Cortisol secretion in saliva and hair: methodological considerations and relationships with state and trait well-being

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Abstract

Cortisol secretion follows a distinct circadian rhythm characterised by a nadir in early sleep, gradually increasing concentrations during late sleep, peak levels at 30-45 minutes post awakening (the cortisol awakening response: CAR) and a declining pattern thereafter. Salivary cortisol enables determination of the diurnal pattern within the domestