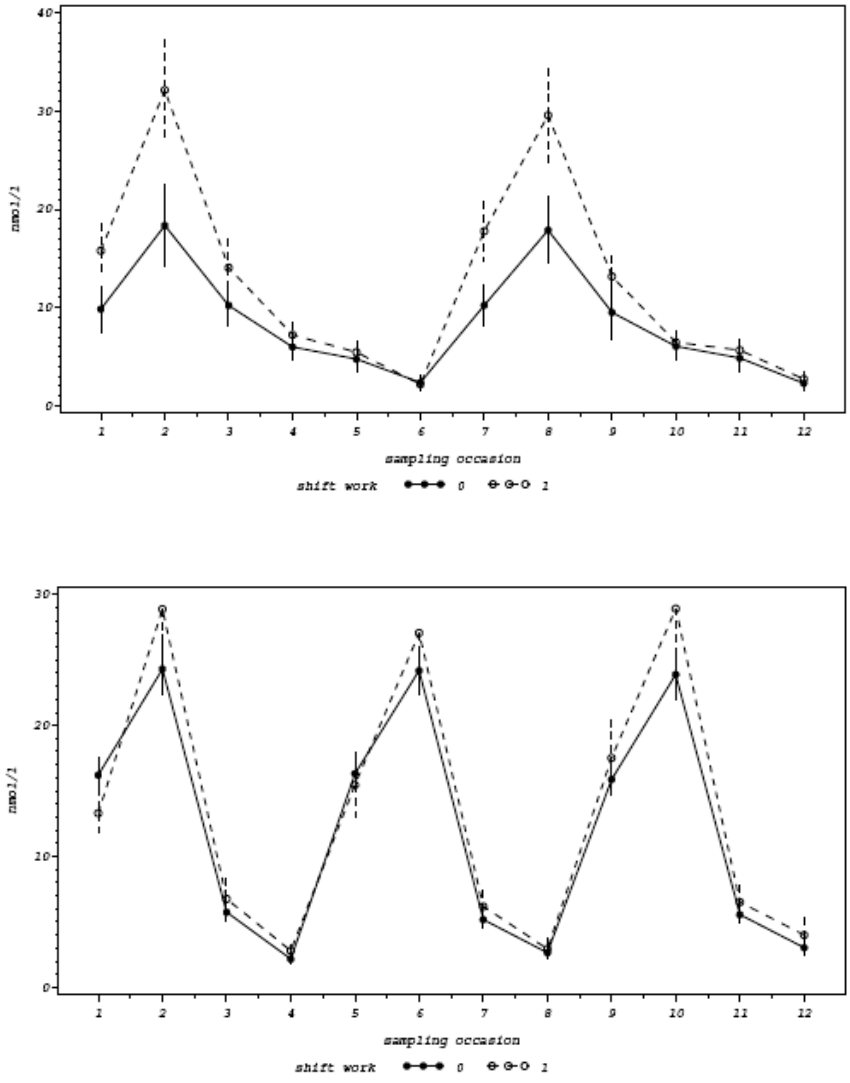

Figure 12: Diurnal salivary cortisol profiles in shift work. (1= shift workers; 0= normal hours; above: study 1, below: study2).

Mixed model analyses for repeated measures revealed a significant main effect of shift work on cortisol levels of single values ($F= 11.69$, $p< 0.001$) as well as a significant interaction effect of shift work × sampling occasion ($F= 7.10$, $p< 0.0001$; Table 22). Decomposing the significant interaction effect by examining the effect slices, significant differences emerged between participants working shift and those working normal hours at morning cortisol samples (t(+0): $F= 22.26$, $p< 0.0001$; t(+30): $F= 22.91$, $p< 0.0001$; t(08:00): $F= 9.05$, $p< 0.01$; Table 22): participants working shifts had significantly higher cortisol levels at these sampling times compared to participants working normal hours (Figure 12).

Mixed model analyses for repeated measures revealed a significant main effect of shift work on all cortisol parameters (Table 22): participants working shifts had consistently higher cortisol levels with regard to all composite cortisol measures.

In study 2, 86 participants (26 %; nurses: n= 74; hotel staff: n= 8; social service assistants: n= 4; 6 participants failed to specify scheduled working hours) reported to work shifts. Descriptive statistics of cortisol levels of single values and parameters in participants working shifts and those working normal hours are given in Appendix 1, Table 39 and 40.

In contrast to study 1, mixed model analyses for repeated measures failed to reveal a significant main effect of shift work on cortisol levels of single values in study 2, however, a significant interaction effect of shift work × sampling occasion ($F= 7.39$, $p< 0.0001$) could be observed (Table 23). Decomposing the significant interaction effect by examining the effect slices, significant differences emerged between participants working shift and those working normal hours for samples collected directly after awakening and 30 minutes thereafter and in the evening (Figure 12): participants working shifts had slightly lower cortisol levels directly after awakening (t(+0): $F= 4.08$, $p< 0.05$), but significantly higher cortisol levels 30 minutes after awakening (t(+30): $F= 5.70$, $p< 0.01$) and in the evening (t(20:00): $F= 7.42$, $p< 0.01$). However, afternoon cortisol levels did not differ significantly between groups (t(16:00): $F= 2.32$, $p> 0.1$).

Moreover, mixed model analyses for repeated measures did not reveal significant main effects of shift on cortisol levels of composite parameters, with the exception of the *cortisol awakening rise* ($F= 10.09$, $p< 0.01$) and *AUC including t(+30)* ($F= 6.86$, $p< 0.05$; Table 23).

Table 22: Effect of shift work on cortisol levels in study 1

Dependent variable	Effect	NDF	DDF	F	p
Study 1					
Single samples: t(+0), t(+30), t(08:00), t(11:00), t(15:00), t(20:00)	DAY	1	83	0.01	
	TIME	5	417	343.91	**
	SHIFT	1	86	11.69	**
	DAY × SHIFT	1	83	0.35	
	TIME × SHIFT	5	417	7.10	**
Cortisol awakening rise	DAY	1	74	2.32	
	SHIFT	1	86	5.08	*
	DAY × SHIFT	1	74	0.95	
Total morning cortisol release	DAY	1	80	0.06	
	SHIFT	1	86	17.45	**
	DAY × SHIFT	1	80	0.24	
Total diurnal cortisol release					
Mean [with t(+30)]	DAY	1	72	0.32	
	SHIFT	1	82	15.01	**
	DAY × SHIFT	1	72	0.47	
Mean [without t(+30)]	DAY	1	72	0.01	
	SHIFT	1	82	12.14	**
	DAY × SHIFT	1	72	2.37	
AUC [with t(+30)]	DAY	1	72	4.27	*
	SHIFT	1	82	4.02	*
	DAY × SHIFT	1	72	0.33	
AUC [without t(+30)]	DAY	1	72	0.22	
	SHIFT	1	82	4.50	*
	DAY × SHIFT	1	72	0.86	
Diurnal cortisol decline					
Slope [without t(+0)]	DAY	1	72	1.57	
	SHIFT	1	82	11.78	*
	DAY × SHIFT	1	72	0.35	
Slope [without t(+30)]	DAY	1	72	0.18	
	SHIFT	1	82	12.21	**
	DAY × SHIFT	1	72	0.81	
Delta [t(+30) – t(20:00)]	DAY	1	64	1.00	
	SHIFT	1	80	20.98	**
	DAY × SHIFT	1	64	0.52	
Delta [t(+0) – t(20:00)]	DAY	1	64	1.99	
	SHIFT	1	81	20.27	**
	DAY × SHIFT	1	64	1.46	

NDF= numerator degrees of freedom; DDF= denominator degrees of freedom; DAY= sampling day; SHIFT= shift work (yes/no); TIME= sampling time (t(+0) to t(20:00)); mixed models for repeated measures: *p<0.05; ** p<0.01

Table 23: Effect of shift work on cortisol levels in study 2

Dependent variable	Effect	NDF	DDF	F	p
Study 2					
Single samples:	DAY	2	643	2.28	
t(+0), t(+30), t(16:00), t(20:00)	TIME	3	964	1387.67	**
	SHIFT	1	323	2.55	
	DAY × SHIFT	2	643	1.28	
	TIME × SHIFT	3	964	7.39	**
Cortisol awakening rise	DAY	2	582	1.76	
	SHIFT	1	319	10.09	**
	DAY × SHIFT	2	582	1.15	
Total morning cortisol release	DAY	2	611	0.43	
	SHIFT	1	322	0.60	
	DAY × SHIFT	2	611	1.17	
Total diurnal cortisol release					
Mean [with t(+30)]	DAY	2	618	0.15	
	SHIFT	1	321	0.82	
	DAY × SHIFT	2	618	0.24	
Mean [without t(+30)]	DAY	2	618	2.67	
	SHIFT	1	321	0.07	
	DAY × SHIFT	2	618	2.64	
AUC [with t(+30)]	DAY	2	618	0.68	
	SHIFT	1	321	6.86	**
	DAY × SHIFT	2	618	0.01	
AUC [without t(+30)]	DAY	2	598	0.50	
	SHIFT	1	320	0.19	
	DAY × SHIFT	2	598	0.55	
Diurnal cortisol decline					
Slope [without t(+0)]	DAY	2	618	0.61	
	SHIFT	1	321	2.27	
	DAY × SHIFT	2	618	0.11	
Slope [without t(+30)]	DAY	2	618	2.17	
	SHIFT	1	321	2.04	
	DAY × SHIFT	2	618	4.35	*
Delta [t(+30) – t(20:00)]	DAY	2	550	1.03	
	SHIFT	1	318	3.82	
	DAY × SHIFT	2	550	0.07	
Delta [t(+0) – t(20:00)]	DAY	2	558	0.62	
	SHIFT	1	318	2.88	
	DAY × SHIFT	2	558	3.27	*

NDF= numerator degrees of freedom; DDF= denominator degrees of freedom; DAY= sampling day; SHIFT= shift work (yes/no); TIME= sampling time (t(+0) to t(20:00)); mixed models for repeated measures: *p<0.05; ** p<0.01

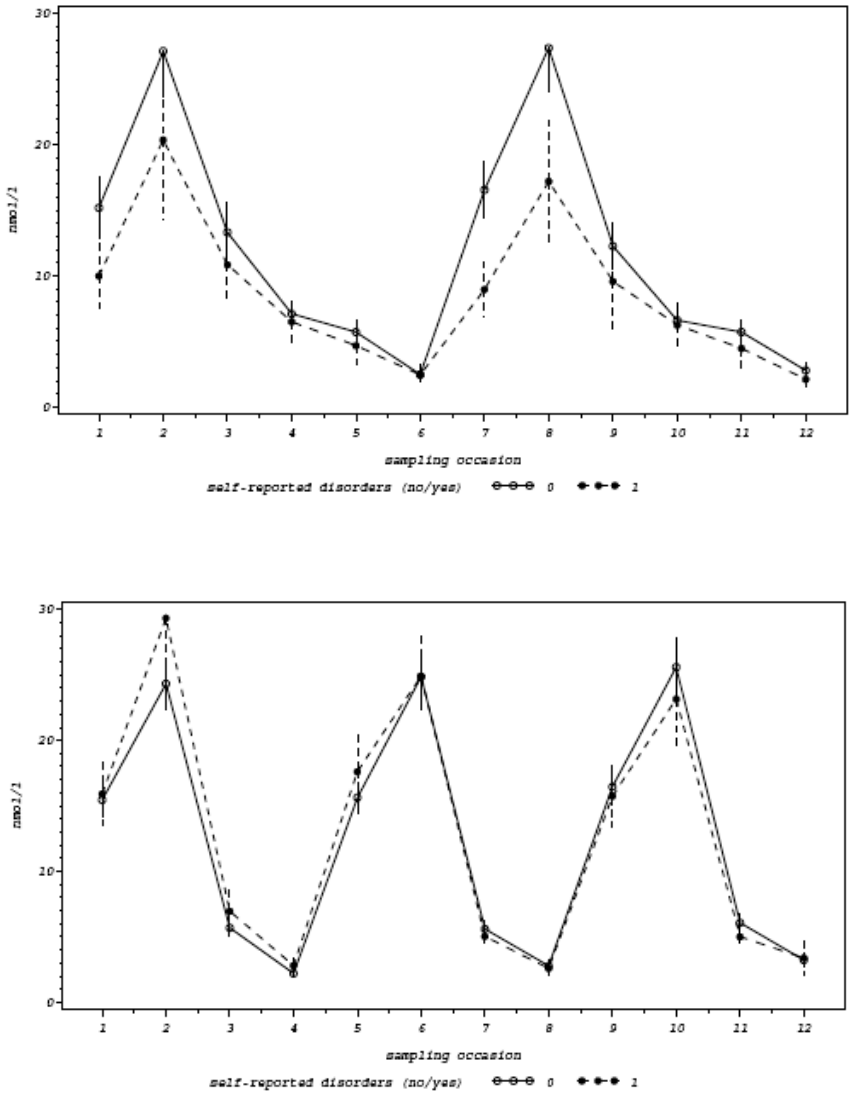

Figure 13: Diurnal salivary cortisol profiles in participants with and without self-reported disorders (above: study 1, below: study 2).

4.2.7 Somatic health status

a) Self-reported disorders and diseases

In study 1, 32 (31%) of 102 participants reported to have at least one of the following disorders: endocrine, psychiatric, neurological and/or sleep disorder (Appendix 1, Table 41). They did not differ significantly in age, gender, depression and anxiety scores, and somatic complaints from participants without self-reported disorders (Appendix 1, Table 42). There was, however, a significant difference regarding occupational groups: 30 of all 32 partici-

pants reporting to have at least one disorder were from the nursing group. While demographic and psychosocial variables failed to discriminate between participants reporting at least one disorder and those reporting none, cortisol profiles of both groups varied significantly (Appendix 1, Table 43 and Figure 13): Mixed models for repeated measures revealed a significant main effect for the two groups ($F= 9.98$, $p< 0.01$) and significant interaction effects of group × sampling day ($F= 5.05$, $p< 0.03$) and group × sampling time ($F=6.35$, $p<0.0001$). Decomposing the significant interaction effects by examining the effect slices, significant differences emerged between both groups on both sampling days (day 1: $F= 4.88$, $p< 0.05$; day 2: $F= 13.96$, $p< 0.001$). Participants reporting at least one disorder had significantly lower cortisol levels at the time of awakening ($F= 22.62$, $p< 0.0001$), 30 minutes after awakening ($F= 20.01$, $p< 0.0001$), at 08:00 h ($F=3.98$, $p< 0.05$), and at 15:00 h ($F= 4.17$, $p< 0.05$). Cortisol levels at sampling times 11:00 h ($F= 0.76$, $p> 0.3$) and 20:00 h ($F=0.50$, $p> 0.4$) did not vary between groups. While cortisol profiles on both sampling days were similar for participants without self-reported disorders ($F= 1.38$, n.s.), they varied significantly for participants reporting at least one disorder ($F= 3.68$, $p= 0.05$).

In study 2, 83 (25%) of 331 participants reported at least one disorder (Appendix 1, Table 41). Although they did not differ significantly in gender from participants without self-reported disorders, in contrast to study 1 substantial differences were observed for age ($F= 5.04$, $p< 0.05$), depression ($F= 16.64$, $p< 0.0001$), trait anxiety ($F= 22.93$, $p< 0.0001$), and musculoskeletal complaints ($F= 8.06$, $p< 0.01$). Participants reporting at least one disorder were older, more depressive, had more trait anxiety and more musculoskeletal complaints. Most participants with at least one self-reported disorder were from the nursing group (28 of 83) and from the group of teachers (35 of 83; Appendix 1, Table 42). While substantial differences in demographic and psychosocial variables were found in study 2, mixed models for repeated measures analyses did not reveal significant differences in cortisol profiles compared to participants without self-reported disorders (day of sampling: $F=0.01$, $p> 0.9$; self-reported disorder: $F= 0.07$, $p> 0.7$; interaction effects: group × sampling day: $F= 2.18$, $p> 0.1$; group × sampling time: $F= 0.46$, $p> 0.7$; Appendix 1, Table 43 and Figure 13).

b) Somatic complaints

In study 1, somatic complaints (i.e. overall distress/indulgence to complaining) were uncorrelated to cortisol levels of single values and parameters (Appendix 1, Table 44). Subsequent mixed model analyses for repeated measures failed to reveal significant main effects of somatic complaints on cortisol levels of single values and composite measures (Appendix 1, Table 45).

In study 2, these results could be replicated: there was no significant relationship between somatic complaints and cortisol levels of single values and parameters (Appendix 1, Table 44). Subsequent mixed model analyses for repeated measures failed to yield significant main effects of somatic complaints on cortisol levels of single values and composite measures (Appendix 1, Table 46).

c) Smoking status

In study 1, 38 participants (38 %; 3 participants failed to specify smoking status) reported to be current smokers. Descriptive statistics of cortisol levels in current smokers and non-smokers are given in Appendix 1, Table 47 and 48.

Mixed model analyses for repeated measures failed to reveal a significant main effect of smoking status (current smoker vs. non-smoker; Figure 14) on cortisol levels of single values ($F= 2.46$, $p> 0.1$), however, a significant interaction effect of smoking status × sampling occasion could be observed ($F= 5.43$, $p< 0.0001$; Table 24). Decomposing the significant interaction effect by examining the effect slices, significant differences emerged between smokers and non-smokers at t(+0) only: current smokers had significantly lower cortisol levels directly after awakening ($F= 16.24$, $p< 0.0001$). Cortisol levels at ensuing sampling times did not vary significantly between smokers and non-smokers (t(+30): $F= 2.32$, $p> 0.1$; t(08:00): $F= 0.70$, $p> 0.4$; t(11:00): $F= 1.19$, $p> 0.2$; t(15:00): $F= 0.68$, $p> 0.4$; t(20:00): $F= 0.66$, $p> 0.4$).

Compared to non-smokers current smokers had lower cortisol levels and flatter diurnal declines with regard to composite cortisol measures: mixed model analyses for repeated measures yielded a significant main effect of smoking status on *total morning cortisol release* ($F= 4.30$, $p< 0.05$), *diurnal mean excluding t(+30)* ($F= 3.96$, $p< 0.05$), *slope of change excluding t(+30)* ($F= 9.62$, $p< 0.01$), *decline from t(+30) to t(20:00)* ($F= 4.05$, $p< 0.05$), and *decline from t(+0) to t(20:00)* ($F= 12.97$, $p< 0.001$; Table 24).

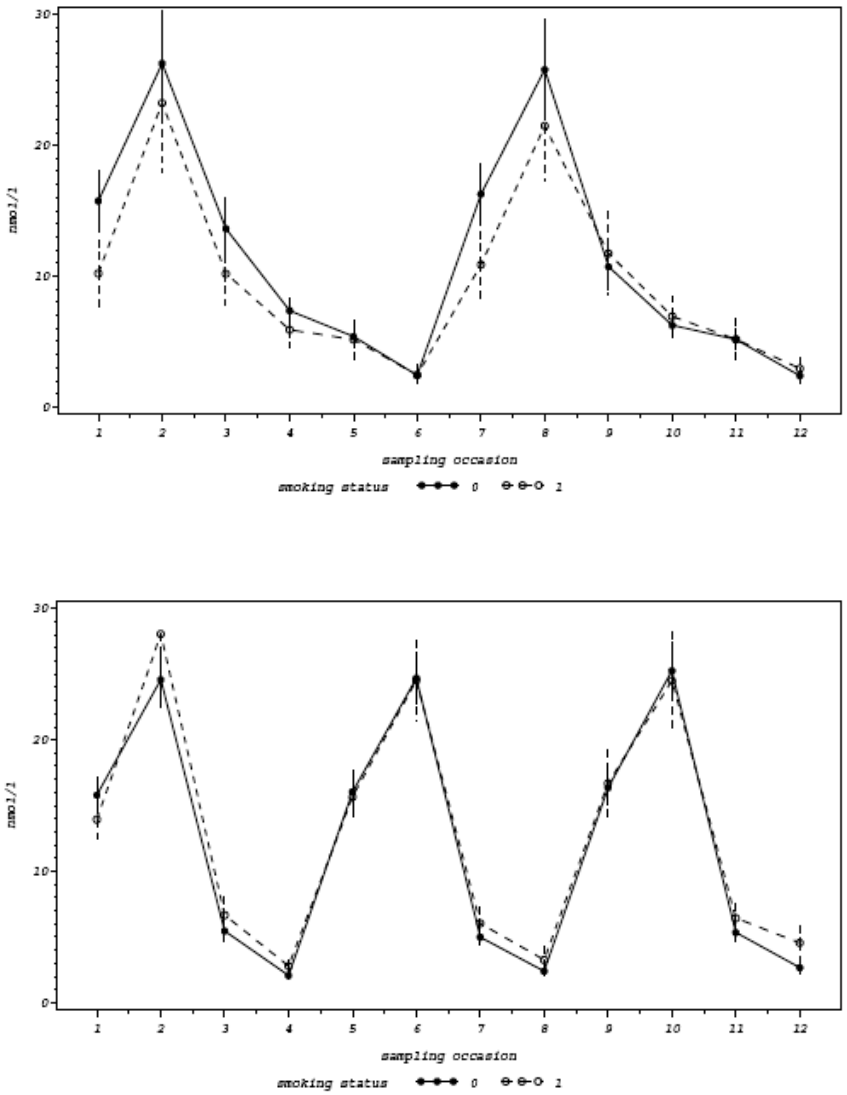

Figure 14: Diurnal salivary cortisol profiles in current smokers and non-smokers (above: study 1, below: study 2.).

Table 24: Effect of smoking status on cortisol levels in study 1

Dependent variable	Effect	NDF	DDF	F	p
Study 1					
Single samples: t(+0), t(+30), t(08:00), t(11:00), t(15:00), t(20:00)	DAY	1	94	0.05	
	TIME	5	471	351.21	**
	SMO	1	97	2.46	
	DAY × SMO	1	94	2.65	
	TIME × SMO	5	471	5.43	**
Cortisol awakening rise	DAY	1	86	0.70	
	SMO	1	97	1.23	
	DAY × SMO	1	86	0.11	
Total morning cortisol release	DAY	1	91	0.03	
	SMO	1	97	4.30	*
	DAY × SMO	1	91	0.18	
Total diurnal cortisol release					
Mean [with t(+30)]	DAY	1	83	0.06	
	SMO	1	93	3.01	
	DAY × SMO	1	83	0.50	
Mean [without t(+30)]	DAY	1	83	0.02	
	SMO	1	93	3.96	*
	DAY × SMO	1	83	4.11	*
AUC [with t(+30)]	DAY	1	83	5.03	*
	SMO	1	93	0.99	
	DAY × SMO	1	83	2.25	
AUC [without t(+30)]	DAY	1	83	0.11	
	SMO	1	93	2.94	
	DAY × SMO	1	83	5.05	*
Diurnal cortisol decline					
Slope [without t(+0)]	DAY	1	83	0.36	
	SMO	1	93	1.66	
	DAY × SMO	1	83	0.01	
Slope [without t(+30)]	DAY	1	93	0.10	
	SMO	1	93	9.62	**
	DAY × SMO	1	93	0.54	
Delta [t(+30) – t(20:00)]	DAY	1	75	0.77	
	SMO	1	91	4.05	*
	DAY × SMO	1	75	0.96	
Delta [t(+0) – t(20:00)]	DAY	1	75	0.53	
	SMO	1	92	12.97	**
	DAY × SMO	1	75	0.00	

NDF= numerator degrees of freedom; DDF= denominator degrees of freedom; DAY= sampling day; SMO= smoker (yes/no); TIME= sampling time (t(+0) to t(20:00)); mixed models for repeated measures: *p<0.05; ** p<0.01

Table 25: Effect of smoking status on cortisol levels in study 2

Dependent variable	Effect	NDF	DDF	F	p
Study 2					
Single samples:	DAY	2	615	2.44	
t(+0), t(+30), t(16:00), t(20:00)	TIME	3	922	1438.42	**
	SMO	1	309	2.30	
	DAY × SMO	2	615	0.39	
	TIME × SMO	3	922	10.23	**
Cortisol awakening rise	DAY	2	557	3.26	*
	SMO	1	305	1.29	
	DAY × SMO	2	557	3.12	*
Total morning cortisol release	DAY	2	585	0.21	
	SMO	1	308	0.21	
	DAY × SMO	2	585	0.02	
Total diurnal cortisol release					
Mean [with t(+30)]	DAY	2	590	0.16	
	SMO	1	307	0.32	
	DAY × SMO	2	590	0.06	
Mean [without t(+30)]	DAY	2	590	2.99	
	SMO	1	307	0.64	
	DAY × SMO	2	590	1.11	
AUC [with t(+30)]	DAY	2	590	0.57	
	SMO	1	307	2.71	
	DAY × SMO	2	590	0.17	
AUC [without t(+30)]	DAY	2	570	1.23	
	SMO	1	306	0.05	
	DAY × SMO	2	570	1.27	
Diurnal cortisol decline					
Slope [without t(+0)]	DAY	2	590	1.95	
	SMO	1	307	0.17	
	DAY × SMO	2	590	1.62	
Slope [without t(+30)]	DAY	2	590	1.21	
	SMO	1	307	3.32	
	DAY × SMO	2	590	1.13	
Delta [t(+30) – t(20:00)]	DAY	2	522	3.20	*
	SMO	1	304	0.22	
	DAY × SMO	2	522	3.99	*
Delta [t(+0) – t(20:00)]	DAY	2	531	0.22	
	SMO	1	304	4.58	*
	DAY × SMO	2	531	0.28	

NDF= numerator degrees of freedom; DDF= denominator degrees of freedom; DAY= sampling day; SMO= smoker (yes/no); TIME= sampling time (t(+0) to t(20:00)); mixed models for repeated measures: *p<0.05; ** p<0.01

In study 2, 95 participants (30 %; 20 participants failed to specify smoking status) reported to be current smokers. Descriptive statistics of cortisol levels in current smokers and non-smokers are given in Appendix 1, Table 47 and 48.

In line with study 1, mixed model analyses for repeated measures failed to yield a significant main effect of smoking status on cortisol levels of single values ($F= 2.30$, $p> 0.1$), but a significant interaction effect of smoking × sampling occasion ($F= 10.23$, $p< 0.0001$; Table 25). Decomposing the significant interaction effect by examining the effect slices, significant differences emerged between smokers and non-smokers for afternoon and evening cortisol samples (Figure 14): current smokers had significantly higher cortisol levels at t(16:00) ($F= 4.74$, $p< 0.05$) and at t(20:00) ($F= 17.08$, $p< 0.0001$). Cortisol levels after awakening and thirty minutes later did not vary significantly between smokers and non-smokers (t(+0): $F= 1.94$, $p> 0.1$; t(+30): $F= 0.27$, $p> 0.6$).

In contrast to study 1, cortisol levels of composite measures did not vary between current smokers and non-smokers, except for *decline from t(+0) to t(20:00)* ($F= 4.58$, $p< 0.05$; Table 25): compared to non-smokers current smokers had a flatter diurnal cortisol decline.

d) Cardiovascular risk factors

The following section focuses on the relationship of cortisol levels and cardiovascular risk factors (body mass index, waist-to-hip ratio, blood pressure). Intercorrelations of measures of cardiovascular risks in both studies are given in Table 26; descriptive statistics of CVD risk factors are given in Table 2.

In study 1, waist-to-hip ratio (WHR) was not recorded. Body mass index (BMI) did not correlate with blood pressure (systolic and diastolic), but – as expected – systolic blood pressure correlated substantially with diastolic (Table 26). Seven participants (7 %) were classified obese (BMI ≥ 30), 23 participants (22 %) were hypertensive (either systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg, 12 participants with systolic blood pressure ≥ 140 mmHg as well as diastolic blood pressure ≥ 90 mmHg). Body mass index and cortisol levels were uncorrelated (Appendix 1, Table 49), however, systolic as well as diastolic blood pressure were positively correlated with morning cortisol levels (t(+0), t(+30), *total morning cortisol release*, but not with *cortisol awakening rise*; Appendix 1, Table 50) and with average diurnal cortisol release (*diurnal mean including/excluding t(+30)*, but not

with *AUC measures*). Additionally, systolic as well as diastolic blood pressure were significantly correlated with diurnal cortisol decline (negatively with *slope of change*, indicating rapid diurnal decline; positively with *decline from t(+0)/t(+30) to t(20:00)*, indicating rapid diurnal decline; Appendix 1, Table 50). However, significant correlation coefficients were rather small, ranging from -.31 to .32 with maximum coefficients of determination of .10, i.e. 10 % of variance in cortisol levels could be attributed to variance in blood pressure at most.

Table 26: Intercorrelation of cardiovascular risk factors

	1	2	3
Study 1			
1 Body mass index[1]	--		
2 Waist-to-hip ratio[2]	--	--	
3 Systolic blood pressure[3]	.14	--	--
4 Diastolic blood pressure[4]	.09	--	.77***
Study 2			
1 Body mass index[1]	--		
2 Waist-to-hip ratio[2]	.23**	--	
3 Systolic blood pressure[3]	.23***	.09	--
4 Diastolic blood pressure[4]	.28***	.10	.80***

Pearson correlation coefficients; *p< 0.05; **p< 0.01; ***p< 0.0001;
[1]body mass index: weight (kg) / height squared (m²); [2]waist-to-hip ratio: waist circumference (cm) / hip circumference (cm);
[3s]ystolic blood pressure: mmHg; [4]diastolic blood pressure: mmHg

In study 2, body mass index correlated significantly with waist-to-hip ratio, as well as with systolic and diastolic blood pressure (Table 26). Systolic and diastolic blood pressure intercorrelated substantially, while waist-to-hip ratio (WHR) was not associated with blood pressure (Table 26). 32 participants (10 %) were classified obese (BMI ≥ 30); 99 participants (29 %) had elevated values for WHR (waist-to-hip ratio was recorded for 173 participants (52 %) only; men: WHR ≥ 0.90; women: ≥ 0.80; Dobbelsteyn et al., 2001). 51 participants (19 %) were hypertensive (either systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg, 39 participants with systolic blood pressure ≥ 140 mmHg as well as dia-

stolic blood pressure ≥ 90 mmHg). Consistent with study 1, body mass index as well as waist-to-hip ratio were not associated with cortisol levels of single samples and composite measures (Appendix 1, Table 49 and 51), but in contrast to study 1, systolic and diastolic blood pressure were also unrelated to cortisol levels of single samples and composite measures (Appendix 1, Table 50).

Table 27: Intercorrelation of mental health scales

	1	2	3	4	5	6
Study 1						
1 Depressive symptoms[1]	--					
2 Trait anxiety[2]	.76***	--				
3 Somatic complaints[3]	.59***	.63***	--			
4 Emotional exhaustion[4]	.67***	.66***	.53***	--		
5 Depersonalization[5]	.45***	.49***	.32**	.49***	--	
6 Personal accomplishment[6]	.34**	.41***	.20	.38**	.43***	--
7 Self-efficacy[7]	-.67***	-.38**	-.26*	-.29**	-.15	-.24*
Study 2						
1 Depressive symptoms[1]	--					
2 Trait anxiety[2]	.81***	--				
3 Somatic complaints[3]	.59***	.58***	--			
4 Emotional exhaustion[4]	.70***	.68***	.58***	--		
5 Depersonalization[5]	.28***	.25***	.25***	.38***	--	
6 Personal accomplishment[6]	.36***	.40***	.23***	.39***	.30***	--
7 Self-efficacy[7]	-.49***	-.55***	-.36***	-.51***	-.17**	-.39***

Pearson correlation coefficients; *p< 0.05; **p< 0.01; ***p< 0.0001; [1]percentile rank (ADS; Hautzinger & Bailer, 1995); [2]percentile rank (STAI-T; Laux et al., 1981); [3]percentile rank (GBBB; Braehler & Scheer, 1995); [4]T-values (MBI; Enzmann & Kleiber, 1989); [5]T-values (MBI; Enzmann & Kleiber, 1989); [6]T-values (MBI; Enzmann & Kleiber, 1989); [7]T-values (SWE; Schwarzer & Jerusalem, 1995).

4.2.8 Mental health status

The following section addresses the impact of mental health status on cortisol levels of single values and composite measures. Intercorrelations of psychometric scales in both studies are given in Table 27. In both studies, scales reflecting emotional and somatic exhaustion (depressive symptoms, anxiety, somatic complaints/overall distress, and emotional exhaus-

tion) intercorrelated substantially, whereas intercorrelations of scales involving work-related individual resources (depersonalization, personal accomplishment, and self-efficacy) were only small to medium-sized (Table 27). Descriptive statistics of psychometric scales in both studies are given in Table 2.

a) Depressive symptoms

In both studies, depressive symptoms failed to correlate substantially and systematically with cortisol levels of single samples and composite measures (Appendix 1, Table 52).

Seven participants (7 % in study 1; 2 % in study 2) had above-average scores of depressive symptoms (percentile rank ≥ 98, which corresponds to two standard deviations above the mean). Due to the small sample size significance testing of differences in cortisol levels was inappropriate.

b) Trait anxiety

In both studies, trait anxiety failed to correlate substantially and systematically with cortisol levels of single samples and composite measures (Appendix 1, Table 53).

In study 1, 12 participants (12 %) and in study 2 24 participants (7 %) had above-average trait anxiety (percentile rank ≥ 98, which corresponds to two standard deviations above the mean). Due to the small sample size significance testing of differences in cortisol levels was inappropriate.

c) Burnout

In both studies, burnout scales (emotional exhaustion, depersonalization, personal accomplishment) failed to correlate substantially and systematically with cortisol levels of single samples and composite measures (Appendix 1, Table 54-56).

In study 1, eight participants (8 %) had above-average scores of emotional exhaustion (T-value ≥ 70, which corresponds to two standard deviations above the mean), 11 participants (11 %) had above-average scores of depersonalization, and 7 participants (7 %) had above-average scores of lack of personal accomplishment (2 participants with above-average scores in all three burnout scales). In study 2, 10 participants (3 %) had above-average scores of emotional exhaustion, 23 participants (8 %) had above average scores of depersonalization, and 22 participants (7 %) had above-average scores of lack of personal accomplishment (2

participants with above-average scores in all three burnout scales). Due to the small sample size of participants with above-average scores in these scales significance testing of differences in cortisol levels was inappropriate.

d) Self-efficacy

In both studies, self-efficacy failed to correlate substantially and systematically with cortisol levels of single samples and composite measures (Appendix 1, Table 57).

In study 1, 14 participants (14 %) and in study 2, 4 participants (1 %) had below-average scores of self-efficacy (T-value ≤ 30, which corresponds to two standard deviations below the mean). Due to the small sample size of participants with below-average scores with regard to self-efficacy significance testing of differences in cortisol levels was inappropriate.

4.3 Synopsis of internal structure and confounding factors

The preceding sections investigate the internal structure of cortisol profiles and factors influencing cortisol levels of single samples and composite measures in two independent studies. This section summarizes the preceding results to determine which confounding factors need to be accounted for when assessing the stability of cortisol profiles.

The following factors either completely failed to be significantly associated or revealed merely weak and unsystematic associations (i.e. not consistently across sampling days and study samples) with cortisol levels of single samples and composite measures:

Sampling time interval between t(+0) and t(+30). The length of the sampling time interval between awakening cortisol samples (directly after awakening and 30 minutes thereafter) was not associated with the magnitude of the *cortisol awakening rise* and *total morning cortisol release* in both studies (Table 12; Appendix 1, Table 4), although adherence to the stipulated sampling time interval was not given in all participants (Table 13).

Sampling time of cortisol values. In both studies, there was no systematic impact of adherence vs. non-adherence to stipulated sampling times (clock time and sampling time synchronised to awakening) on cortisol levels of single samples (Table 14-17; Appendix 1, Table 5 and 6).

Time of awakening. In both studies alike, awakening time was neither systematically nor substantially associated to cortisol levels of single samples and composite measures. In study 1, awakening time correlated negatively with the *AUC*, but not with the *diurnal mean* of cortisol levels (Table 18; Appendix 1, Table 11). In study 2, participants who woke up later in the morning had slightly flatter diurnal rhythms (Table 18; Appendix 1, Table 11). A systematic impact of awakening time, especially on morning cortisol levels, could not be observed in either study.

Sleep duration and quality of sleep. *Sleep duration* was recorded in study 2, only. Sleep duration was positively correlated with cortisol levels directly after awakening across all three sampling days, yet significant correlation coefficients were rather small. Systematic or substantial associations with all other cortisol levels of single samples and composite measures could not be observed (Table 19; Appendix 1, Table 12). In both studies, *quality of sleep* (study 1: self-reported sleep deprivation; study 2: frequency and duration of self-reported sleep disruption episodes) did not prove to yield a systematic and substantial impact on cortisol levels of single samples and composite measures (Table 20-22; Appendix 1, Table 13 and 15).

Intake of oral contraceptives (OC) . In both studies, there was no significant difference in cortisol levels of single samples and composite measures between women reporting to take oral contraceptives and those reporting not to (Table 25-28; Appendix 1, Table 17).

Age In study 1, older participants tended to have higher levels of morning cortisol as well as flatter diurnal cortisol rhythms; in study 2, however, age was entirely uncorrelated with cortisol levels (Table 17-19; Appendix 1, Table 29).

Somatic health status. a) *Self-reported disorders* (endocrine, psychiatric, neurological and/or sleep disorders) did not prove to exert a systematic impact on cortisol levels: although participants in study 1 reporting at least one disorder had significantly lower cortisol levels compared to those without self-reported disorders (Appendix 1, Table 41-43), the majority of participants with self-reported disorders were from the nursing group, and occupational groups indeed proved to reveal significant differences in cortisol levels. In study 2, significant differences in cortisol levels between participants with and without self-reported disorders could not be observed (Appendix 1, Table 41-43). b) In both studies *self-*

reported somatic complaints were entirely uncorrelated to cortisol levels of single samples and composite measures (Appendix 1, Table 44-46). c) *Cardiovascular risk factors* (body mass index, waist-to-hip ratio, blood pressure) failed to provide systematic associations with cortisol levels across both study samples: there was no significant relationship between body mass index and cortisol levels in both studies. While in study 1 blood pressure was positively correlated with morning cortisol levels and the *average diurnal cortisol release* as well as with the *diurnal cortisol decline*, no such relationship could be observed in study 2 (Table 26; Appendix 1, 49 and 50).

Mental health status. In both studies depressive symptoms, trait anxiety, burnout, as well as self-efficacy failed to be significantly associated with cortisol levels of single samples and composite measures (Table 27; Appendix 1, Table 52-57).

The following factors proved to account for significant differences in cortisol levels of single samples and composite measures in both studies:

Gender. In both studies, men had significantly higher cortisol levels with regard to afternoon samples (study 1: t(15:00); study 2: t(16:00)) and average diurnal cortisol output. In study 2 only, women displayed a larger *cortisol awakening rise* compared to men (Table 15 and 16; Appendix 1, Table 23 and 24).

Occupational group. In study 1, morning cortisol levels (t(+0) to t(11:00)) and composite measures of cortisol were lowest in nurses, while evening cortisol levels (t(20:00)) were lowest in hotel staff. In study 2, hotel staff had lowest morning cortisol levels (t(+0), t(+30)), while afternoon and evening levels (t(16:00); t(20:00)) were lowest in teachers and social service assistants (Appendix 1, Table 30-38).

Shift work. In both studies, participants working shifts tended to display higher cortisol levels of single samples and composite measures (Table 23; Appendix 1, Table 39 and 40). While participants working shifts were more or less evenly distributed among occupational groups in study 1, the majority of participants working shifts in study 2 were from the nursing group (74 of 86 shift workers). Thus, the effect of shift work in study 2 might be mainly due to the impact of occupation.

Smoking status. In both studies, current smokers had significantly lower cortisol levels compared to non-smokers (Table 24 and 25; Appendix 1, Table 47 and 48). In study 1, current smokers displayed lowest cortisol levels directly after awakening (t(+0), in study 2, however, cortisol levels were lower in current smokers with regard to afternoon and evening levels (t(16:00); t(20:00)). Compared to non-smokers, current smokers displayed a flatter diurnal cortisol decline in both studies.

A comprehensive outline on the effects of these influencing factors on cortisol levels of particular single samples and composite measures is given in Table 28. Occupational groups accounted strongest for differences in cortisol levels, while the impact of gender, shift work, and smoking status on cortisol levels was comparatively moderate. These factors are included and allowed for in the subsequent assessment of cortisol profile stability presented in the following chapter.

Table 28: Outline of significant confounding factors regarding cortisol levels in study 1 and 2

	Influencing factors			
Cortisol levels	**Gender**	**Occupational groups**	**Shift work**	**Smoking status**
t(+0)	Study 2	Study 1 / 2	Study 1 / 2	n.s.
t(+30)	n.s.	Study 1 / 2	Study 1 / 2	n.s.
t(08:00)	Study 1	Study 1	Study 1	n.s.
t(11:00)	Study 1	Study 1	n.s.	n.s.
t(15:00)	Study 1 / 2	Study 1 / 2	n.s.	Study 2
t(20:00)	n.s.	Study 1 / 2	Study 2	Study 2
Cortisol awakening rise	Study 2	Study 1 / 2	Study 1 / 2	n.s.
Total morning cortisol release	n.s.	Study 1	Study 1	Study 1
Total diurnal cortisol release				
Mean [with t(+30)]	n.s.	Study 1	Study 1	Study 1
Mean [without t(+30)]	Study 1 / 2	Study 1	Study 1	n.s.
AUC [with t(+30)]	n.s.	Study 1 / 2	Study 1 / 2	n.s.
AUC [without t(+30)]	Study 1	Study 1	Study 1	n.s.
Diurnal cortisol decline				
Slope [without t(+0)]	n.s.	Study 1	Study 1	n.s.
Slope [without t(+30)]	Study 2	Study 1	Study 1	Study 1
Delta [t(+30) – t(20:00)]	n.s.	Study 1 / 2	Study 1	Study 1
Delta [t(+0) – t(20:00)]	n.s.	Study 1 / 2	Study 1	Study 1 / 2

4.4 Diurnal mood and subjective well-being

4.4.1 Diurnal profiles of mood and subjective well-being

Self-rated current psychological strain (study 1) and self-rated mood and well-being (study 2) were simultaneously recorded with cortisol samples.

In both studies, profiles of psychological strain and mood/well-being varied within sampling days: mixed model analyses for repeated measures revealed a significant main effect of sampling time (study 1: $F= 25.73$, $p< 0.0001$, Appendix 1, Table 58; study 2: a) good mood: $F= 26.57$, $p< 0.0001$, b) alertness: $F= 23.37$, $p< 0.0001$, c) relaxation: $F= 9.59$, $p< 0.0001$, Appendix 1, Table 68), but no interaction of sampling day and sampling time (study 1: $F= 1.02$, $p> 0.4$; study 2: a) good mood: $F= 0.50$, $p> 0.7$; b) alertness: $F= 0.23$, $p> 0.9$; c) relaxation: $F= 0.50$, $p> 0.07$).

a) Diurnal profiles of mood and subjective well-being: differential aspects

In study 1, self-rated current psychological strain profiles did not differ between men and women (Appendix 1, Table 59 and 78), occupational groups (Appendix 1, Table 60 and 78) and shift work (Appendix 1, Table 61 and 78). There were, however, significant group differences in diurnal psychological strain profiles between participants with high and low somatic and psychological distress (median-split of the relevant scales): somatic symptoms (Appendix 1, Table 62 and 78), depressive symptoms (Appendix 1, Table 63 and 78), trait anxiety (Appendix 1, Table 64 and 78), and burnout scales (emotional exhaustion: Appendix 1, Table 65 and 78; depersonalization: Appendix 1, Table 66 and 78; personal accomplishment: Appendix 1, Table 67 and 78). As anticipated, participants with high somatic and psychological distress reported higher diurnal levels of psychological strain.

In study 2, diurnal profiles of mood and well-being did not differ between men and women (Appendix 1, Table 69 and 79), but – in contrast to study 1 – between occupational groups (Appendix 1, Table 70 and 80) and shift work (Appendix 1, Table 71 and 81): with regard to occupational groups teachers had lowest diurnal levels of good mood, alertness and relaxation. Shift workers – rather unexpectedly – had higher levels of good mood, alertness, and relaxation than participants working normal hours. Comparable to study 1, significant differences in diurnal profiles of mood and subjective well-being could be observed between participants with low and high somatic and psychological distress (median-split of the rele-

vant scales): somatic symptoms (Appendix 1, Table 72 and 82), depressive symptoms (Appendix 1, Table 73 and 83), trait anxiety (Appendix 1, Table 74 and 84), and burnout scales (emotional exhaustion: Appendix 1, Table 75 and 85; personal accomplishment: Appendix 1, Table 77 and 87); except for depersonalization: Appendix 1, Table 76 and 86). As in study 1, participants with high somatic and psychological distress reported lower levels of good mood, alertness, and relaxation compared to participants with low somatic and psychological distress. Appendix 4.1 displays diurnal profiles of psychological strain (study 1) and mood and subjective well-being (study 2).

b) Diurnal profiles of mood and subjective well-being: association with cortisol profiles

In a next step we examined the association of diurnal profiles of psychological strain (study 1) and mood / well-being (study 2) with diurnal cortisol profiles.

In study 1, mixed model analyses for repeated measures revealed a significant main effect of psychological strain profiles on diurnal cortisol profiles ($F= 12.08$, $p< 0.001$), but no interaction effects of strain profiles with time of sampling ($F= 0.12$, $p> 0.9$; Table 29) in the total sample. Separate analyses with gender, occupational groups, and shift work as group factors yielded comparable results: with regard to each group factor (gender, occupational group, and shift work) there was a significant main effect of psychological strain profiles on cortisol profiles, however, no interaction of strain and group and/or time of sampling, respectively (Table 29). Appendix 4.2 shows scatter diagrams of cortisol and psychological strain with fitted regression lines of each sampling time (averaged across both sampling days) for the total sample as well as for groups (gender, occupational groups, shift work) displaying a weak positive, yet unsystematic relationship between cortisol secretion and self-rated psychological strain.

In study 2, mixed model analyses for repeated measures failed to reveal a significant association of diurnal profiles of good mood/alertness/relaxation on diurnal cortisol profiles (Table 30). Appendix 4.2 shows scatter diagrams of cortisol and mood/well-being (good mood / alertness / relaxation) with fitted regression lines of each sampling time (averaged across all three sampling days) for the total sample as well as for groups (gender, occupational groups, shift work) illustrating the lack of association between cortisol secretion and self-rated mood and well-being.

Table 29: Effect of diurnal strain on cortisol levels in study 1

Cortisol samples / group	Effect	NDF	DDF	F	p
Sample – study 1					
Single samples:	DAY	1	85	1.86	
t(+0), t(+30), t(08:00), t(11:00),	TIME	5	435	29.28	**
t(15:00), t(20:00)	KAB	1	843	12.08	**
	DAY × KAB	1	843	2.33	
	TIME × KAB	5	843	0.12	
GENDER					
Single samples:	DAY	1	84	0.45	
t(+0), t(+30), t(08:00), t(11:00),	TIME	5	430	25.73	**
t(15:00), t(20:00)	KAB	1	820	9.27	**
	SEX	1	91	0.54	
	SEX × TIME	5	430	1.93	
	KAB × TIME	5	820	0.25	
	KAB × SEX	1	820	0.04	
	KAB × SEX × TIME	5	820	1.55	
OCCUPATIONAL GROUPS					
Single samples:	DAY	1	82	0.78	
t(+0), t(+30), t(08:00), t(11:00),	TIME	5	420	21.16	**
t(15:00), t(20:00)	KAB	1	774	7.56	**
	OCC	3	89	4.23	
	OCC × TIME	15	420	1.59	
	KAB × TIME	5	774	1.45	
	KAB × OCC	3	774	0.43	
	KAB × OCC × TIME	15	774	1.34	
SHIFT WORK					
Single samples:	DAY	1	72	1.94	
t(+0), t(+30), t(08:00), t(11:00),	TIME	5	368	25.77	**
t(15:00), t(20:00)	KAB	1	693	11.03	**
	SHIFT	1	78	5.32	*
	SHIFT × TIME	5	368	0.54	
	KAB × TIME	5	693	0.18	
	KAB × SHIFT	1	693	0.13	
	KAB × SHIFT × TIME	5	693	0.46	

NDF= numerator degrees of freedom; DDF= denominator degrees of freedom; DAY= sampling day; TIME= sampling time (t(+0) to t(20:00)); KAB = current psychological strain (KAB, Mueller & Basler, 1993); SEX = gender; OCC = occupational groups (nurses, teachers, hotel staff, social service assistants); SHIFT = shift work (yes/no); mixed models for repeated measures: *p<0.05; ** p<0.01

Table 30: Effect of diurnal mood and well-being on cortisol levels in study 2

Cortisol samples / group	Effect	NDF	DDF	F	p
Sample – study 2					
Single samples:	DAY	2	651	1.61	
t(+30), t(16:00), t(20:00)	TIME	2	652	166.47	**
	MOOD	1	2423	2.15	
	ALERT	1	2423	2.07	
	CALM	1	2423	0.61	
	MOOD × TIME	2	2423	1.18	
	ALERT × TIME	2	2423	1.43	
	CALM × TIME	2	2423	0.10	
GENDER					
Single samples:	DAY	2	651	1.60	
t(+30), t(16:00), t(20:00)	TIME	2	652	139.32	**
	MOOD	1	2416	1.21	
	ALERT	1	2416	1.60	
	CALM	1	2416	0.87	
	SEX	1	327	0.43	
	SEX × TIME	2	650	1.40	
	MOOD × TIME	2	2416	0.38	
	ALERT × TIME	2	2416	0.39	
	CALM × TIME	2	2416	0.37	
	MOOD × SEX	1	2416	0.06	
	ALERT × SEX	1	2416	0.00	
	CALM × SEX	1	2416	0.54	
	MOOD × SEX × TIME	2	2416	0.32	
	ALERT × SEX × TIME	2	2416	1.26	
	CALM × SEX × TIME	2	2416	0.80	
OCCUPATIONAL GROUPS					
Single samples:	DAY	2	651	1.79	
t(+30), t(16:00), t(20:00)	TIME	2	644	108.22	**
	MOOD	1	2383	0.94	
	ALERT	1	2383	2.60	
	CALM	1	2383	0.77	
	OCC	4	324	0.51	
	OCC × TIME	8	644	1.33	
	MOOD × TIME	2	2383	0.98	
	ALERT × TIME	2	2383	0.05	
	CALM × TIME	2	2383	1.85	
	MOOD × OCC	4	2383	0.58	
	ALERT × OCC	4	2383	1.83	
	CALM × OCC	4	2383	0.88	
	MOOD × OCC × TIME	8	2383	1.23	
	ALERT × OCC × TIME	8	2383	1.36	
	CALM × OCC × TIME	8	2383	2.14	

Table 30 (continued)

Cortisol samples / groups	Effect	NDF	DDF	F	p
SHIFT WORK					
Single samples:	DAY	2	639	1.67	
t(+30), t(16:00), t(20:00)	TIME	1	638	88.85	**
	MOOD	1	2375	1.65	
	ALERT	1	2375	2.92	
	CALM	1	2375	0.30	
	SHIFT	1	321	0.04	
	SHIFT × TIME	2	638	1.20	
	MOOD × TIME	2	2375	0.64	
	ALERT × TIME	2	2375	0.66	
	CALM × TIME	2	2375	0.43	
	MOOD × SHIFT	1	2375	0.25	
	ALERT × SHIFT	1	2375	2.08	
	CALM × SHIFT	1	2375	0.17	
	MOOD × SHIFT × TIME	2	2375	0.06	
	ALERT × SHIFT × TIME	2	2375	0.78	
	CALM × SHIFT × TIME	1	2375	0.93	

NDF= numerator degrees of freedom; DDF= denominator degrees of freedom; DAY= sampling day; TIME= sampling time (t(+0) to t(20:00)); MOOD = NRS: very bad mood – very good mood (0 – 10; MDBF, Steyer et al., 1997); ALERT = NRS: very tired – very alert (0 – 10; MDBF, Steyer et al., 1997); CALM = NRS: very restless – very calm (0 – 10; MDBF, Steyer et al., 1997); SEX = gender; OCC = occupational groups (nurses, teachers, hotel staff, social service assistants); SHIFT = shift work (yes/no); mixed models for repeated measures: *p<0.05; ** p<0.01

4.4.2 Mean diurnal mood and subjective well-being

Diurnal mean scores of self-rated psychological strain (study 1) and mood/well-being (study 2) were calculated from diurnal profiles of psychological strain (study 1) and mood/well-being (study 2).

a) Mean diurnal mood and subjective well-being: differential aspects

In study 1, average diurnal psychological strain did not differ significantly between sampling days (mixed models for repeated measures: F=3.86, p>0.06; Appendix 1, Table 88) in the total sample. As observed in analyses of diurnal profiles of psychological strain, there were no significant differences in average diurnal psychological strain between men and women (Appendix 1, Table 89), occupational groups (Appendix 1, Table 89), and shift workers and participants reporting to work normal hours (Appendix 1, Table 89): separate mixed

model analyses for repeated measures failed to reveal significant overall effects of gender (F= 0.45, p> 0.5), occupational group (F= 0.64, p> 0.5), shift work (F=0.06, p> 0.8) and there were no significant interaction effects of gender × sampling day (F= 0.12, p> 0.7), occupational group × sampling day (F= 0.21, p> 0.8), and shift work × sampling day (F= 0.38, p> 0.5). Corresponding to analyses of diurnal profiles, average diurnal psychological strain levels differed significantly between subjects with low and with high somatic and psychological distress (median-split of the relevant scales), i.e. participants with high somatic and psychological distress reported higher levels of average diurnal psychological strain: a) *somatic complaints* (low/high; Appendix 1, Table 90*)*: significant overall effect of group (F= 9.29, p< 0.01), no significant interaction effect of group × sampling day (F= 2.45, p> 0.1); b) *depressive symptoms* (low/high; Appendix 1, Table 90): significant overall effect of group (F= 21.49, p< 0.0001), no significant interaction effect of group × sampling day (F= 0.81, p> 0.3); c) *trait anxiety* (low/high; Appendix 1, Table 90): significant overall effect of group (F= 28.84, p< 0.0001), no significant interaction effect of group × sampling day (F= 0.75, p> 0.3); d) *emotional exhaustion* (low/high, Appendix 1, Table 90): significant overall effect of group (F= 12.04, p< 0.001), no significant interaction effect of group × sampling day (F= 1.64, p> 0.2); e) *depersonalization* (low/high; Appendix 1, Table 90): significant overall group effect (F= 9.31, p< 0.01), no significant interaction effect of group × sampling day (F= 0.96, p> 0.3); f) *personal accomplishment* (low/high; Appendix 1, Table 90): significant overall group effect (F= 10.39, p< 0.01), no significant interaction effect of group × sampling day (F= 0.03, p> 0.8).

In study 2, average levels of diurnal mood, alertness, and relaxation intercorrelated substantially (Pearson correlation coefficients .46 - .67; Appendix 1, Table 91), thus a mean score of diurnal mood/well-being was calculated and used in subsequent analyses to avoid excessive multiple testing. Average diurnal levels of mood/well-being did not differ significantly between sampling days (mixed models for repeated measures: F=1.16, p>0.3; Appendix 1, Table 92) across all participants. As observed in analyses of diurnal profiles of mood and well-being, there were no significant differences in average diurnal psychological strain between men and women (mixed models for repeated measures, overall effect of gender: F= 0.35, p> 0.4; interaction effect of gender × sampling day: F= 1.25, p> 0.2; Appendix 1, Table

93), but significant differences in all subsequent group analyses. As for occupational groups, teachers had lowest levels of average diurnal mood/well-being (overall effect occupational group: F= 5.77, p< 0.001; interaction effect of group × sampling day: F= 2.33, p> 0.05; Appendix 1, Table 93). Shift workers had higher levels of good mood/well-being compared to participants working normal hours (overall group effect: F= 17.13, p< 0.0001; interaction effect of group × sampling day: F= 1.48, p> 0.2). Participants with high somatic and psychological distress had significantly lower levels of good mood/well-being compared to participants with low somatic and psychological distress (median-split of the relevant scales): a) *somatic complaints* (low/high; Appendix 1, Table 94): significant overall effect of group (F= 46.60, p< 0.0001), no significant interaction effect of group × sampling day (F= 0.07, p> 0.9); b) *depressive symptoms* (low/high; Appendix 1, Table 94): significant overall effect of group (F= 90.17, p< 0.001), no significant interaction effect of group × sampling day (F= 1.66, p> 0.1); c) *trait anxiety* (low/high; Appendix 1, Table 94): significant overall effect of group (F= 134.31, p< 0.0001), no significant interaction effect of group × sampling day (F= 0.32, p> 0.7); d) *emotional exhaustion* (low/high; Appendix 1, Table 94): significant overall effect of group (F= 83.26, p< 0.0001), no significant interaction effect of group × sampling day (F= 0.35, p> 0.7); e) *depersonalization* (low/high; Appendix 1, Table 94): no significant group effect (F= 3.36, p> 0.06), no significant interaction effect of group × sampling day (F= 3.26, p> 0.06); f) *personal accomplishment* (low/high; Appendix 1, Table 94): significant overall group effect (F= 22.95, p< 0.0001), no significant interaction effect of group × sampling day (F= 1.97, p> 0.1).

b) Mean diurnal mood and subjective well-being: association with cortisol parameters

In a next step, the association between average diurnal mood and subjective well-being and cortisol composite measures was investigated.

In study 1, average self-rated diurnal psychological strain was not substantially and unsystematically correlated to cortisol composite measures (*cortisol awakening rise, total morning cortisol, total diurnal cortisol release, and diurnal cortisol decrease*; Appendix 1, Table 95) in the total samples and with regard to gender (Appendix 1, Table 96), occupational groups (Appendix 1, Table 97), and shift work (yes/no; Appendix 1, Table 98).

In study 2, average self-rated mood/well-being (mean score calculated from average levels of mood, alertness, and relaxation) was completely uncorrelated to cortisol parameters (*cortisol awakening rise*, *total morning cortisol release*, *total diurnal cortisol release*, and *diurnal cortisol decline*; Appendix 1, Table 99) across participants, with regard to gender (Appendix 1, Table 100), occupational groups (Appendix 1, Table 101), and shift work (yes/no; Appendix 1, Table 102).

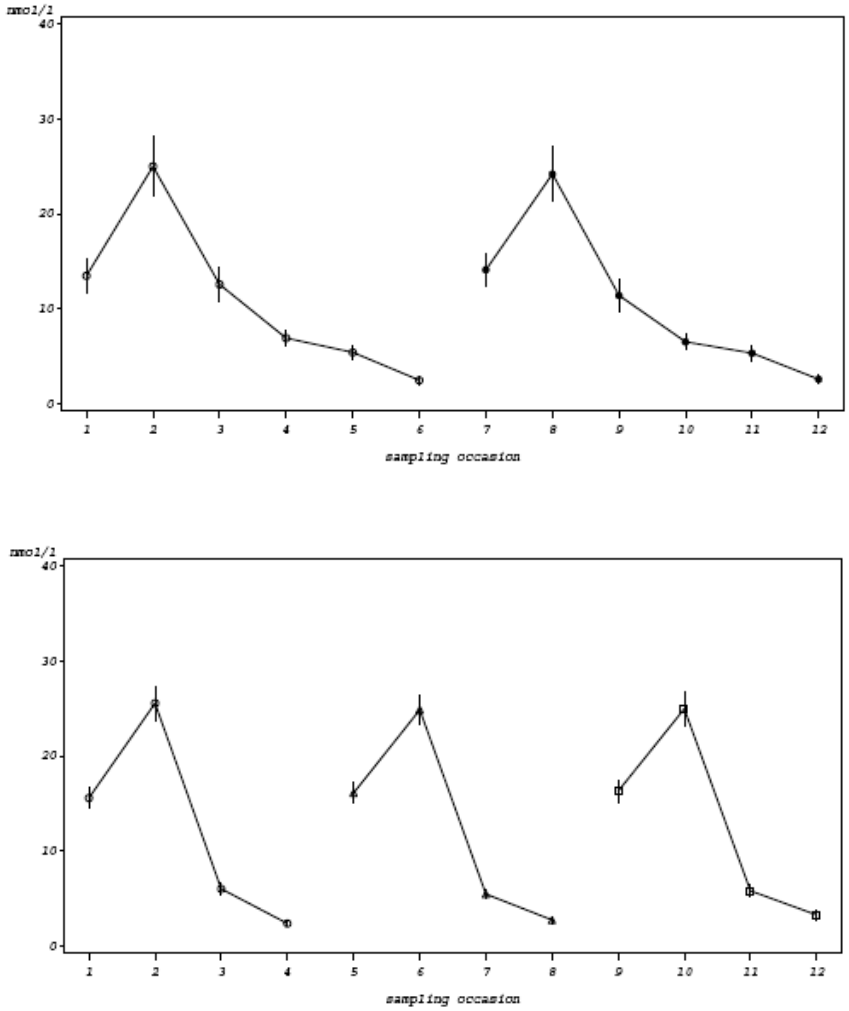

Figure 15: Stability of diurnal salivary cortisol profiles across sampling days (above: study 1, below: study 2).

Table 31: Stability of diurnal cortisol profiles

		Study 1				Study 2			
	Effect	**NDF**	**DDF**	**F**	**p**	**NDF**	**DDF**	**F**	**p**
Sample	DAY	1	98	0.02		2	657	1.11	
	TIME	5	490	372.79	**	3	985	1854.30	**
	DAY × TIME	5	424	0.63		6	1801	1.23	
Men	DAY	1	20	0.03		2	210	0.96	
	TIME	5	103	88.54	**	3	314	583.86	**
	DAY × TIME	5	86	1.42		6	578	0.49	
Women	DAY	1	77	0.03		2	445	1.12	
	TIME	5	382	297.02	**	3	668	1282.71	**
	DAY × TIME	5	333	0.28		6	1217	1.03	
Nurses	DAY	1	48	0.62		2	160	2.90	
	TIME	5	232	125.85	**	3	242	408.26	**
	DAY × TIME	5	194	0.31		6	436	0.32	
Teachers	DAY	1	20	0.39		2	213	0.59	
	TIME	5	99	199.78	**	3	319	733.84	**
	DAY × TIME	5	93	0.62		6	575	1.40	
Hotel staff	DAY	1	6	1.95		2	32	0.61	
	TIME	5	35	30.92	**	3	48	57.41	**
	DAY × TIME	5	23	1.57		6	94	0.60	
Social servcie	DAY	1	21	0.41		2	176	0.63	
	TIME	5	109	109.64	**	3	263	600.67	**
	DAY × TIME	5	99	0.67		6	490	0.32	
Mixed group	DAY					2	68	1.08	
	TIME	--	--	--		3	101	142.64	**
	DAY × TIME					6	182	0.49	
Shift / yes	DAY	1	42	0.27		2	168	1.98	
	TIME	5	210	241.04	**	3	254	402.90	**
	DAY × TIME	5	179	0.82		6	456	0.57	
Shift / no	DAY	1	41	0.11		2	475	0.60	
	TIME	5	207	119.40	**	3	710	1454.82	**
	DAY × TIME	5	178	0.51		6	1309	1.35	
Current	DAY	1	35	1.22		2	187	1.40	
smoker	TIME	5	176	107.84	**	3	280	329.95	**
	DAY × TIME	5	147	0.41		6	513	1.59	
Non-smoker	DAY	1	59	1.43		2	428	1.15	
	TIME	5	295	295.94	**	3	642	1449.82	**
	DAY × TIME	5	263	1.00		6	1170	0.38	

NDF= numerator degrees of freedom; DDF= denominator degrees of freedom; DAY= sampling day; TIME= sampling occasion (t(+0) to t(20:00)); mixed models for repeated measures: *p<0.05; ** p<0.01

4.5 Stability of cortisol profiles

4.5.1 Stability of diurnal cortisol profiles

Stability of the average diurnal pattern (i.e. diurnal cortisol profiles) was examined using mixed model analyses for repeated measures with sampling time and day as independent variables and cortisol output as the outcome variable. Separate analyses were applied in both studies for the total sample (across all participants of the relevant study), for men and women, for occupational groups and participants working shifts and those working normal hours.

In both studies, the average diurnal cortisol profiles in the total sample and in the separate groups respectively proved to be highly stable across sampling days: mixed model analyses for repeated measures did not reveal significant main effects of sampling day or significant interaction effects of day × sampling time/occasion (Table 31). Cortisol profiles of the total samples in both studies are displayed in Figure 15; Appendix 5 illustrates the cortisol profiles of all subgroups.

4.5.2 Stability of single cortisol samples

Additionally, the stability of single cortisol samples, i.e. t(+0) to t(20:00), was examined using intraclass correlation coefficients (ICC) to determine the degree of agreement or concordance of cortisol output between sampling days. The ICC values (including 95% confidence intervals) of single cortisol samples for the total sample and groups in both studies (gender, occupational groups, shift work, smoking status) are given in Appendix 1, Table 103 and 104.

a) Awakening cortisol samples: t(+0) and t(+30)

In study 1, awakening cortisol samples across all subjects showed moderate stability, although the ICC value of cortisol samples measured 30 minutes after awakening (ICC= 0.69) was higher compared to the ICC of samples directly after awakening (ICC= 0.59). In study 2, however, ICC values were smaller compared to study 1 with a higher ICC value of cortisol samples directly after awakening (ICC= 0.47) than cortisol samples measured 30 minutes after awakening (ICC= 0.37; Appendix 1, Table 103), indicating low to moderate stability.

This pattern (higher ICC values for t(+30) in study 1; lower ICC values for t(+30) in study 2; and in general: ICC values in study 1 slightly higher compared to study 2) persisted in separate analyses of groups with regard to gender, shift work, and smoking status (Appendix 1, Table 103). In both studies ICC values were higher in women than in men, whereas ICC values were comparable among shift workers and participants working normal hours as well as among current smokers and non-smokers. With regard to occupational groups, marked differences could be observed: while nurses and teachers in study 1 displayed the aforementioned pattern of ICC values (t(+30) > t(+0)), in hotel staff and social service assistants ICC values of cortisol samples directly after awakening were substantially higher than ICC values of cortisol samples 30 minutes after awakening (Appendix 1, Table 104). In study 2, nurses, hotel staff, social service assistants, and the mixed group displayed the aforementioned pattern (t(+30) < t(+0)), while in teachers ICC values directly after awakening were comparable to those of cortisol samples 30 minutes after awakening (Appendix 1, Table 104).

b) Diurnal cortisol samples: t(08:00) to t(20:00)

In contrast to study 2, antemeridian cortisol samples – t(08:00) and t(11:00) – were collected in study 1. The corresponding ICC values displayed a moderate stability of antemeridian cortisol samples in the total sample with slightly higher ICC values for t(08:00) (ICC= 0.62) compared to t(11:00) (ICC= 0.55; Appendix 1, Table 103), which in terms of size were comparable to ICC values of awakening cortisol samples. This pattern persisted in group analyses, with the exception of constant ICC values for t(08:00) and t(11:00) in participants working normal hours, non-smokers, and nurses (Appendix 1, Table 103).

In study 1, ICC values for cortisol samples at t(15:00) were consistently and substantially higher than those for cortisol samples at t(20:00) with regard to the total sample (t(15:00): ICC= 0.68; t(20:00): ICC= 0.33) and with regard to separate groups (Appendix 1, Table 103 and 104), indicating a moderate stability for samples at t(15:00) and rather low stability for samples at t(20:00).

In study 2, however, both ICC values were of comparable magnitude (t(16:00): ICC= 0.27; t(20:00): ICC= 0.32; Appendix 1, Table 103), indicating a rather low stability of after-

noon/evening cortisol samples. This pattern could be observed in group analyses as well (Appendix 1, Table 103 and 104).

4.5.3 Stability of composite measures of the diurnal cortisol pattern

The stability of cortisol parameters (*cortisol awakening rise, total morning cortisol release, total diurnal cortisol release, and diurnal cortisol decline*) was examined using intraclass correlation coefficients (ICC) to determine the degree of agreement or concordance of cortisol output between sampling days. The ICC values (including 95% confidence intervals) of single cortisol samples for the total sample and for groups (gender, occupational groups, shift work, smoking status) in both studies are given in Appendix 1, Table 105-107.

a) Parameters reflecting the average level of diurnal cortisol output

In study 1, parameters reflecting the average level of cortisol output (*total morning cortisol release, total diurnal release*) proved to be of moderate to high stability (with ICC values ranging from 0.68 to 0.83 in the total sample; Appendix 1, Table 105). The stability of *total morning cortisol release* was comparable to parameters reflecting the *total diurnal release*, except for ICC values of the *AUC measures*, which were slightly lower (Appendix 1, Table 105). This pattern persisted among groups, apart from hotel staff and social service assistants with very low to moderate stability coefficients (ICC values ranging from 0.05 to 0.46 in the hotel group; 0.28 to 0.49 in social service assistants; Appendix 1, Table 106).

In study 2, stability coefficients of parameters reflecting the average diurnal level of cortisol output were low to moderate (with ICC values ranging from 0.24 to 0.50 in the total sample; Appendix 1, Table 105). The stability of *total morning cortisol release* was comparable to parameters reflecting the *total diurnal release*, yet ICC values of *AUC measures* were slightly smaller (Appendix 1, Table 105). This pattern could also be observed with regard to groups (gender, shift work, smoking status, occupational groups; Appendix 1, Table 105 and 106).

b) Parameters reflecting dynamic changes of diurnal cortisol output

In study 1, stability coefficients of parameters reflecting dynamic aspects of the cortisol output (*cortisol awakening rise, diurnal decline*) were low to moderate (with ICC values ranging from 0.37 to 0.66 in the total sample; Appendix 1, Table 108). The *cortisol awakening*

rise proved to be of poorer stability (ICC= 0.37) compared to parameters reflecting the *diurnal decrease in cortisol* (Appendix 1, Table 108). This pattern as well as the size of stability coefficients persisted in all groups, apart from current smokers with comparable stability coefficients for the *cortisol awakening rise* and the *diurnal decline* (Appendix 1, Table 108).

In study 2, stability coefficients reflecting dynamic aspects of cortisol secretion were very low to scarcely moderate compared to study 1 (with ICC values ranging from 0.12 to 0.46 in the total sample and in groups; Appendix 1, Table 107 and 108) without substantial differences between ICC values of the *cortisol awakening rise* and parameters reflecting the *diurnal decrease in cortisol*.

In summary, although average diurnal cortisol profiles proved to be highly stable across sampling days in both studies, single samples of cortisol in the morning and throughout the day were only moderately stable and rather unstable in the evening in study 1. In study 2, stability coefficients of single cortisol samples with regard to morning and afternoon/evening measures were of comparable size, yet rather small, indicating poor stability. With regard to composite measures of the diurnal cortisol pattern: parameters reflecting the average level of cortisol output (*total morning cortisol release, total diurnal cortisol release*) proved to be of moderate to high stability, whereas parameters reflecting dynamic changes of cortisol output (*cortisol awakening rise, diurnal cortisol decline*) revealed comparatively moderate to poor stability. In general, stability coefficients were higher in study 1 compared to study 2, and composite measures, particularly those reflecting the average level of diurnal cortisol output, proved to be of higher stability than single cortisol samples.

4.5.4 Replication of studies

a) Comparison of diurnal cortisol profiles

Average cortisol output across sampling days was calculated for each sampling occasion and subsequently cortisol samples collected at the same time in both studies, i.e. t(+0), t(+30), and t(20:00), as well as cortisol samples collected at t(15:00) in study 1 and cortisol samples at t(16:00) in study 2 were compared (Figure 16). Descriptive statistics of cortisol secretion at each sampling occasion common in both studies – t(+0), t(+30), t(15:00)/t(16:00), t(20:00) – for both samples are given in Appendix 1, Table 109.

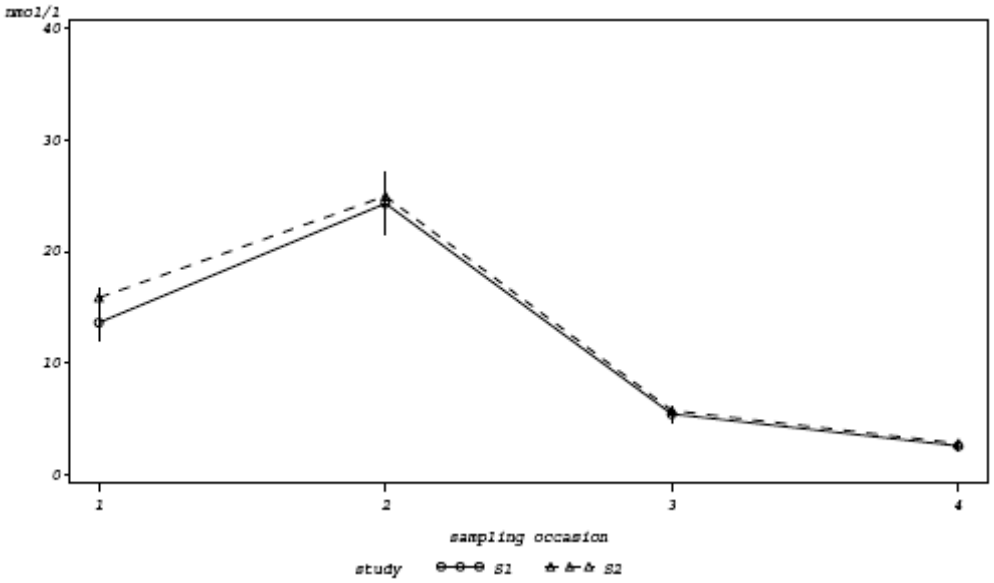

Figure 16: Mean diurnal salivary cortisol profiles: study 1 vs. study 2. Sampling occasion: 1= directly after awakening, 2= 30 minutes after awakening; 3= 15:00-16:00h, 4= 20:00h.

Table 32: Descriptive statistics of cortisol levels: normal values

	Salivary cortisol (nmol/l)				
	N	**Mean ± SD**	**Median**	**Min**	**Max**
t(+0)	432	15.33 ± 8.18	14.51	0.20	54.07
t(+30)	432	24.81 ± 12.88	22.80	1.70	68.53
t(15:00/16:00)	429	5.66 ± 3.84	4.87	0.60	28.30
t(20:00)	427	2.74 ± 2.55	2.20	0.30	24.40

Comparing cortisol secretion in study 1 with study 2, mixed model analyses for repeated measures failed to reveal a significant main effect of group (study 1 vs. study 2; F= 2.97, p>0.1) or a significant interaction effect of group × sampling occasion (F= 1.41, p> 0.2), indicating that there were no significant differences in cortisol output between study 1 and study 2 with regard to equally timed sampling occasions, i.e. t(+0), t(+30), t(20:00), as well as with regard to deviating sampling occasions, i.e. t(15:00) in study 1 compared to t(16:00) in study 2. Thus, both samples of study 1 and study 2 were pooled (descriptive statistics Table 32) and group differences in cortisol output were examined with regard to gender, occupa-

tional groups, shift work, and smoking status. Descriptive statistics of cortisol output at each sampling occasion common to both studies are given in Appendix 1, Table 110 (gender), Appendix 1, Table 111 (occupational groups), Appendix 1, Table 112 (shift work), and Appendix 1, Table 113 (smoking status).

Mixed model analyses for repeated measures revealed a significant main effect of gender ($F= 4.02$, $p< 0.05$) and a significant interaction effect of gender × sampling occasion ($F= 4.48$, $p< 0.01$). Decomposing the significant interaction effect by examining the effect slices, significant differences in cortisol output between men and women emerged for samples collected directly after awakening (t(+0); $F= 5.93$, $p< 0.05$) and in the afternoon (t(15:00)/t(16:00); $F= 6.94$, $p< 0.01$) with significantly lower cortisol levels in women (Appendix 1, Table 110; Figure 17).

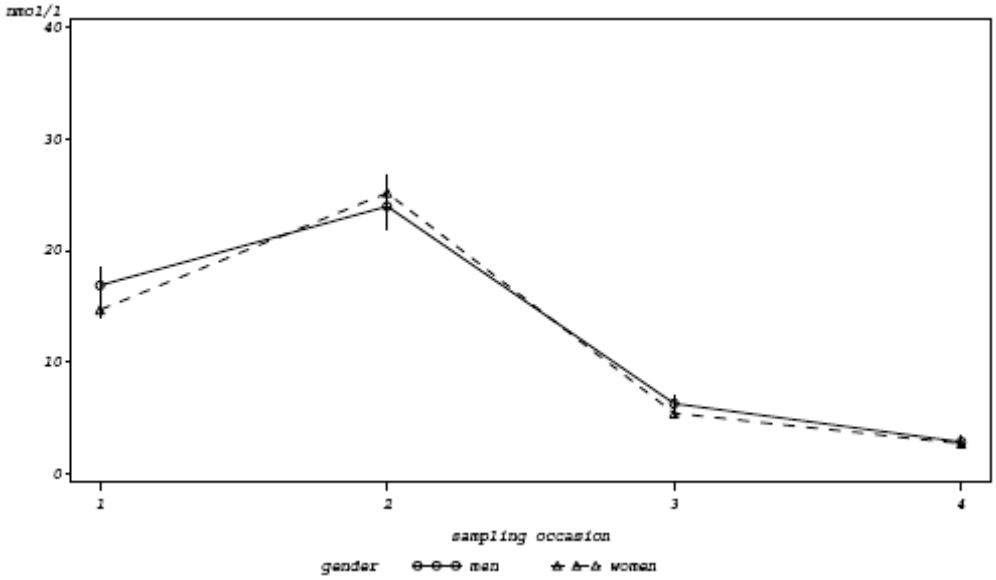

Figure 17: Mean diurnal salivary cortisol profiles by gender. Sampling occasion: 1= directly after awakening, 2= 30 minutes after awakening; 3= 15:00-16:00h, 4= 20:00h.

With regard to occupational groups, mixed model analyses for repeated measures revealed a significant main effect of occupational group ($F= 3.42$, $p< 0.01$) and a significant interaction effect of group × sampling occasion ($F= 3.15$, $p< 0.001$). Post-hoc tests (differences of least square means) revealed significant differences in cortisol output between oc-

cupational groups for samples collected directly after awakening (t(+0); nurses < teachers: t= -3.97, p< 0.0001; nurses < social service assistants: t= -5.27, p< 0.0001; nurses < mixed group: t= -2.09, p< 0.05; hotel staff < social service assistants: t= -2.36, p< 0.05), for samples collected 30 minutes subsequent to awakening (t(+30); nurses < social service assistants: t= -2.58, p< 0.01), and for evening cortisol samples (t(20:00); teachers < nurses, t= 2.18, p< 0.05; teachers < hotel staff: t= -2.48, p< 0.05; teachers < social service assistants: t= -2.32, p< 0.05; teachers < mixed group: t= -3.29, p< 0.01) with significantly lower cortisol levels in nurses subsequent to awakening and 30 minutes thereafter and significantly lower cortisol levels in teachers in the evening (Appendix 1, Table 111; Figure 18).

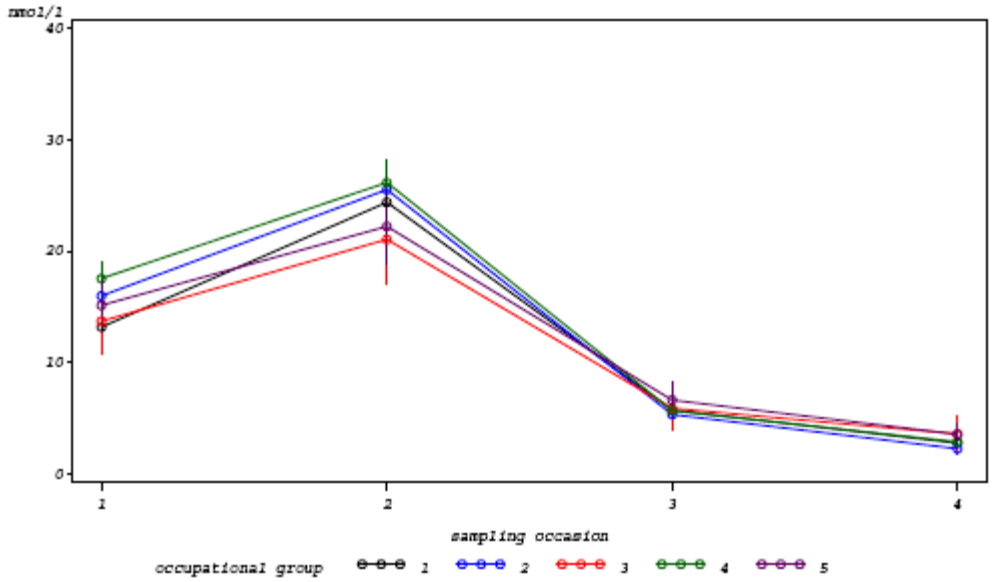

Figure 18: Mean diurnal salivary cortisol profiles by occupational groups (1= nurses; 2= teachers; 3= hotel staff; 4= social service assistants; 5= mixed group). Sampling occasion: 1= directly after awakening, 2= 30 minutes after awakening; 3= 15:00-16:00h, 4= 20:00h.

With regard to shift work (yes/no), mixed model analyses for repeated measures revealed a significant main effect of group (shift work vs. normal hours; F= 7.87, p< 0.01) and a significant interaction effect of group × sampling occasion (F= 2.84, p< 0.05). Decomposing the significant interaction effect by examining the effect slices, significant differences in cortisol output between participants working shift and those working normal hours emerged for

samples collected at 30 minutes subsequent to awakening (t(+30); F= 11.17, p< 0.001) and in the afternoon (t(15:00)/t(16:00); F= 4.63, p< 0.05) with significantly lower cortisol levels in shift workers (Appendix 1, Table 112; Figure 19).

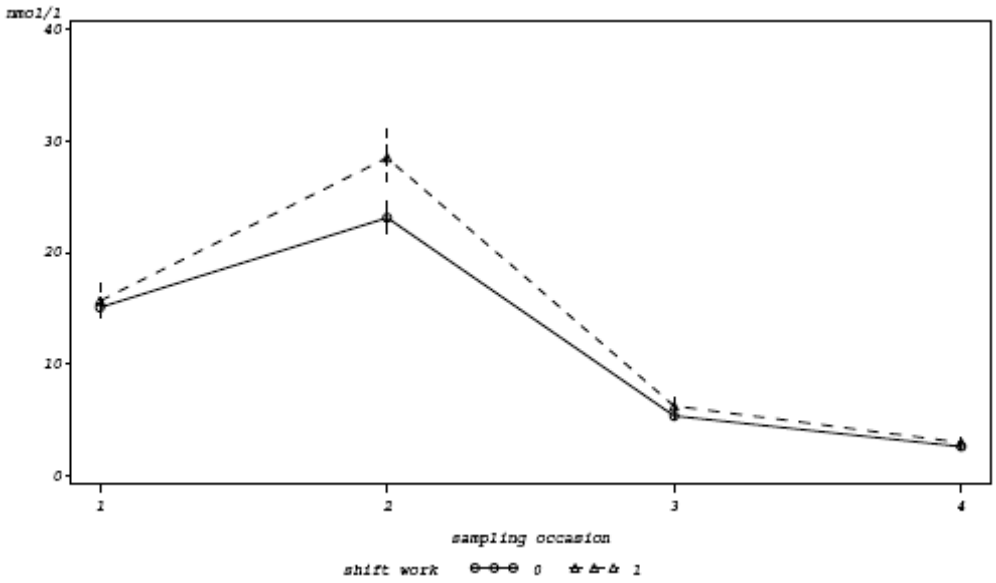

Figure 19: Mean diurnal salivary cortisol profiles by shift work (0= normal hours; 1= shift work). Sampling occasion: 1= directly after awakening, 2= 30 minutes after awakening; 3= 15:00-16:00h, 4= 20:00h.

With regard to smoking status (current smokers vs. non-smokers), mixed model analyses for repeated measures failed to reveal a significant main effect of smoking status (F= 0.20, p> 0.6), but yielded a significant interaction effect of group × sampling occasion (F= 7.91, p< 0.0001). Decomposing the significant interaction effect by examining the effect slices, significant differences in cortisol output between current smokers and non-smokers emerged for samples collected directly after awakening (t(+0); F= 8.93, p< 0.01) and samples collected in the evening (t(20:00); F= 13.67, p< 0.001) with significantly lower cortisol levels in current smokers directly after awakening and significantly higher levels in the evening (Appendix 1, Table 113; Figure 20).

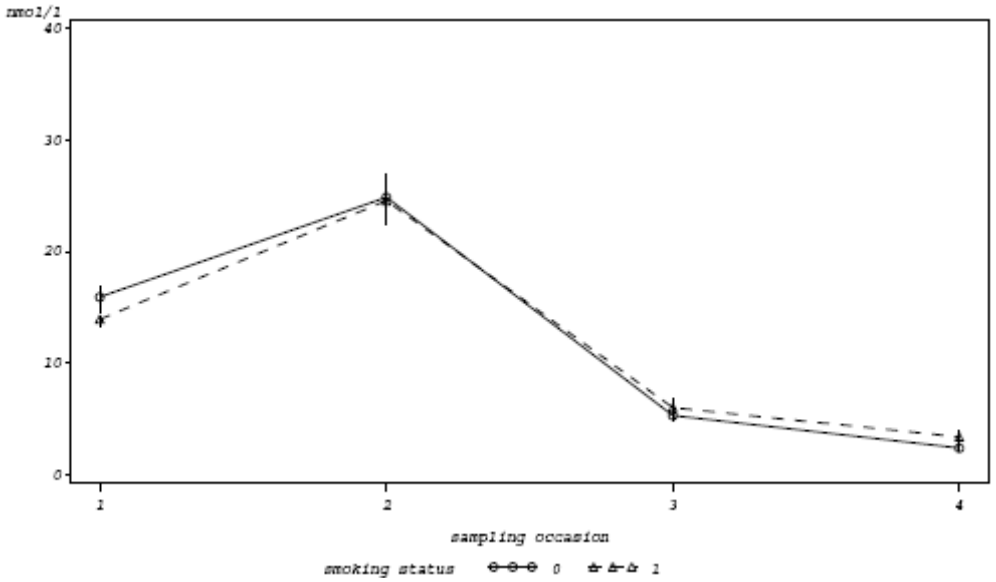

Figure 20: Mean diurnal salivary cortisol profiles by smoking status (0= non-smoker; 1= current smoker). Sampling occasion: 1= directly after awakening, 2= 30 minutes after awakening; 3= 15:00-16:00h, 4= 20:00h.

b) Comparison of composite measures of diurnal cortisol profiles

In a next step, composite measures of mean diurnal cortisol profiles (averaged across sampling days) were compared between study 1 and study 2. Only composite measures, that were calculated from sampling occasions common in both studies, were chosen for comparison, i.e. the *cortisol awakening rise* [t(+30) minus t(+0)], the *total morning cortisol release* [mean of t(+0) and t(+30)] and the *diurnal decrease in cortisol* [a) t(+30) minus t(20:00); b) t(+0) minus t(20:00)]. Descriptive statistics of the relevant cortisol parameters in both studies are given in Appendix 1, Table 114.

Separate one-way ANOVAs for each relevant cortisol parameter were calculated: there was no significant difference in the *cortisol awakening rise* ($F= 1.88$, $p> 0.1$), the *total morning cortisol release* ($F= 3.59$, $p< 0.06$), and both measures of *diurnal decrease in cortisol* ([t(+30) minus t(20:00)]: $F= 0.01$, $p< 0.9$; [t(+0) minus t(20:00)]: $F= 3.49$, $p< 0.06$) between study 1 and study 2. Thus, both samples of study 1 and study 2 were pooled (descriptive statistics Table 33; Figure 21) and group differences in composite measures of mean diurnal cortisol profiles were examined with regard to gender, occupational groups, shift work, and

smoking status. Descriptive statistics of cortisol parameters in both studies are given in Appendix 1, Table 115 (gender), Appendix 1, Table 116 (occupational groups), Appendix 1, Table 117 (shift work), and Appendix 1, Table 118 (smoking status).

Table 33: Descriptive statistics of cortisol levels (parameters): normal values

	Salivary cortisol (nmol/l)				
	N	**Mean ± SD**	**Median**	**Min**	**Max**
Cortisol awakening rise	429	9.54 ± 11.64	8.17	-30.27	51.80
Total morning cortisol release	432	19.90 ± 9.18	19.00	1.65	52.13
Diurnal cortisol decline					
Delta [t(+30) – t(20:00)]	422	22.18 ± 12.96	20.74	-6.20	67.35
Delta [t(+0) – t(20:00)]	423	12.82 ± 8.26	12.00	-9.00	52.97

With regard to gender, men and women differed significantly in the *cortisol awakening rise* (F= 8.56, p< 0.01) and in the *diurnal decrease in cortisol* from directly after awakening to the evening ([t(+0) minus t(20:00)]: F= 6.63, p< 0.01), but not in the *total morning cortisol release* (F= 0.13, p> 0.7) and the *diurnal decrease in cortisol* from 30 minutes subsequent to awakening to the evening (([t(+30) minus t(20:00)]: F= 0.88, p< 0.3) with a larger *cortisol awakening rise*, but flatter diurnal rhythm in women (Appendix 1, Table 115; Figure 22).

Significant differences in cortisol parameters between occupational groups could be observed for the *total morning cortisol release* (F= 4.14, p< 0.01; significant post-hoc Scheffé's tests: nurses < social service assistants) and the *diurnal decrease in cortisol* from awakening to the evening ([t(+0) minus t(20:00)]: F= 5.83, p< 0.0001; significant post-hoc Scheffé's tests: nurses < social service assistants; nurses < teachers) with lowest levels in nurses, but not for the *cortisol awakening rise* (F= 1.46, p> 0.2) and the *diurnal decrease in cortisol* from directly after awakening to the evening ([t(+0) minus t(20:00)]: F= 1.73, p> 0.1) with lowest levels of total morning cortisol release and flatter diurnal cortisol decline in nurses (Appendix 1, Table 117; Figure 23).

Compared to participants working normal hours, participants working shifts had significantly larger *cortisol awakening rises* (F= 15.39, p< 0.0001), higher levels in *total morning cortisol release* (F= 6.25, p< 0.05), and a steeper *decline in cortisol* from directly after awak-

ening to evening ([t(+0) minus t(20:00)] F= 14.54, p< 0.001). However, there were no significant differences between both groups in the *diurnal decrease in cortisol* from 30 minutes subsequent to awakening to evening ([t(+30) minus t(20:00)]: F= 0.00, p> 0.9; Appendix 1, Table 117; Figure 24).

With regard to smoking status, significant differences between current smokers and non-smokers emerged solely in the *diurnal decrease in cortisol* from directly after awakening to evening (([t(+0) minus t(20:00)] F= 12.25, p< 0.001) with a flatter diurnal decline in current smokers (Appendix 1, Table 118; Figure 25). There were no significant differences between both groups with regard to the *cortisol awakening rise* (F= 2.31, p> 0.1), the *total morning cortisol release* (F= 2.72, p> 0.1), and the *diurnal decrease in cortisol* from 30 minutes subsequent to awakening to evening (([t(+30) minus t(20:00)] F= 1.82, p> 0.1).

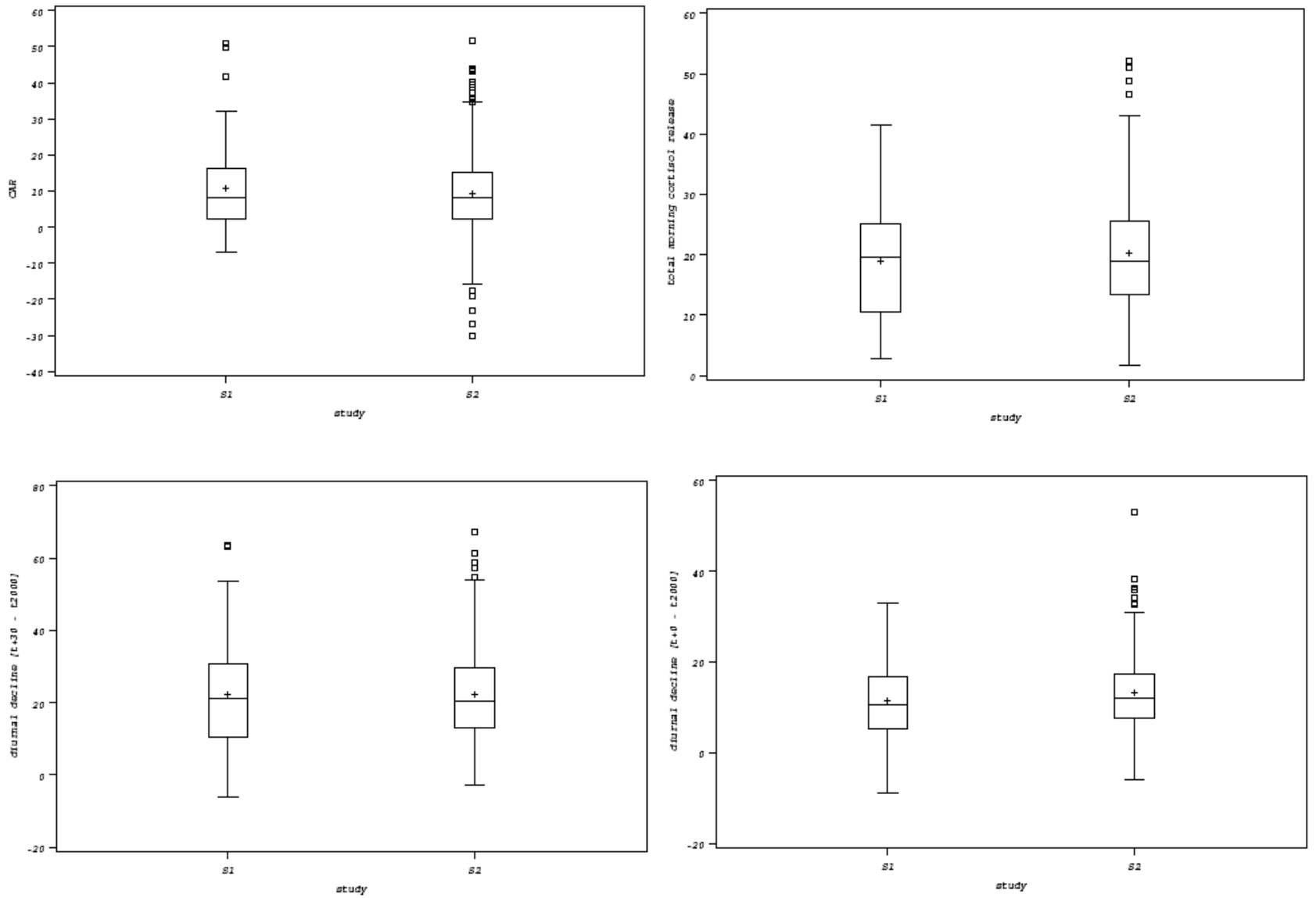

Figure 21: Composite measures of salivary cortisol levels: study 1 vs. study 2. Clockwise: cortisol awakening response; total morning cortisol release; diurnal decline in cortisol from 30 minutes after awakening to 20:00h; diurnal decline in cortisol from directly after awakening to 20:00h.

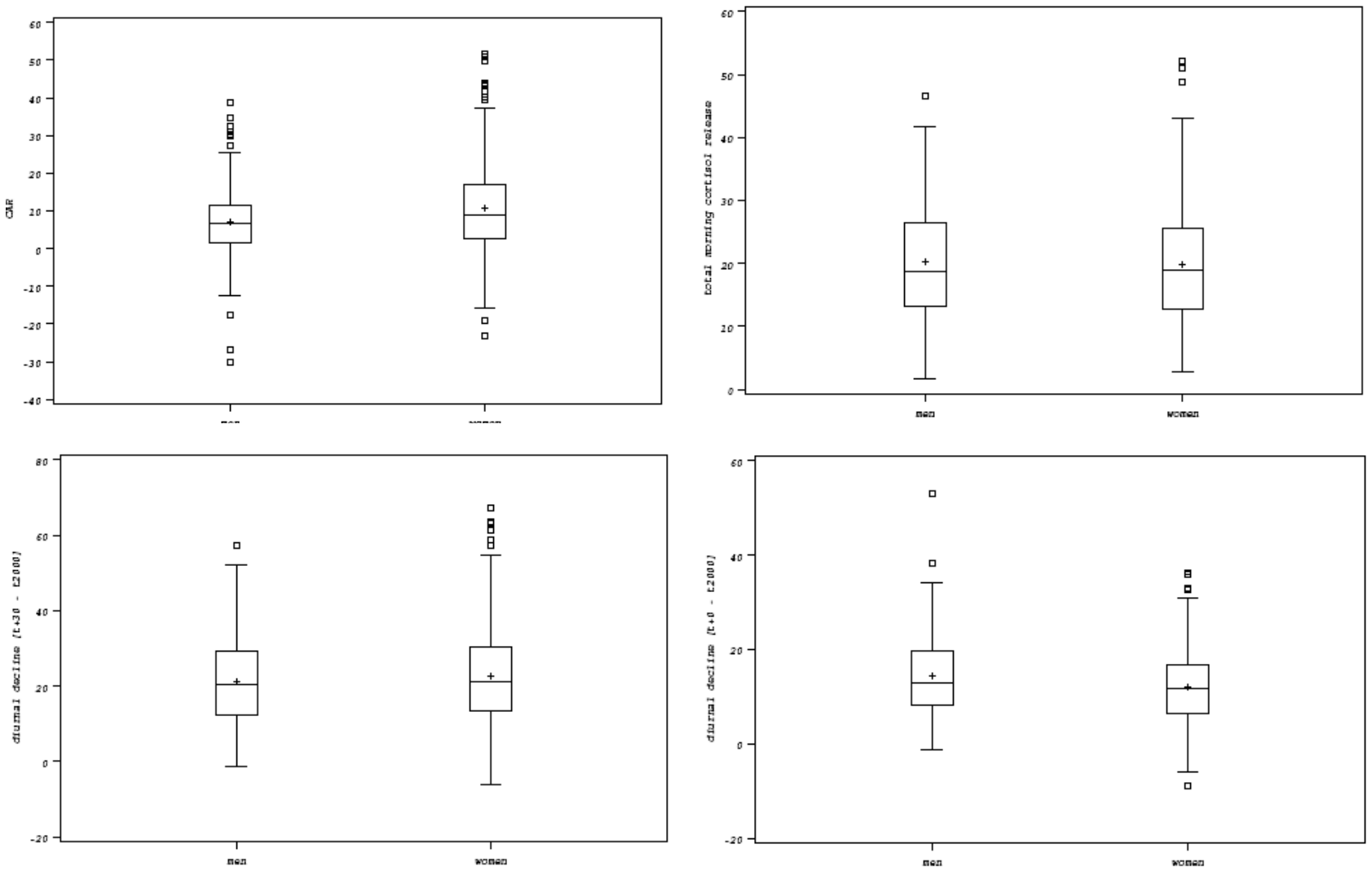

Figure 22: Composite measures of salivary cortisol levels by gender. Clockwise: cortisol awakening response; total morning cortisol release; diurnal decline in cortisol from 30 minutes after awakening to 20:00h; diurnal decline in cortisol from directly after awakening to 20:00h.

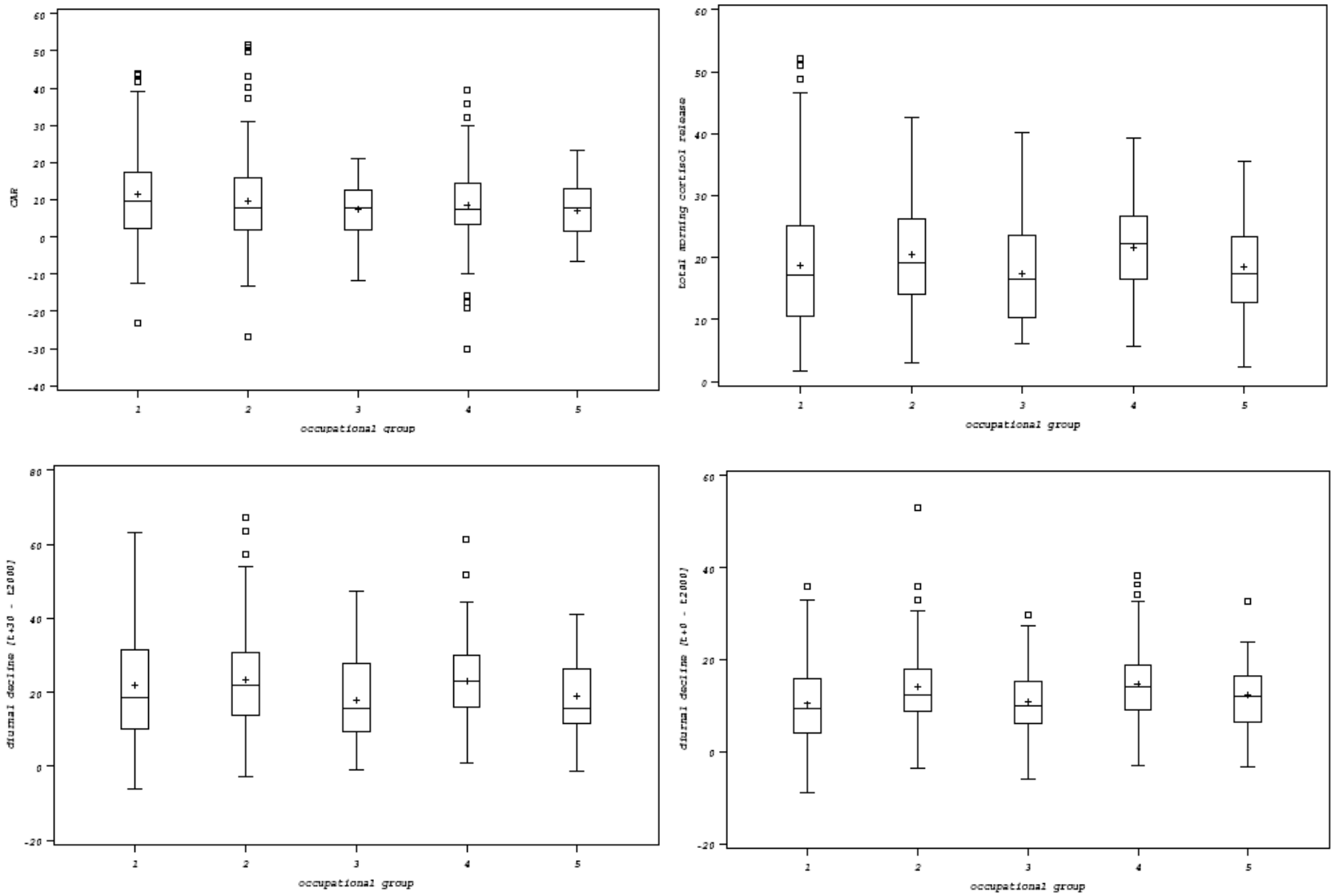

Figure 23: Composite measures of salivary cortisol levels by occupational group. (1= nurses; 2= teachers; 3= hotel staff; 4= social service assistants; 5= mixed group). Clockwise: cortisol awakening response; total morning cortisol release; diurnal decline in cortisol from 30 minutes after awakening to 20:00h; diurnal decline in cortisol from directly after awakening to 20:00h.

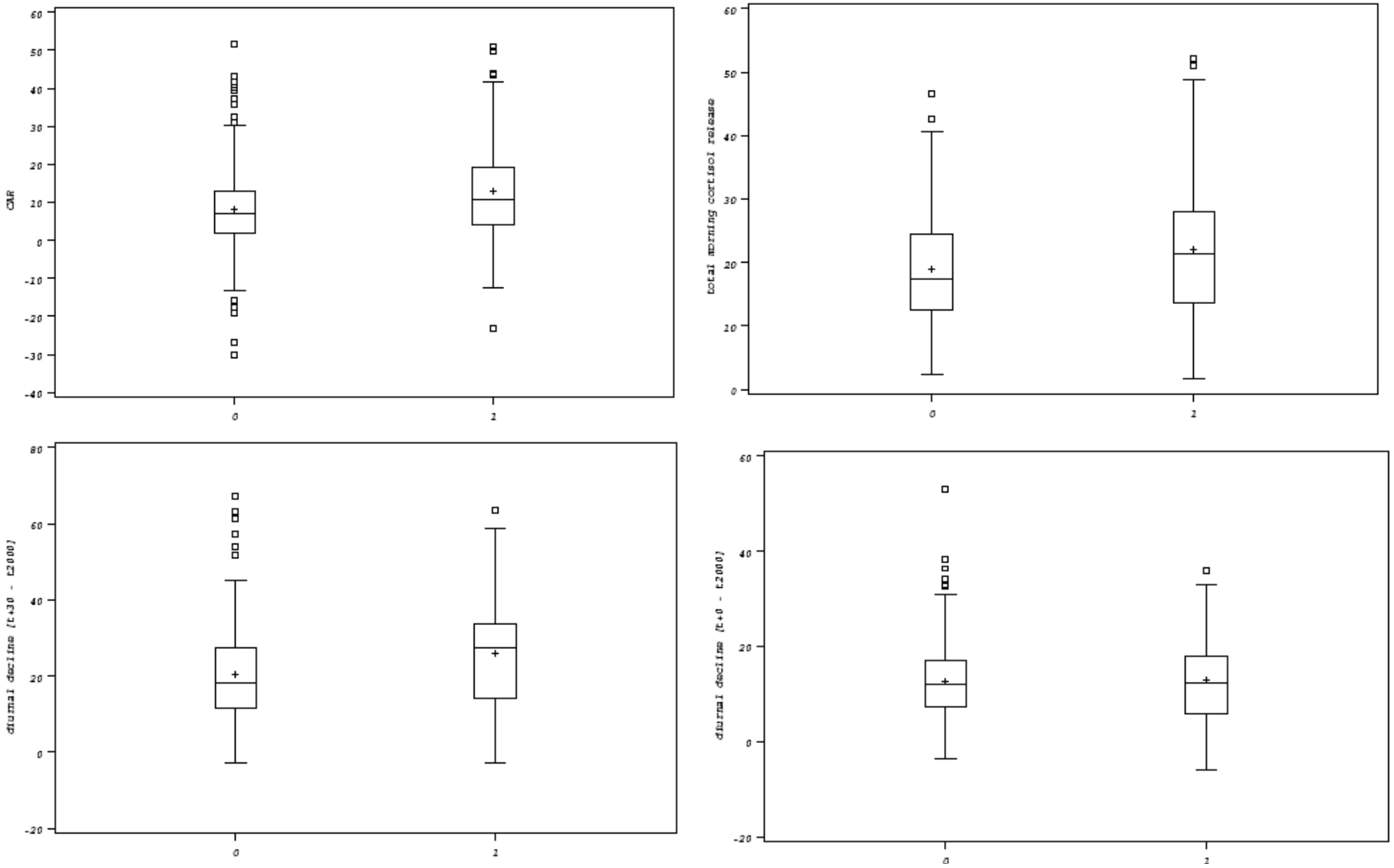

Figure 24: Composite measures of salivary cortisol levels by shift work (0= normal hours; 1= shift work). Clockwise: cortisol awakening response; total morning cortisol release; diurnal decline in cortisol from 30 minutes after awakening to 20:00h; diurnal decline in cortisol from directly after awakening to 20:00h.

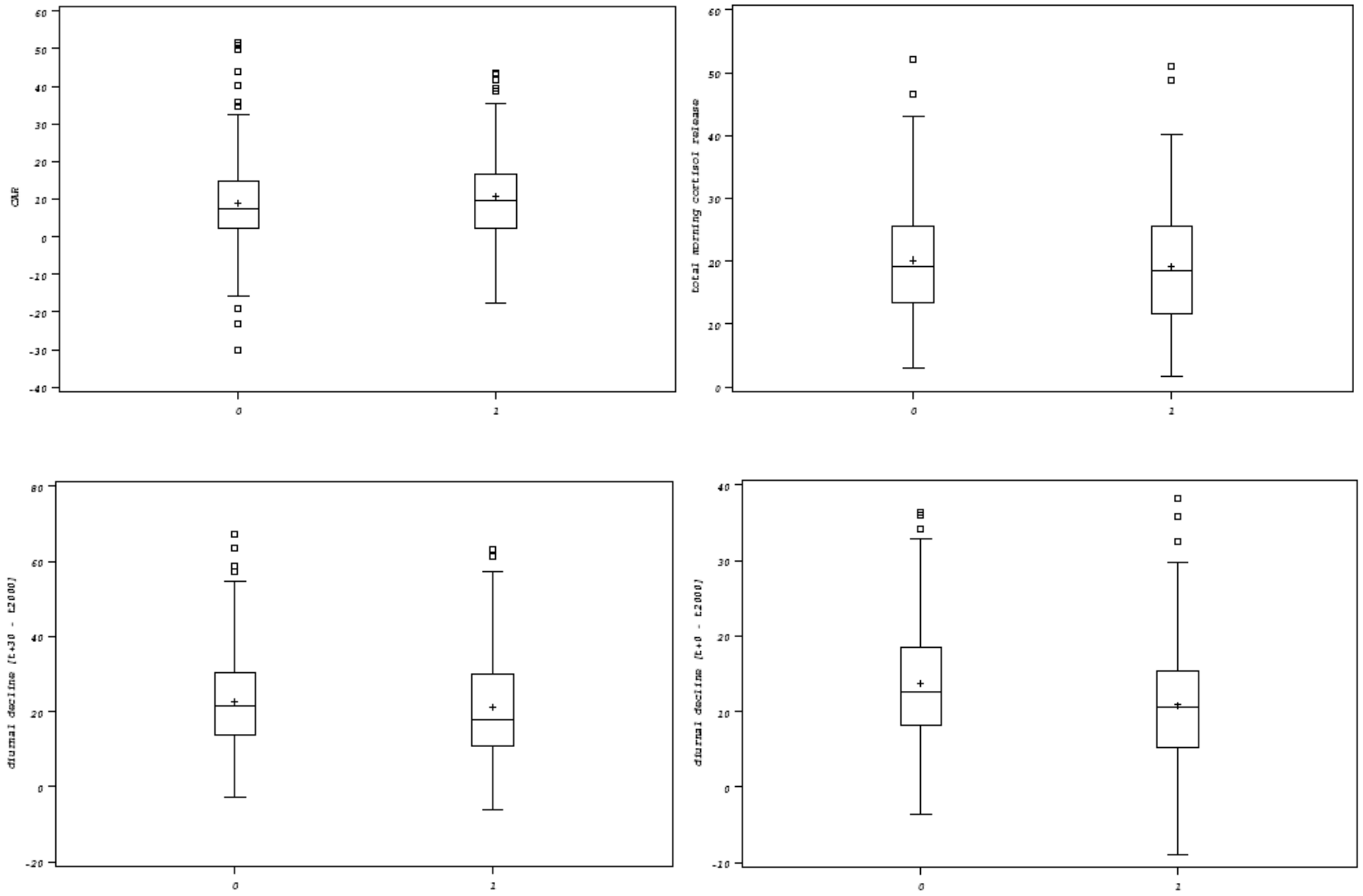

Figure 25: Composite measures of salivary cortisol levels by smoking status (0= non-smoker; 1= current smoker). Clockwise: cortisol awakening response; total morning cortisol release; diurnal decline in cortisol from 30 minutes after awakening to 20:00h; diurnal decline in cortisol from directly after awakening to 20:00h.

5. Discussion

5.1 Internal structure of cortisol samples

Pattern of missing data. Missing values (i.e. missing cortisol samples as well as missing specification of sampling times) were evenly distributed across sampling days, but more likely to occur at later sampling time points (afternoon/evening). In study 1, men had more missing values than women, while in study 2 there were no significant gender differences. With regard to occupational groups hotel staff accounted for significant differences in missing values in both studies: in study 1 missing values accumulated in the group hotel staff, while in study 2 hotel staff provided the fewest missing values. A total of seven participants had to be excluded from subsequent analyses: in study 1 one participant and in study 2 five participants consistently failed to indicate sampling times, in study 2 one participant was excluded due to substantial deviation from the sampling protocol on all three sampling days.

Extreme cortisol outliers. Extreme cortisol values were assessed for each timed sample and sampling day separately using three criteria with increasing strictness: a) cortisol values ranging more than four standard deviations above the sample mean; b) cortisol values ranging more than three interquartile ranges above the 75th percentile; c) cortisol values ranging 4(Q3-Q2) above the sample mean (Appendix 1, Table 6). With regard to all three criteria there was no systematic bias in extreme values: extreme cortisol values were evenly distributed across sampling times and days, gender, and occupational groups. There was no systematic bias regarding depression and anxiety scores, somatic complaints and disorders, thus extreme values were not excluded from data sets.

Adherence to the sampling protocol: compliance rates and deviation in minutes. In study 1, 69 participants used drug exposure monitors to electronically verify sampling times and additionally provided self-reported sampling times in their stress diary. The remaining 33 participants in study 1 as well as the total sample in study 2 provided self-reported sampling times only. Mean compliance rates and mean deviation in minutes were calculated across sampling occasions, sampling days and participants. In study 1, verified average compliance rates and average deviation in minutes were significantly lower than self-reported sampling times in electronically monitored participants. However, average compliance rates and de-

viation in minutes derived from self-reported sampling times did not vary significantly between participants in study 1 using drug exposure monitors and those providing self-reported sampling times only. Additionally, average compliance rates of self-reported sampling times in study 2 did not differ significantly from those in study 1 with or without drug exposure monitors. However, average self-reported deviation in minutes in study 2 was considerably lower compared study 1 with or without drug exposure monitors.

As observed in other studies (Broderick et al., 2004; Kudielka et al., 2003) self-report of compliance considerably overestimates the actual objective compliance derived from electronic monitors. Thus, the use of drug exposure monitors seems to be advisable to obtain precise information about compliance rates and deviation in minutes from the scheduled sampling protocol. However, studies examining the effect of adherence to the saliva sampling protocol on cortisol profiles (Broderick et al., 2004; Kudielka et al., 2003) usually classify participants non-compliant in case of one or more non-compliant single cortisol samples. We think that this method results in unfavourable confounding of individual cortisol profiles with a combination of compliant and non-compliant measures and consequently prefer to investigate the effect of non-adherence to the saliva sampling protocol on each single cortisol sample separately.

Adherence to the sampling protocol: sampling time interval between t(+0) and t(+30). Variations in the length of the time interval between awakening cortisol samples, i.e. directly after awakening and thirty minutes thereafter, due to non-adherence to the scheduled sampling protocol were not associated with the magnitude of the cortisol awakening rise and total morning cortisol release in both studies. Subdividing the sampling time interval into four categories (*0 to 19 minutes*, *20 to 40 minutes*, *40 to 60 minutes*, and *more than 60 minutes*) did not result in significant differences in cortisol levels with regard to single samples (t(+0), t(+30)) and composite measures (*cortisol awakening rise*, *total morning cortisol release*). In addition, non-responders of the cortisol awakening rise (defined by an increase in cortisol of less than 2.5 nmol/l according to Wuest et al., 2000) did not accumulate in categories of sampling time intervals outside the scheduled range of 20 to 40 minutes.

These findings do not agree with results obtained by Broderick et al. (2004), Kudielka et al. (2003), Kunz-Ebrecht et al. (2004b) and Thorn et al. (2003), who consistently found that

non-compliant samples either failed to show the usual rise in cortisol from baseline at 30 minutes after awakening or had a considerably weaker rise in cortisol compared to compliant samples. To resolve these contradictory findings, studies investigating the cortisol awakening rise with a more detailed schedule, i.e. cortisol samples collected directly after awakening (baseline level) and then in subsequent intervals of 10 or 15 minutes up to 60 minutes after awakening, need to be considered: within the first hour after awakening increases in cortisol were observed with peaks in cortisol 30 to 45 minutes after awakening and a gradual decrease in cortisol 60 minutes after awakening, usually without reaching baseline levels (e.g. Edwards et al., 2001a,b; Pruessner et al., 1997; Schmidt-Reinwald et al., 1999; Wuest et al., 2000). The study by Wuest and co-workers (2000) provides normal values of salivary cortisol levels in the post-awakening period derived from a sample comparable to our studies (n= 509 adult participants; mean age 37.3 yrs; 319 females and 190 males): average cortisol levels (mean ± SD) at t(+0) were 15.12 ± 6.25 nmol/l, at t(+30) 22.95 ± 9.13 nmol/l, at t(+45) 22.31 ± 9.33 nmol/l, and at t(+60) 20.23 ± 8.25 nmol/l. Thus, a significant increase of cortisol 30 minutes after awakening was evident, followed by a slight decrease in cortisol levels thereafter.

Although in contrast to other studies (Broderick et al, 2004; Kudielka et al., 2003; Kunz-Ebrecht et al., 2004b; Thorn et al., 2003) significant difference in cortisol levels were not obtained comparing compliant to non-compliant samples in our studies, it is still advisable not to disregard compliance issues to avoid missing the peak in cortisol after awakening, and thus underestimating the actual magnitude of the cortisol awakening rise.

Adherence to the sampling protocol: Sampling time. In both studies, there was no systematic impact of adherence vs. non-adherence to the stipulated sampling protocol: Neither absolute sampling times (clock-time) nor relative sampling times (time intervals synchronised to awakening) were significantly and systematically associated with cortisol levels of single samples (study 1: t(08:00) to t(20:00); study 2: t(16:00), t(20:00)). Considerably less than 10% of all cortisol samples in both studies were collected outside the scheduled range (less than 60 minutes before or more than 60 minutes after the stipulated sampling time), thus non-compliant samples could not be reliably compared to compliant cortisol samples.

In general, although there were significant differences in compliance rates and deviation in minutes between electronically verified and self-reported sampling times, indicating that the exclusive use of self-reported adherence to the sampling protocol overestimates the actual degree of adherence, the effect of on cortisol samples in our studies was rather weak and unsystematic. However, with regard to the cortisol awakening rise adherence to the stipulated sampling protocol, and in particular objectifying adherence by using drug exposure monitors or similar devices seems to be essential as not to miss the cortisol peak after awakening and not to underestimate the size of the cortisol awakening response.

Effect of morning cortisol release on subsequent cortisol samples and parameters. In study 1, cortisol samples directly after awakening (t(+0)) were positively correlated with samples 30 minutes later (t(+30) and the diurnal mean (mean of t(08:00), t(11:00), t(15:00), t(20:00), i.e. participants with higher levels of cortisol after awakening had higher cortisol levels 30 minutes later as well as higher diurnal levels. Additionally, participants with higher levels of cortisol after awakening (i.e. cortisol awakening rise and total morning cortisol release) had higher diurnal levels. In study 2, however, these findings could not be replicated: the association between cortisol levels directly after awakening and 30 minutes later was considerably weaker, and cortisol levels after awakening (t(+0), t(+30), cortisol awakening rise, total morning cortisol release) were uncorrelated to diurnal levels (mean of t(16:00), t(20:00).

Thus, the findings of Edwards et al. (2001a) indicating that cortisol levels subsequent to awakening reliably predict mean cortisol levels throughout the remainder of the day could be replicated to some extent only. To check whether the lack of association between awakening cortisol levels and the diurnal mean in study 2 was due to the different sampling protocol, correlation analyses in study 1 were rerun using the diurnal mean calculated analogous to study 2 (mean of t(15:00) and t(20:00), thus excluding antemeridian samples (t(08:00) and t(11:00)). Although to a lesser extent, the positive relationship between cortisol levels in the post-awakening period and diurnal levels remained unaffected. In addition, a more recent study by Hucklebridge et al. (2005) – associated to the same study group as Edwards - revealed that same day correlations between awakening secretory activity and day secretory activity (mean of post-awakening cortisol and mean diurnal levels for two sampling days separately) were low on day 1 and non-significant for day 2. These findings

strongly suggest that the association of post-awakening and diurnal cortisol levels depends on the sample under investigation and can thus not be generalized.

Relationship of single samples and diurnal mean. The predictive power of single cortisol samples in representing mean diurnal cortisol levels was examined. In study 1, each single cortisol sample was positively correlated to the mean of the remaining cortisol samples indicating a substantial relationship. In study 2, however, this could not be replicated: the association between single samples and the mean of all remaining samples was very low, indicating an almost negligible relationship.

Thus, results from a study by Edwards and co-workers (2001a) suggesting that single cortisol samples are indicative of diurnal levels could only be replicated in our studies to a certain extent. In study 1, the associations were weaker compared to those found by Edwards et al. (2001a), in study 2, the association of single samples and diurnal levels was almost negligible. To check whether the lack of association in study 2 was due to a shorter sampling protocol, correlation analyses in study 1 were rerun excluding antemeridian cortisol samples from analyses. Although to a lesser extent, single samples were still indicative of diurnal levels. These findings suggest that the predictive power of single samples in representing diurnal cortisol levels may depend on the sample under investigation and can not reliably be generalized. However, the sampling protocol in the study by Edwards et al. (2001a) was synchronised to awakening using relative sampling times (i.e. 3, 6, 9, 12 hours post-awakening), while in our studies sampling times were standardized clock times. Consequently, results may not be comparable.

5.2 Confounding factors

Time of awakening. In both studies, awakening time was neither systematically nor substantially correlated to cortisol levels of single samples and composite measures. In study 1, awakening time was negatively correlated to the AUC, but not to the diurnal mean, while in study 2, participants who woke up later in the morning had slightly flatter diurnal rhythms. However, a systematic effect of awakening time on cortisol levels, particularly on morning cortisol levels, could not be observed.

Empirical evidence of other study groups regarding the effect of awakening time on cortisol samples is inconsistent: while no association between cortisol levels and awakening time could be found in a multitude of studies (e.g. Brooke-Wavell et al., 2002; Kunz-Ebrecht et al., 2004b; Pruessner et al., 1997; Wuest et al., 2000), other studies in contrast report significant associations between cortisol levels and the time of awakening: In a study by Edwards et al. (2001b) a 12-hour diurnal profile of salivary cortisol was measured in 40 healthy adults on two consecutive days. A median split at 07:21 h awakening time was performed and cortisol profiles were associated with early and late awakeners. The awakening mean increase in cortisol, the AUC of the post-awakening period as well as the diurnal mean of cortisol were negatively correlated with awakening time with higher cortisol levels in early awakeners. Kudielka & Kirschbaum (2003) investigated the cortisol awakening response in 64 early and 38 late awakeners (classified by a cluster analysis of awakening time). Cortisol profiles differed significantly between wake-up groups, the increase in cortisol during the post-awakening period as well as the AUC was higher in early compared to late awakeners. Hucklebridge and co-workers (2000) compared the cortisol awakening response after nocturnal (4 hours prior to the participants' usual awakening time) and normal awakening in 12 healthy adults. While the cortisol awakening rise was similar in both conditions, the AUC was smaller in nocturnal compared to normal awakening. Federenko et al. (2004) investigated the impact of awakening time on the cortisol awakening rise during early, late, and night shifts in 24 nurses. The cortisol awakening rise on early shift days (with early awakening times) was more pronounced and differed significantly from late and night shift. Schlotz and co-workers (2004) assessed the 60 minute post-awakening period in 219 healthy adults on six consecutive days comparing weekdays to weekends. Although there was no significant correlation of cortisol levels averaged across sampling days and time of awakening averaged across sampling days, significant differences emerged when calculating both awakening time and cortisol differences between weekday and weekend days. Lower cortisol levels in the post-awakening period could be observed on weekends and the group with high awakening time differences had more pronounced cortisol differences.

Most studies, including our present studies, that failed to uncover a significant association of awakening time and cortisol levels did not account for awakening time as an explicit

group factor in the study design, i.e. early and late awakening were not systematically varied. Thus, potential effects may not be detectable in contrast to studies with a systematic variation of early and late awakening (e.g. Federenko et al., 2004; Schlotz et al., 2004).

Sleep duration and quality of sleep. Sleep duration at night (i.e. total hours slept) was recorded in study 2, only. Sleep duration was systematically, yet weakly, positively correlated to cortisol levels directly after awakening across all sampling days and negatively correlated to the diurnal decline in cortisol (slope of change excluding t(+30)). Thus, participants with longer sleep duration had higher cortisol levels directly after awakening and a steeper decline in diurnal cortisol. However, this association was not reflected in similar cortisol parameters, such as the cortisol awakening rise or the total morning cortisol release or all other measures of diurnal decline (i.e. slope of change excluding t(+0), decrease from t(+0) to t(20:00) and from t(+30) to t(20:00)).

Empirical evidence regarding the association of sleep duration and cortisol levels is as inconsistent as the results obtained in our study: a study by Pruessner et al. (1997) investigating post-awakening cortisol levels in three independent studies (total n= 152) failed to uncover a significant association of total hours slept and awakening cortisol levels. However, Wuest and co-workers (2000) found a rather weak negative correlation between sleep duration and mean increase of cortisol after awakening, suggesting a slightly weaker cortisol awakening rise after shorter sleep length, though this effect accounted for less than one percent of variability in post-awakening cortisol levels.

In both of our studies, self-reported quality of sleep (study 1: self-reported sleep deprivation / subjective lack of sufficient sleep; study 2: frequency and duration of sleep disruption episodes) failed to yield a systematic and substantial impact on cortisol levels of single samples and composite measures.

While complete sleep deprivation is reported to result in a discrete increase in nocturnal plasma cortisol, i.e. an elevation of the nocturnal nadir, and a significant rise in post-awakening plasma cortisol levels compared to undisturbed sleep (Schultes & Fehm, 2004; Spaeth-Schwalbe et al., 1991), decreased salivary cortisol levels after awakening were found in patients with primary insomnia (Backhaus et al., 2004). In a pilot study by Waye et al.

(2003) the cortisol awakening response was found to be altered following nocturnal exposure to low frequency noise (40 dBA; 12 male participants): levels of cortisol failed to peak 30 minutes after awakening. Additionally, impaired subjective sleep quality due to exposure to traffic noise resulted in decreased cortisol levels 30 minutes after awakening. A more recent study by Kirschbaum and co-workers (2005) revealed that the morning cortisol response after disturbed sleep did not differ from the response following undisturbed sleep; even if sleep was repeatedly interrupted (15 healthy adult women were woken up three times in each of three consecutive nights).

Gender. In both studies, men displayed higher cortisol levels with regard to afternoon samples (study 1: t(15:00); study 2: t(16:00)). In study 1, men had higher cortisol levels with regard to both diurnal mean excluding t(+30) and the AUC excluding t(+30); in study 2 men had higher cortisol levels regarding the diurnal mean excluding t(+30) and steeper decline in cortisol, while women displayed a larger cortisol awakening rise.

Empirical evidence regarding gender differences in basal cortisol levels is inconsistent: while hardly any baseline differences are found in most studies, a distinct impact of gender on cortisol levels is usually found when exposed to laboratory stressors (e.g. Kirschbaum et al., 1992; Kudielka & Kirschbaum, 2005; Zimmer et al., 2003). No gender differences in the cortisol awakening response (measured in 15-minutes intervals within the first hour of the post-awakening period) were found in a study by Kudielka and Kirschbaum (2003) who also reported no differences in basal cortisol levels among women regarding the menstrual cycle phase (follicular vs. luteal phase). An earlier study by Kirschbaum and co-workers (1999) investigating the cortisol awakening response and diurnal cortisol profiles in 81 healthy adults failed to find baseline differences in cortisol levels with regard to gender, but found a clear impact of gender when exposed to psychosocial stressors in a laboratory setting. Kunz-Ebrecht et al. (2004b) investigated the cortisol awakening response on work days and weekends in 196 participants drawn from the Whitehall II cohort (Marmot et al., 1991) and found that, although there were no gender differences in cortisol levels directly after awakening, the cortisol awakening rise was larger in women on work days, but not on weekends. A study by Schulz and Merck (1997) investigating diurnal cortisol profiles in 48 laboratory employees on two days (salivary cortisol measured before starting work, before noon, in the

afternoon and before the end of the working day) revealed no gender differences in the diurnal cortisol pattern, but women displayed higher cortisol levels directly before work and lower levels before noon, thus indicating a steeper decline in cortisol from morning to noon.

Given the rather inconsistent empirical evidence regarding baseline levels of cortisol output in men and women as well as the apparent evidence of gender differences in cortisol levels subsequent to psychosocial stressors, field studies, and in particular those investigating the effect of stressful events on diurnal cortisol profiles, should account for potential gender differences.

Oral contraceptives. In both studies, there was no significant difference in cortisol levels of single samples and composite measures between women reporting to take oral contraceptives and those reporting not to.

An early review on salivary cortisol as a biomarker of stress (Kirschbaum & Hellhammer, 1989) concluded that unstimulated cortisol concentrations (i.e. without the presence of a stressor) are usually not affected by the use of oral contraceptives in women. This finding was replicated in our studies and in several others (e.g. Kirschbaum et al., 1995, 1999), apart from a study by Pruessner et al. (1999) who investigated the cortisol awakening response in 66 teachers finding overall lower levels in morning salivary cortisol in women using oral contraceptives. Experimental studies applying psychosocial stressors consistently found lower cortisol levels subsequent to stressors in women using oral contraceptives (e.g. Kirschbaum et al., 1995, 1999; Kudielka & Kirschbaum, 2005), thus there is clear evidence that oral contraceptives reduce HPA-axis responses to stress, while basal, unstimulated levels remain unaffected.

Age. In study 1, older participants tended to have higher levels of cortisol in the post-awakening period as well as flatter diurnal cortisol rhythms. In study 2, however, age was entirely unrelated to cortisol levels. Apart from the exclusive effect of age on cortisol levels, the interaction effect of age and gender on cortisol levels was investigated, yet in neither study a significant interaction effect could be observed.

Nicolson et al. (1997) found higher basal salivary cortisol levels in older age groups (i.e. above 60 years in age) indicating moderate increases in basal cortisol levels associated with

age, while Lupien et al. (1996) conducted a six-year longitudinal study investigating basal plasma (free and total) cortisol levels in healthy elderly adults and revealed that age was uncorrelated to cortisol levels and to the pattern of change over years. A study by Kudielka et al. (1999) also failed to uncover age-related differences in basal salivary morning cortisol levels comparing 15 post-menopausal women (60-75 years) to 15 young women (20-31 years). A more recent study by Kudielka and Kirschbaum (2003), however, found a weak correlation between age and the cortisol response after awakening.

In our studies, participants were drawn from the active working population, thus participants were younger than 65 years. Age-related changes in HPA-axis functioning, i.e. typically elevated cortisol levels, are usually reported in samples above 70 or 80 years of age (e.g. Collaziol et al., 2004; Raff et al., 1999).

Occupational group. In study 1, morning cortisol levels (t(+0) to t(+30)) and composite measures of cortisol reflecting level and dynamic aspects of cortisol profiles were lowest in nurses, while evening cortisol levels (t(20:00)) were lowest in hotel staff. In study 2, hotel staff displayed lowest morning cortisol levels (t(+0), t(+30)), while afternoon and evening levels (t(16:00), t(20:00)) were lowest in teachers and social service assistants. Hotel staff had lowest, while nurses had highest levels in cortisol with regard to composite measures of cortisol profiles. However, no distinct pattern in cortisol secretion discriminating clearly between occupational groups could be found which might also be due to the fact that participants were not evenly distributed among occupational groups. In our studies, occupational groups (nurses, teachers, hotel staff, and social service assistants) were chosen because of their potential high job strain and work-related emotional demands in human service professions (e.g. Dormann & Zapf, 2004; Zapf 2002; Zapf et al., 2001). In study 2 only, a control group ("mixed group") was drawn from occupations other than human service professions, yet systematic endocrine-related differences between service and non-service professions could not be observed. Thus, future studies should include explicit measures of job strain when comparing endocrine measures in occupational groups, as the perceived degree of work-related strain has proved to be a powerful criterion.

A study by Steptoe et al. (2000) examined salivary cortisol profiles (at 2-hour intervals along a working day) in 105 school teachers. Participants reporting high job strain had

higher cortisol levels in the morning, yet there were no significant differences in cortisol levels regarding subsequent diurnal samples. However, awakening time was not recorded, thus it remained unclear how much time had elapsed between awakening and the first morning measure of cortisol and whether differences in cortisol levels could reliably be attributed to strain-related effects. Empirical evidence from Whitehall studies revealed that high job demands were associated with high awakening salivary cortisol levels, yet attenuated by higher socio-economic status and that low job control was related to elevated diurnal cortisol in men, but not in women (Kunz-Ebrecht et al., 2004a). An associated study (Steptoe et al., 2004) revealed that overcommitment (i.e. effort-reward-imbalance) resulted in higher cortisol awakening responses and higher diurnal cortisol levels in men.

Shift work. In study 1, participants working shifts tended to display higher cortisol levels of single samples and composite measures. In study 2, shift workers displayed higher cortisol levels at t(+30) and in the evening (t(20:00)), had a larger cortisol awakening rise and higher diurnal cortisol levels regarding the AUC including t(+30), only. While shift workers were more or less evenly distributed among occupational groups in study 1, the majority of shift workers in study 2 came from the nursing group. Thus, the effect of shift work in study 2 may be confounded by the impact of occupational groups. It should also be noted that cortisol levels were measured on normal working days (i.e. non-shift or day shift) and participants were asked to state whether they usually worked shifts. Thus, the effect of shift work in our samples reflects chronic changes in HPA-axis functioning in contrast to acute responses during shift work.

A reversal of the circadian rhythm with higher evening than morning salivary cortisol levels was found in nurses after the fifth night of night shifts, yet normal circadian rhythms were observed during early shifts. Six out of the 24 participants failed to show changes in cortisol secretion in the course of night shifts and were classified as non-adapters, i.e. intolerant to shift work (Hennig et al., 1998). A study by Lac and Chamoux (2004) compared 16 male day-workers to 32 male workers with two different shift work rotas. Shift work proved to induce a marked disturbance in the cortisol circadian rhythm for the night shift period in rapidly changing shift schedules with slightly lower cortisol levels in the evening and a global flattening of the diurnal cortisol curve. While circadian disturbances due to shift

work were found in several other studies (e.g. Goh et al., 2000; Leese et al., 1996; Motohashi, 1992; Munakata et al., 2001), physiological rhythms could successfully be adapted to night shift work when the pattern of light and darkness exposure was controlled (James et al., 2004).

Somatic health status. **Self-reported disorders** (endocrine, psychiatric, neurological and/or sleep disorders) did not prove to exert a systematic impact on cortisol levels: although participants in study 1 reporting at least one disorder had significantly lower cortisol levels (in particular directly after awakening, 30 minutes thereafter, at 08:00h and 15:00h), the majority of participants with self-reported disorders were from the nursing group, and occupational groups indeed proved to yield significant differences in cortisol levels (nurses with lowest cortisol levels compared to other occupational groups). In study 2, significant differences in cortisol levels between participants with and without self-reported disorders could not be observed. While demographic and psychosocial variables failed to discriminate between participants reporting at least one disorder and those reporting none in study 1, participants in study 2 reporting at least one disorder were older, more depressive had more trait anxiety and more somatic complaints. The majority of participants with at least one disorder in study 2 were nurses (34%) and teachers (42%).

Since cortisol levels were unaffected in participants with self-reported diseases in study 2 and attenuated cortisol levels of participants with self-reported disorders in study 1 may be due to occupational affiliation (the majority of participants with self-reported diseases were nurses), it remains unclear to which extent self-reported health problems affect salivary cortisol levels. A study by Kudielka and Kirschbaum (2003) investigating the cortisol awakening response in 179 adults revealed that 74 participants reporting health problems (i.e. cardiovascular, autoimmune/atopic/allergic, psychiatric, and others) displayed blunted cortisol levels with regard to the profile of the cortisol awakening response and the increase in the post-awakening period. These participants were excluded from subsequent analyses. In our two studies, participants of study 1 with self-reported diseases were not excluded from further analyses, since their attenuated cortisol levels are suspected to be – at least partly – associated with occupational affiliation and since the same effect on cortisol levels was not observed in study 2.

In both our studies, **self-reported somatic complaints**, i.e. the extent of somatic distress or the indulgence to complaining, were entirely uncorrelated to cortisol levels of single samples and composite measures indicating that perceived somatic distress was not reflected in changes of HPA-axis functioning. Empirical evidence regarding the effect of self-reported somatic complaints on basal salivary cortisol levels is scarce: Schulz and Merck (1997) found that low diurnal cortisol levels in 48 healthy laboratory employees were associated with more somatic complaints, while Rief and Auer (2000) failed to uncover a significant relationship between somatic symptoms and cortisol measures (diurnal profiles of salivary cortisol, night-time urinary cortisol levels and serum cortisol subsequent to dexamethasone suppression test) in 22 participants with somatisation syndrome, 33 participants with somatisation syndrome and comorbid major depression and 22 healthy controls.

In both our studies, **current smokers** had significantly lower cortisol levels compared to non-smokers. In study 1, current smokers displayed lower cortisol levels directly after awakening, had lower levels with regard to total morning cortisol release and the diurnal mean (excluding t(+30)), and a flatter diurnal cortisol decline. In study 2, awakening cortisol levels did not discriminate current smokers from non-smokers, yet current smokers had higher cortisol levels in the afternoon and evening and thus displayed a flatter diurnal decline of cortisol throughout the day.

There is broad evidence regarding the acute effects of cigarette smoking on cortisol levels, i.e. significant increases in cortisol output as a response to the smoking of at least two cigarettes (Kirschbaum et al., 1992b, Mendelson et al., 2005; for a concise review: Rohleder & Kirschbaum, 2006) and blunted responsiveness of the HPA-axis in habitual smokers to psychosocial stressors (Kirschbaum et al., 1992b, 1993b; Rohleder & Kirschbaum, 2006). However, studies investigating basal cortisol levels in habitual smokers compared to non-smokers have produced mixed results: while some studies failed to uncover significant differences in basal, unstimulated circadian cortisol levels between smokers and non-smokers (e.g. Edwards et al., 2001a; Hansen et al., 2001; Kirschbaum et al., 1994; Pruessner et al., 1997), significantly elevated basal cortisol levels were found in habitual smokers by Kirschbaum et al. (1992b), by Wuest et al. (2000) and two recent studies. Olff et al. (2006) investigated circadian salivary cortisol levels in 40 patients with posttraumatic stress disorder

(PTSD), 17 patients with posttraumatic major depressive disorder (MDD) and 38 healthy survivors of the Enschede fireworks disaster. Salivary cortisol levels were higher in smokers and habitual smoking mediated the relationship between traumatic stress and HPA-axis functioning, since patients with posttraumatic MDD tended to consume more tobacco per day and had a flatter diurnal decline in cortisol (differences in cortisol levels could only be obtained between healthy survivors, PTSD and posttraumatic MDD patients when the quantity of smoking was adjusted). Steptoe and Ussher (2006) investigated circadian salivary cortisol profiles on weekdays and weekends in 196 participants drawn from the Whitehall II cohort. Slightly elevated diurnal cortisol levels and a larger cortisol awakening rise on weekdays and weekends were found in 15 habitual smokers compared to 152 non-smokers.

Cardiovascular risk factors, such as body mass index (BMI), waist-to-hip ratio (WHR), and blood pressure, failed to provide systematic associations with cortisol levels in both studies. There was no significant relationship between BMI and cortisol levels; while in study 1 higher cortisol levels were associated with higher blood pressure (in particular with regard to morning cortisol levels, the diurnal mean, and a steeper decline), no such relationship could be observed in study 2. In study 1, BMI was unrelated to blood pressure, while in study 2 BMI positively correlated with WHR and blood pressure.

Given that only 1/5 of all participants in both studies were classified as hypertensive (either systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg; Dobbelsteyn et al., 2001) and only 1/10 as obese (BMI ≥ 30) the lack of systematic association between salivary cortisol levels and cardiovascular risk factors was not unexpected. Several studies, even those that systematically investigated the association of cardiovascular risk factors and HPA-axis functioning, failed to yield significant relationships: salivary cortisol levels in the morning are reported to be unrelated to BMI and WHR (three patient cohorts: BMI: 27-32; BMI: 33-39; BMI: above 40; Laederach-Hofmann et al., 2000) and Gold et al. (2005) failed to reveal significant differences in basal urinary cortisol (12-hour nocturnal cortisol) in 27 hypertensive and 27 normotensive subjects. Mark et al. (2006), however, found that the diurnal decline in salivary cortisol from awakening to noon was inversely related to BMI in 154 female blue-collar workers, yet the magnitude of the association between cortisol decline and BMI was buffered by higher education.

Mental health status. In both studies depressive symptoms, trait anxiety, burnout as well as self-efficacy failed to be significantly associated with cortisol levels of single samples and composite measures which could be due to the small number of participants with significantly reduced emotional well-being: less than 2% (n= 7) of all participants from study 1 and 2 had above-average scores of depressive symptoms, 8% (n= 36) had above-average scores of trait anxiety, 4% (n= 18) had above-average scores of emotional exhaustion, 5% (n= 24) had above-average scores of depersonalization, 7% (n= 29) had above-average scores of diminished personal accomplishment, and 3% (n= 15) had below-average scores of self-efficacy.

While there is clear and consistent evidence for significantly elevated cortisol levels in patients with major depressive disorder (e.g. Burke et al., 2005a, 2005b; Gould & Chrousos, 2002; Holsboer 2001; Plotsky et al., 1998), studies investigating the association of subclinical depressive symptoms and cortisol levels are scarce: a study by Pruessner et al. (2003a) revealed a rather weak positive association of elevated salivary cortisol levels after awakening and severity of self-reported depressive symptoms in 40 healthy males. While correlation coefficients were low in the total sample and in participants with mild depressive symptomatology (after excluding participants in the subclinical and clinical range of depression), significantly higher cortisol levels in the post-awakening period were found in the high depressive group (median split) with persistent significant group effects after the exclusion participants in the subclinical and clinical range of depression.

Trait anxiety and depressive symptoms, however, were reported to be unrelated to diurnal salivary cortisol levels in 54 women with suspected breast disease attending a one-stop diagnostic breast clinic (Vedhara et al., 2003). Salivary morning cortisol levels in 22 patients with somatisation syndrome, 33 patients with somatisation syndrome comorbid with major depressive disorder and 22 healthy controls were unrelated to trait anxiety and depressive symptoms, yet positively related to higher afternoon cortisol levels (Rief & Auer, 2000).

The effect of burnout on HPA-axis functioning was investigated in a multitude of studies, although yielding mixed results and failing to provide convincing evidence for HPA-axis dysregulation in burnout: significantly blunted salivary cortisol levels during the first hour after awakening and an increased suppression of cortisol levels after the dexamethasone

suppression test were found in teachers with high levels of burnout independent of the amount of perceived stress (Pruessner et al., 1999); significantly elevated salivary cortisol levels within the first hour after awakening were observed in female burnout patients on sick leave, yet among male participants increased cortisol levels were found in participants with moderate levels of burnout compared to burnout patients and healthy controls (Grossi et al., 2005). Mommersteeg et al. (2006a) reported significantly reduced cortisol awakening responses in 22 participants with clinical burnout compared to 21 healthy controls, but no differences with regard to diurnal cortisol levels. Subsequent to psychotherapeutic intervention, which led to a an increase of the initially lowered morning cortisol levels in burnout patients and to a significant reduction in complaints, no systematic or substantial association between the changes in complaints and the change in cortisol levels was found. The same study group investigated diurnal salivary cortisol levels in 74 patients with clinical burnout and 35 healthy controls and failed to find significant basal cortisol levels as well as significant differences in the feedback efficacy of the HPA-axis (dexamethasone suppression test) between burnout patients and healthy controls concluding that the HPA-axis functioning in clinically diagnosed burnout patients seems to be normal (Mommersteeg et al., 2006b). Additionally, basal salivary cortisol levels as well as cortisol reactivity and recovery measures after laboratory sessions involving psychosocial stressors were comparable among burnout patients and healthy controls apart from slightly elevated salivary cortisol levels in burnout patients during the first hour of the post-awakening period (De Vente et al., 2003).

Interestingly, perceived self-efficacy to cope with stressors as a buffering factor in physiological stress responses has only rarely been examined. In our studies, the degree of self-efficacy was unrelated to unstimulated diurnal cortisol levels. Lower cortisol levels have been shown to be related to increasing self-efficacy in studies investigating the rate of growth of self-efficacy in phobic stress paradigms (Wiedenfeld et al., 1990) or the physiological response to exercise-induced affective states (Rudolph & McAuley, 1995, 1997). Polk et al. (2005) examined diurnal levels of salivary cortisol in 334 healthy adults and revealed that cortisol levels for men low in trait positive affect did not decrease in the afternoon, resulting in a relatively high, flat diurnal cortisol rhythm. Women, in contrast, high in trait positive affect had lower morning cortisol levels resulting in a low flat diurnal rhythm.

Only a few confounding factors proved to account for significant differences in cortisol levels of single samples and composite measures in our two present studies (i.e. gender, occupational group affiliation, shift work and smoking status), while most confounding factors failed to be significantly associated or revealed only weak and unsystematic associations with basal diurnal cortisol levels. It should be noted, however, that in our two present studies participants were drawn from four occupational groups of the human service sector (nurses, teachers, hotel staff, social service assistants) without obtaining equal sized groups and with a rather weak control group in study 2 (employees from other professions than the human service sector). Thus, confounding factors could not be varied systematically and accounted for as an explicit group factor in the study design, but were rather investigated post hoc in a given sample of employees of human service professions. In all probability confounding factors, such as adherence to the sampling protocol, awakening time, sleep duration and quality, age and somatic and mental health status, were not significantly and systematically associated with cortisol levels in our samples, because they varied within a very low range. Furthermore, most confounding factors to which HPA-axis functioning is reported to be sensitive were empirically investigated in experimental studies that systematically vary the independent variables. In our two studies, however, the effectiveness of these experimentally proved confounding factors was investigated with respect to basal salivary cortisol levels measured in the natural environment of the participants, thus to determine whether basal salivary cortisol levels measured in field studies are sensitive to these confounding factors and therefore need to be accounted for.

Significant group differences in cortisol levels in our present studies with regard to gender, occupational groups, shift work and smoking status could not be observed in all composite measures of diurnal cortisol profiles: the *total morning cortisol release* (i.e. the average cortisol output in the post-awakening period), the *diurnal mean including t(+30)* (i.e. the average diurnal cortisol output calculated from all cortisol samples), the *AUC excluding t(+30)* (i.e. area under the curve excluding cortisol samples measured at t(+30)), and both regression measures of the *diurnal decrease in cortisol* (i.e. linear slopes of the diurnal change in cortisol levels excluding both t(+0) and t(+30)) did not differ significantly between groups in both studies in parallel.

In both our studies, significant group differences could be observed in the *cortisol awakening response* (among occupational groups and shift work), in the *diurnal mean excluding t(+30)* (gender differences), in the *AUC including t(+30)* (among occupational groups and shift work), and in both measures of the *diurnal decrease in cortisol from morning* (t(+) or t(+30)) *to evening* (t(20:00)) (among occupational groups and smoking status).

It should thus be noted that parameters of diurnal cortisol profiles reflecting the average output and those reflecting dynamic changes of cortisol output are equally affected by confounding factors, although there is no clear evidence which parameterisation should be preferred. For example, the total diurnal cortisol release can be calculated as the arithmetic mean or the AUC either taking into account all diurnal cortisol samples or excluding the awakening response sample (thirty minutes after awakening: t(+30)); the diurnal decline in cortisol could be assessed as the linear slope of the diurnal change in cortisol or as a difference value from morning and evening cortisol values and both measures can be assessed with or without taking into account the awakening response sample measured thirty minutes after awakening.

5.3 Diurnal mood and subjective well-being

Diurnal profiles of mood and subjective well-being. Current psychological strain (study 1) and mood/subjective well-being (study 2) varied significantly within sampling days, representing a considerable diurnal fluctuation. Across all participants in study 1 psychological strain increased continually until t(11:00) and decreased thereafter with slightly lower levels in the evening compared to those directly after awakening. In study 2, good mood increased continuously from post awakening to evening, while alertness was highest in the afternoon with a slight decrease in the evening. Relaxation remained stable throughout the day with a slight increase in the evening. In both studies, diurnal profiles of mood and well-being were quite stable across sampling days.

In study 1, diurnal profiles of current psychological strain did not vary significantly with regard to gender, occupational groups or shift work, but participants with a high degree of somatic and psychological distress displayed higher levels of self-rated strain. In study 2, however, diurnal profiles of mood and well-being did not vary significantly between men

and women, but in contrast to study 1 between occupational groups (teachers had lowest diurnal levels of good mood and well-being). Surprisingly, shift workers reported higher levels of good mood and well-being compared to participants working normal hours; however this effect may be due to the fact that teachers, who had lowest diurnal levels of good mood and well-being, were not in the shift worker group. Comparable to study 1, participants with a high degree of somatic and psychological distress had lower levels of good mood, alertness, and relaxation.

There was no significant systematic association between diurnal profiles of salivary cortisol and diurnal profiles of psychological strain (study 1) and mood/well-being (study 2): in study 1 only, a mostly weak positive, yet unsystematic association between cortisol profiles and concurrent psychological strain was found. Scatter diagrams (including fitted regression lines) for each sampling occasion separately on each sampling day (Appendix 4.2) failed to reveal a systematic and substantial relationship between endocrine and self-reported strain profiles.

However, two studies examining the effect of mood states on salivary cortisol levels found significantly elevated cortisol levels related to negative affective states: van Eck et al. (1996) investigated salivary cortisol profiles (10 samples per day over five consecutive days) and concurrent mood states using the Experience Sampling Method (participants received auditory signals after which they filled in questionnaires and collected saliva). While positive mood was unrelated to diurnal cortisol levels, negative affect and agitation were associated with higher cortisol levels, although the observed effects were relatively small (indicating increases in cortisol of about 5% above mean levels). Smyth et al. (1998) obtained comparable results on salivary cortisol levels (measured six times a day in response to a preprogrammed wristwatch): positive affect was significantly associated with lower salivary cortisol levels (10% decrease in average cortisol levels), while negative affect was significantly associated with higher salivary cortisol levels (12% increase in average cortisol levels). The authors of both studies, however, concede, that given these relatively weak effects of negative mood, it remains unclear whether the observed small increase in cortisol levels is biologically significant, i.e. in respect to illness susceptibility, and whether the small increase in cortisol in response to negative mood states constitutes a normal adaptation process

(i.e. allostatic state according to McEwen & Stellar, 1993) or involves long-term effects of HPA-axis dysregulation (i.e. allostatic load according to McEwen & Stellar, 1993).

Average diurnal mood and well-being. Average diurnal psychological strain (study 1) and mood/well-being (study 2) were calculated from the diurnal profiles. Results reflected the effects derived from profile analyses of diurnal strain and mood: as expected, there was no significant difference in average psychological strain with regard to gender, occupational groups, and shift work in study 1; participants with a high degree of somatic and psychological distress had higher levels of average psychological strain. In study 2, significant differences in average diurnal mood emerged for occupational groups (teachers with lowest average levels of mood and well-being), shift work (shift workers with higher average levels of mood and well-being), and as in study 1 participants with a high degree of somatic and psychological distress had lower average levels of good mood/well-being. There were no gender differences in average mood and well-being.

In both studies average levels of psychological strain or mood/well-being were uncorrelated to composite measures of cortisol profiles (i.e. cortisol awakening rise, total morning cortisol release, total diurnal cortisol release, diurnal cortisol decline). These results were not entirely unexpected, given that in both our studies diurnal profiles of mood and subjective well-being as well as affective trait measures (i.e. depressive symptoms, trait anxiety, measures of work-related burnout, and self-efficacy) were unrelated to diurnal cortisol profiles.

5.4 Stability of cortisol profiles

Stability of diurnal cortisol profiles. In both studies, average diurnal cortisol profiles in the total sample and in separate group analyses (i.e. gender, occupational groups, shift work, smoking status) proved to be highly stable across sampling days: mixed model analyses for repeated measures did not reveal a significant main effect of sampling day. Average cortisol profiles across participants and groups reflected the expected circadian cortisol rhythm with higher cortisol levels in the post-awakening period and a subsequent significant decline in cortisol for the remainder of the day.

Stability of single cortisol samples. The stability of single cortisol samples was assessed using intraclass correlation coefficients (ICC, Shrout & Fleiss, 1979) to determine the concordance of cortisol output between sampling days for each sampling occasion.

Calculated across the total sample, **awakening cortisol samples** (t(+0) and t(+30)) showed moderate stability in study 1 and low to moderate stability in study 2, indicating moderate to high within-subject variance. In study 1, cortisol samples directly after awakening were less stable than those sampled 30 minutes thereafter; in study 2, however this pattern was reversed: cortisol samples directly after awakening were slightly more stable than those 30 minutes thereafter. While in both studies the stability of awakening cortisol samples with regard to shift work and smoking status was comparable in size to the stability calculated across all participants, marked differences with regard to gender and occupational groups could be observed: in both studies awakening cortisol samples were slightly more stable in women compared to men (stability values were higher in study 1 compared to study 2); in study 1, stability coefficients of nurses were comparable to those of the total sample, while the other occupational groups presented considerably lower stability. In study 2, stability coefficients in occupational groups were more or less comparable.

Antemeridian cortisol samples (t(08:00) and t(11:00)) were recorded in study 1 only, showing reasonable stability in the total sample with slightly higher stability for samples collected at t(08:00) compared to t(11:00). With regard to group analyses no marked differences could be observed.

Afternoon cortisol samples (study 1: t(15:00), study 2: t(16:00)) were consistently higher in study 1 compared to study 2 with regard to the total samples and group analyses, indicating moderate stability in study 1 and low stability in study 2. Compared to afternoon samples, stability coefficients of **evening cortisol samples** (t(20:00)) were substantially lower, but comparable among total samples and groups in study 1 and study 2, indicating rather low stability of evening cortisol samples.

Stability of composite measures of the diurnal cortisol pattern. The stability of cortisol parameters (cortisol awakening rise, total morning cortisol release, diurnal cortisol release,

and diurnal cortisol decline) was examined using intraclass correlation coefficients (ICC) to determine the degree of concordance of cortisol output between sampling days.

Parameters reflecting the average level of cortisol output (i.e. total morning cortisol release and average diurnal release) proved to be of moderate to high stability in study 1 and of low to moderate stability in study 2 across the total samples and with regard to group analyses.

Parameters reflecting dynamic aspects of diurnal cortisol release (i.e. cortisol awakening rise and diurnal decline in cortisol) proved to be of low to moderate stability in study 1 and very low to scarcely moderate stability in study 2 across the total samples and with regard to group analyses.

In summary, although the average diurnal pattern of cortisol release (i.e. diurnal cortisol profiles) proved to be highly stable across sampling days in both studies, single samples of cortisol in the morning and throughout the day were only moderately stable and rather unstable in the evening in study 1. In study 2, single cortisol samples with regard to morning, afternoon and evening were of comparable size with rather poor stability. Composite measures of cortisol secretion reflecting the average level of diurnal cortisol output proved to be of moderate to high stability, while parameters reflecting dynamic changes of cortisol output indicated comparatively moderate to poor stability. In general, stability coefficients were higher in study 1 compared to study 2, and composite measures, particularly those reflecting the average level of diurnal cortisol output, proved to be of higher stability compared to single samples. In addition, although group differences in cortisol levels could be observed with regard to gender, occupational group, shift work and smoking status, stability coefficients for these allegedly homogeneous subgroups failed to be larger than stability coefficients obtained from the total sample. This applied in particular to occupational groups and may – at least partly – be due to the smaller sample size (especially regarding hotel staff) and to the fact that homogeneity with respect to occupation does not necessarily result in homogeneity with respect to HPA-axis functioning.

Most studies assessing the stability of basal salivary cortisol profiles apply correlation analyses, i.e. test-retest reliability, using either parametric Pearson product-moment coeffi-

cients or non-parametric Spearman rank coefficients (e.g. Edwards et al., 2001b; Federenko et al., 2004; Pruessner et al., 1997; Wuest et al., 2000). However, correlation coefficients are measures of association, i.e. measuring the strength of linear association; they are not measures of agreement (Altman & Bland, 1983; Bland & Altman, 1986). Thus, intraclass correlation coefficients are recommended to assess the degree of agreement (Rankin & Stokes, 1998), i.e. assessing the ratio of within-subject variance to total variance (Shrout & Fleiss, 1979). In our studies stability coefficients of single cortisol samples and composite measures of diurnal cortisol levels as well as the stability of diurnal cortisol profiles were assessed with statistical analyses that average across individuals resulting in measures of interindividual stability. To assess intraindividual stability of cortisol profiles in terms of diagnostic application statistical procedures should be deployed to determine individual variation from averaged profiles.

Nevertheless, stability measures of basal salivary cortisol levels in our studies are well in line with results obtained in other studies: Edwards et al. (2001b) reported low to moderate stability for dynamic measures of the post-awakening period (r= .34 - .50; covering the first 45 minutes subsequent to awakening), the diurnal decline in cortisol levels (r= .45 - .55), and for the diurnal mean (r= .45) based on two sampling days in 40 healthy adults. Federenko et al. (2004) investigated cortisol levels of the post-awakening period within the first 60 minutes in shift working nurses and students with a regular sleep-wake cycle on two sampling days, revealing low to high stability for single samples (nurses: r= .38 - .74; students: r= .53 - .81), and moderate to high stability for dynamic measures of the cortisol awakening response (nurses: r= .56 - .65; students: r= .50 - .84). Pruessner et al. (1997) examined cortisol levels in the post-awakening period covering 30 to 60 minutes after awakening on two and three sampling days in three independent studies, obtaining low to moderate stability (r= .39 - .67) of the cortisol increase within this period. Wuest et al. (2000) pooled salivary cortisol levels measured within the first 60 minutes after awakening of 509 healthy adults from four independent studies and found low to moderate stability of single samples (r= .37 - .66; with the lowest stability for cortisol levels directly after awakening) and moderate stability for the increase in cortisol levels within this period (r= .47 - .63).

Replication of studies. With regard to **average diurnal cortisol profiles** (equivalent sampling times only: t(+0), t(+30), t(15:00)/t(16:00), t(20:00); averaged across sampling days) in the total samples no significant differences were observed between study 1 and 2. Salivary cortisol levels directly after awakening, 30 minutes later, and in the evening did not differ significantly between study 1 and study 2. Interestingly, there was also no significant difference in cortisol levels measured at 15:00h in study 1 and those measured at 16:00h in study 2, indicating that afternoon levels in cortisol output did not vary significantly between 15:00h and 16:00h. Thus, the total samples of both studies were pooled and group differences were examined with regard to gender, occupational groups, shift work and smoking status (i.e. those confounding factors that proved to be significant in both studies separately). Compared to men, women showed significantly lower cortisol levels directly after awakening and in the afternoon. Comparing occupational groups, nurses had lowest awakening cortisol levels (directly after awakening and 30 minutes thereafter), while teachers had lowest levels in the evening. Afternoon levels of cortisol output were comparable among occupational groups. Regarding shift work, significant differences in cortisol output could be observed for cortisol samples 30 minutes after awakening and in the afternoon, with lowest levels in shift workers compared to participants working normal hours. Current smokers had lower levels of cortisol directly after awakening and higher levels in the evening compared to non-smokers resulting in a flatter decline in cortisol throughout the day.

With regard to **composite measures of diurnal cortisol profiles** (averaged across sampling days; only composite measures that were calculated from sampling occasions common in both studies were chosen for comparison, i.e. cortisol awakening rise, total morning cortisol release, and diurnal decrease in cortisol) no significant differences could be observed between the total samples of study 1 and study 2. Thus, samples were pooled and group differences were examined with regard to gender, occupational groups, shift work and smoking status (i.e. those confounding factors that proved to be significant in both studies separately). Compared to men, women had a larger cortisol awakening rise, but flatter diurnal decline in cortisol. Among occupational groups nurses had lowest morning cortisol levels and a flatter diurnal decline in cortisol. Compared to participants working normal hours, shift workers showed a larger cortisol awakening rise, higher levels of total morning cortisol

release and a steeper decline in cortisol throughout the day. With regard to smoking status, current smokers showed a flatter diurnal decline than non-smokers, while awakening cortisol levels were comparable between smokers and non-smokers.

In summary, there were no significant differences in cortisol levels of single values and composite measures between the total samples of study 1 and study 2. Single samples of cortisol levels were compared that were collected at equivalently timed sampling occasions in both studies, i.e. directly after awakening, 30 minutes late, and in the evening at 20:00h, and at almost equivalently timed sampling occasions, i.e. at 15:00h in study 1 and 16:00h in study 2. With regard to composite measures of diurnal cortisol profiles only those parameters were compared that were calculated from sampling occasions common to both studies, i.e. the cortisol awakening rise, the total morning cortisol release, and the diurnal decrease from awakening to evening and from 30 minutes subsequent to awakening to evening (difference value between morning and evening cortisol levels). Since in study 1 saliva samples were collected on two consecutive days and on three consecutive days in study 2, cortisol levels of single samples and composite measures were averaged across sampling days to compare cortisol levels in both studies. There were no significant differences in average cortisol levels between study 1 and study 2, thus both samples were pooled. Examining group differences in average cortisol levels of single samples and composite measures, results obtained from both studies separately could be replicated in the pooled sample: compared to men, women had slightly lower diurnal cortisol profiles and a flatter diurnal decrease in cortisol, but a larger cortisol awakening rise. Compared to all other occupational groups, nurses presented the lowest cortisol levels in the post-awakening period, resulting in a flatter decline in cortisol throughout the day, while lowest cortisol levels in the evening were observed in teachers. Participants who usually work shifts had significantly higher cortisol levels in the post-awakening period and in the afternoon, but did not differ from non-shift workers in evening levels of cortisol. Thus, the diurnal decline in cortisol was significantly steeper in shift workers. Habitual smokers displayed significantly lower cortisol levels directly after awakening, but significantly higher levels in the evening, resulting in a flatter diurnal decline in cortisol compared to non-smokers.

Average salivary cortisol levels in the pooled samples of study 1 and study 2 were comparable to normal values published by other study groups: Wuest et al. (2000) investigated salivary cortisol levels of the post-awakening period in 509 healthy participants of four independent studies and reported normal average salivary cortisol levels directly after awakening (mean: 15.12 nmol/l, SD: 6.25 nmol/l) and thirty minutes thereafter (mean: 22.95 nmol/l; SD: 9.13 nmol/l). Clow et al. (2004) aggregated salivary cortisol levels of the post-awakening period from 12 published studies (with more than 1000 healthy participants in total) and found a wide range of salivary cortisol values directly after awakening (range of all 12 studies: 4.7 – 18.5 nmol/l; aggregated mean ± standard deviation: 11.6 ± 4.6 nmol/l) and thirty minutes later (range of all 12 studies: 8.6 – 29.1 nmol/l; aggregated mean ± standard deviation: 20.0 ± 5.9 nmol/l); however, the cortisol awakening rise, i.e. the increase in cortisol from 0 to 30 minutes post-awakening, was reported to be relatively consistent between studies (range of all 12 studies: 3.9 – 15.0 nmol/l; aggregated mean ± standard deviation: 9.3 ± 3.1 nmol/l). These results are well in line with our findings: salivary cortisol levels averaged across both samples of study 1 and study 2 amounted to 15.33 ± 8.81 (n= 432) directly after awakening, to 24.81 ± 12.88 nmol/l (n= 432) thirty minutes later, and to 9.54 ± 11.64 nmol/l (n= 429) for the cortisol awakening rise. With regard to salivary cortisol levels in the afternoon and evening, normal values were published by Kirschbaum & Hellhammer (1989) amounting to 4.5 ± 3.5 nmol/l (15:00h – 17:00h; n= 708) and 1.96 ± 1.7 nmol/l (20:00h – 22:00h; n= 698). Salivary cortisol levels averaged across our both studies for these time points were well within this range with 5.66 ± 3.84 (15:00h – 16:00h; n= 429) and 2.74 ± 2.55 nmol/l (20:00h; n= 427).

5.5 Concluding synopsis

A total of 440 participants (132 men, 308 women) were investigated for diurnal salivary cortisol levels in their natural environment in two independent studies. In both studies, participants covered four occupational groups of the human service sector (nurses, teachers, hotel staff, and social service assistants) and, in study 2 only, a mixed control group with employees from other professions. In both studies, saliva samples were collected immediately after awakening and thirty minutes later and then at 08:00h, 11:00h, 15:00h, 20:00h on

two consecutive working days in study 1 and at 16:00h and 20:00h on three consecutive working days in study 2.

The aim of the present study was to analyse the internal structure of the cortisol data and to assess the influence of potential confounding factors, to which salivary cortisol levels are reported to be sensitive according to the current research literature, on cortisol levels of single samples and the range of composite measures used in a variety of studies. In addition, the stability of cortisol profiles, single samples and composite measures was examined to determine whether the aggregation of cortisol values, i.e. averaging cortisol values across sampling days, and their appropriateness as trait measures of HPA-axis functioning can be empirically justified.

Internal structure of cortisol data. Missing values, i.e. missing cortisol samples and/or not specified sampling times were evenly distributed on sampling days, but were more likely to occur at sampling occasions in the afternoon or the evening. A total of seven participants from both studies had to be excluded from further analyses due to their large amount of non-specified sampling times and substantial deviations from the stipulated sampling protocol.

Extreme cortisol values (i.e. outliers) were evenly distributed among sampling times and days; there was no significant association with gender, age or somatic and mental health status. Thus, outliers were not excluded from subsequent analyses.

Adherence to the stipulated sampling protocol was examined by determining average compliance rates and deviation in minutes across sampling occasions, sampling days and participants. Significant differences in compliance rates and deviation in minutes emerged between electronically verified and self-reported compliance; thus self-reported compliance overestimated the actual, i.e. objectified compliance. However, studies examining the effect of adherence to the saliva sampling protocol on cortisol profiles (Broderick et al., 2004; Kudielka et al., 2003) usually classify participants non-compliant in case of one or more non-compliant single cortisol samples. We think that this method results in unfavourable confounding of individual cortisol profiles with a combination of compliant and non-compliant measures and consequently preferred to investigate the effect of non-adherence

to the saliva sampling protocol on each single cortisol sample separately: the effect of adherence vs. non-adherence to the sampling protocol on cortisol samples was rather weak and unsystematic in both studies. There was no systematic effect of non-adherence on cortisol levels in the post-awakening period, on the size of the cortisol awakening rise and on diurnal cortisol samples. However, with respect to the cortisol awakening rise adherence to the stipulated sampling protocol, in particular objectified adherence, seems to be essential as not to miss the peak in cortisol 30 minutes after awakening and thus not to underestimate the size of the cortisol awakening response, since cortisol levels are reported to start to slightly to decrease from about 45 minutes after awakening without reaching baseline levels (i.e. those directly after awakening) within the first hour after awakening (e.g. Edwards et al., 2001a,b; Pruessner et al., 1997; Schmidt-Reinwald et al., 1999; Wuest et al., 2000).

In study 1, cortisol levels directly after awakening were indicative of subsequent cortisol levels, i.e. participants with higher levels after awakening had higher levels 30 minutes later and for the remainder of the day. This effect, however, could not be observed in study 2. In addition, the predictive power of single cortisol samples in representing mean diurnal levels could only be observed in study 1, but not in study 2.

Confounding factors. Only a few confounding factors proved to account for significant differences in cortisol levels in both studies: compared to men, women had lower cortisol levels, but a larger cortisol awakening rise. Regarding occupational groups, lowest cortisol levels in the morning were observed in nurses (study 1) and hotel staff (study 2), while lowest cortisol levels in the evening were observed in teachers (study 1 and 2) and social service assistants (study 2). Compared to participants working normal hours, shift workers tended to display higher cortisol levels, but this effect could be mainly due to the impact of occupation, since the majority of participants working shifts in study 2 were from the nursing group. Habitual smokers displayed lower cortisol levels in the morning and higher cortisol levels in the evening, resulting in a flatter diurnal decline of cortisol levels compared to non-smokers.

Time of awakening, sleep duration and quality of sleep, age, somatic and mental health status failed to be significantly associated or revealed only weak and unsystematic associations with diurnal cortisol levels. It should be noted, however, that in both studies, con-

founding factors were investigated post hoc, i.e. they were not varied systematically and were not accounted for as explicit group factors in the study design. Thus, their range of variation was rather narrow: old age, for example, which is reported to be related with elevated levels in cortisol (e.g. Collaziol et al., 2004; Raff et al., 1999) was not accounted for in our studies, since participants were drawn from the active working population. Additionally, less than 10% of all participants of both studies combined had significantly reduced levels of affective well-being; thus the well-known and evidence-based effects of affective disorders on HPA-axis functioning could not be observed in our samples displaying emotional well-being in the normal to subclinical range which might also be due to the well-investigated selection bias, i.e. the healthy worker effect (Li & Sung, 1999), defined by a favourable health status of employed populations in comparison to that of the general population.

Diurnal profiles of psychological strain (study 1) and mood/well-being (study 2) assessed concurrently with saliva samples were unrelated to cortisol levels in both studies. This lack of covariation among different system responses, i.e. physiological and emotional-cognitive stress responses, is a well-known observation in psychological physiology (Fahrenberg, 2000; Lacey, 1967). Although there is evidence from two studies that within diurnal fluctuations negative affective states are associated with higher cortisol levels, the observed effect was rather weak, resulting in 5-12% increase in cortisol above mean levels (Smyth et al., 1998; van Eck et al., 1996). It remains unclear, however, whether the observed small increase in cortisol levels is biologically significant, i.e. in respect to illness susceptibility, and whether the small increase in cortisol in response to negative mood states constitutes a normal adaptation process (i.e. allostatic state according to McEwen & Stellar, 1993) or involves long-term effects of HPA-axis dysregulation (i.e. allostatic load according to McEwen & Stellar, 1993).

Parameterisation of cortisol profiles. Composite measures of diurnal cortisol profiles were subdivided in parameters reflecting average levels of the diurnal cortisol output (i.e. total morning cortisol release, total diurnal cortisol release) and in parameters reflecting dynamic changes of cortisol output (i.e. cortisol awakening response, decrease in cortisol over the day; Table 1), representing the various parameterisations of cortisol profiles used in psy-

choendocrine stress research. In our two studies, parameters of diurnal cortisol profiles reflecting the average output and those reflecting dynamic changes of cortisol output were more or less equally affected by confounding factors, although there was no clear evidence which parameterisation should be preferred. In future studies, it should be well defined which parameterisation is used in which context and why e.g. dynamic measures are preferred to level measures or vice versa.

Stability of cortisol profiles. Average diurnal salivary cortisol profiles proved to be very stable in both studies, i.e. there was no significant variation across sampling days when average diurnal profiles were examined in the total samples or subgroups (gender, occupational group, shift work, smoking status). Average cortisol profiles across participants and groups reflected the expected circadian cortisol rhythm with increasing cortisol levels in the post-awakening period and a subsequent significant decline in cortisol for the remainder of the day. However, stability of profiles corresponded to averaged profiles in groups (i.e. interindividual stability) and did not allow for intraindividual variations. It is of concern that published normal values for salivary cortisol output (e.g. Clow et al., 2004; Kirschbaum & Hellhammer, 1989; Wuest et al., 2000) as well as normal values derived from our studies vary to such a large extent. Thus, with respect to diagnostic application it remains unclear what constitutes e.g. too high a cortisol awakening response, too low the average diurnal cortisol output, or too steep or too flat the diurnal decline in cortisol.

In general, stability coefficients (intraclass correlation coefficients) were larger in study 1 than in study 2. In study 1, single samples of salivary cortisol in the morning and throughout the day were moderately stable, but rather unstable in the evening. In study 2, stability coefficients with regard to morning, afternoon, and evening samples were of comparable size, yet rather small indicating poor stability. Parameters reflecting the average level of cortisol output (total morning cortisol release and total diurnal cortisol release) proved to be of moderate to high stability, while parameters reflecting dynamic changes in diurnal cortisol output (cortical awakening rise and diurnal cortisol decline) revealed comparatively moderate to poor stability.

Thus, it remains unclear, whether the aggregation of cortisol values, i.e. averaging cortisol values across several sampling days, and the appropriateness of using these aggregated

values as trait measures can be empirically justified. It is of concern that stability coefficients in study 1 were larger compared to study 2, while average cortisol levels did not vary between both study samples. In study 1 cortisol levels were measured on two consecutive days, while in study 2 on three consecutive days, which leaves to speculation whether stability of cortisol levels decreases with the number of sampling days or at which number of sampling days stability levels off.

Comparing salivary cortisol levels of sampling time points that were common in both studies (i.e. directly after awakening, 30 minutes thereafter, 20:00h), no significant differences could be observed between the total samples of both studies. In addition, there was also no significant difference in cortisol levels with regard to afternoon measures when comparing cortisol levels measured at 15:00h in study 1 and those at 16:00h in study 2. Thus, cortisol samples were averaged across sampling days (to allow for the different number of sampling days in both studies) and the two samples were pooled. Subsequent group analyses replicated and confirmed the results obtained from both studies separately: compared to men, diurnal cortisol levels in women were lower, displaying a flatter decline in cortisol over the day, but a larger cortisol awakening response. With respect to occupational groups, morning cortisol levels were lowest in nurses who also presented a flatter decline in cortisol over the day, while evening cortisol levels were highest in teachers. Participants working shifts had higher cortisol levels in the morning, and thus a steeper decline in cortisol over the day, while there was no significant difference in cortisol levels in the evening compared to non-shift workers. Compared to non-smokers, current smokers had lower cortisol levels in the morning, but higher levels in the evening, thus displaying a flatter decline in cortisol over the day.

Limitations of the study. There are certain limitations to the present study which may curtail the generalizability of results. First, adherence to the stipulated sampling protocol was objectified, i.e. electronically monitored and verified, in 69 participants of study 1 only and only self-report of sampling times was used in study 2. Our analyses, however, revealed that self-reported compliance consistently overestimates the actual compliance. Furthermore, the first two cortisol samples subsequent to awakening rely on the participants' records of self-reported awakening time which, at least in our study design, was not objecti-

fied. The rapid changes in salivary cortisol levels in the post-awakening period can only be measured reliably if the samples are collected directly after awakening without time lag. Since awakening time was self-reported in all participants it is as vulnerable to non-compliance as all other samples. Although in contrast to other studies (Broderick et al., 2004; Kudielka et al., 2003) non-compliance with the stipulated sampling protocol did not reveal significant effects on cortisol levels in our studies, the use of electronic monitoring devices to objectify adherence is advisable to obtain precise information on compliance rates and deviation in minutes from the stipulated sampling protocol, since self-reported compliance indeed overestimates the actual compliance. Moreover, with regard to the cortisol awakening rise adherence to the sampling protocol is of particular importance as not to miss the peak in cortisol after awakening and, thus, not to underestimate the magnitude of the cortisol awakening rise.

The impact of confounding factors to which HPA-axis functioning is reported to be sensitive was investigated in a given sample within the scope of a field study in contrast to experimental studies that systematically vary the independent variables. This may to a certain extent affect the internal and external validity with regard to the generalizability of results. However, gender, work-related aspects, such as occupational group and shift work, as well as smoking status indeed yielded significant differences in cortisol levels, while confounding factors, such as awakening time, sleep duration and quality of sleep, age, somatic and mental health status failed to account for significant differences in cortisol levels which may be due to the narrow range of these variables. For example, participants were not recruited to obtain an even distribution among different age groups, awakening time was not systematically varied within the samples, and with respect to the somatic and mental health status the participants' status quo was recorded, which did not vary in a wide range and may thus be liable to the healthy worker effect.

The large variability in cortisol values resulting in moderate stability coefficients is of concern regarding the diagnostic appropriateness of cortisol levels. Thus, with respect to diagnostic application it remains unclear what constitutes e.g. too low or too high a cortisol awakening response, too low or too high the average diurnal cortisol output, or too steep or too flat the diurnal decline in cortisol, and to which extent these variations are biologically

significant regarding illness susceptibility. It is of even more concern that cortisol levels in study 1 with two sampling days proved to be more stable than in study 2 with three sampling days which leaves to speculation whether stability of cortisol levels decreases with the number of sampling days or at which number of sampling days stability levels off. Thus, it remains unclear how many sampling days are needed to provide stable measures of cortisol levels and to empirically justify their appropriateness as trait measures of HPA-axis functioning.

Summary

Particularly the last two decades of research on hypothalamus-pituitary-adrenal (HPA) axis activity have established salivary cortisol as a prevalent biological marker of stress. Given the large interindividual variation regarding cortisol levels, various variables or confounding factors doubtlessly account for these differences.

Functioning under basal conditions, diurnal salivary cortisol profiles in healthy adults are characterized by peak values in the post-awakening period and a consecutive decline thereafter. Basically, the variety of published measures quantifying basal salivary cortisol profiles can be classified into *level* and *dynamic parameters* reflecting both state and trait aspects. Level parameters describe the overall or average levels of cortisol output over a specified period of time, while dynamic parameters represent changes of cortisol levels within a specified period of time. State parameters are cortisol parameters of one single day, while trait parameters refer to parameters computed from cortisol values aggregated over several sampling days.

So far, the stability of basal salivary cortisol values over several sampling days has not been investigated systematically; generally, moderate stability of cortisol levels across two sampling days is reported in several studies. Normal values of basal salivary cortisol levels in healthy adults have been published, yet it is of concern that these published normal values vary to such a large extent across studies. Due to the lack of normative values regarding cortisol levels the diagnostic appropriateness of cortisol profiles is challenged: it remains unclear what constitutes too high or too low a cortisol awakening response or too steep or too flat a diurnal decline in cortisol levels.

A total of 440 participants (132 men, 308 women) working in the human service sector were investigated for diurnal salivary cortisol levels in their natural environment in two independent studies.

The aim of the present study was to analyse the internal structure of the cortisol data and to assess the influence of potential confounding factors, to which salivary cortisol levels are reported to be sensitive according to the current research literature, on cortisol levels of single samples and the range of composite measures used in a variety of studies. In addition, the stability of cortisol profiles, single samples and composite measures was examined to determine whether the aggregation of cortisol values, i.e. averaging cortisol values across sampling days, and their appropriateness as trait measures of HPA-axis functioning can be empirically justified.

Regarding the internal structure, missing values were evenly distributed across sampling days, but were more likely to occur in the afternoon and the evening. Extreme cortisol values (i.e. outliers) were also evenly distributed across sampling days and sampling occasions and were unrelated to gender, age or somatic and mental health status. In study 1 only, cortisol levels of the post-awakening period were predictive of subsequent cortisol levels and single cortisol samples were predictive of mean diurnal levels. Examination of adherence to the stipulated sampling protocol revealed that self-reported compliance systematically overestimates the actual, i.e. electronically objectified, compliance. However, the effect of adherence vs. non-adherence to the sampling protocol was rather weak and unsystematic in both studies.

Only few confounding factors proved to account for significant differences in cortisol levels in both studies. Significant differences in cortisol levels could be observed regarding gender, occupational groups, shift work, and smoking status. Time of awakening, sleep duration and quality of sleep, age, somatic and mental health status failed to be significantly associated or revealed only weak and unsystematic associations with diurnal cortisol levels. Diurnal profiles of psychological strain and mood/well-being assessed concurrently with saliva samples were unrelated to cortisol levels in both studies. This lack of covariation among different system responses, i.e. physiological and emotional-cognitive stress responses, is a well-known observation in psychological physiology (Fahrenberg, 2000; Lacey, 1967).

Average diurnal salivary cortisol profiles proved to be very stable in both studies and reflected the expected circadian cortisol rhythm with higher cortisol levels in the post-

awakening period and a subsequent significant decline in cortisol for the remainder of the day. In general, stability coefficients (intraclass correlation coefficients) were larger in study 1 than in study 2. Single samples of salivary cortisol in the morning and throughout the day were moderately stable, but rather unstable in the evening. Parameters reflecting the average level of cortisol output proved to be of moderate to high stability, while parameters reflecting dynamic changes in diurnal cortisol output revealed comparatively moderate to poor stability. Thus, it remains unclear, whether the aggregation of cortisol values, i.e. averaging cortisol values across several sampling days, and the appropriateness of using these aggregated values as trait measures can be empirically justified.

The large variability in cortisol values resulting in moderate stability coefficients is of concern regarding the diagnostic appropriateness of cortisol levels. Thus, with respect to diagnostic application it remains unclear what constitutes e.g. too low or too high a cortisol awakening response, too low or too high the average diurnal cortisol output, or too steep or too flat the diurnal decline in cortisol, and to which extent these variations are biologically significant regarding illness susceptibility. It is of even more concern that cortisol levels in study 1 with two sampling days proved to be more stable than in study 2 with three sampling days which leaves to speculation whether stability of cortisol levels decreases with the number of sampling days or at which number of sampling days stability levels off. Thus, it remains unclear how many sampling days are needed to provide stable measures of cortisol levels and to empirically justify their appropriateness as trait measures of HPA-axis functioning.

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EHRENWÖRTLICHE ERKLÄRUNG

Ich versichere hiermit ehrenwörtlich, dass ich die vorliegende Dissertation selbstständig verfasst und alle benutzten Hilfsmittel angegeben habe. Stellen, die anderen Werken dem Wortlaut oder dem Sinn nach entnommen sind, habe ich in jedem einzelnen Fall durch Angabe der Quelle kenntlich gemacht.

Mannheim, 06.10. 2006 Alexandra Bernhardt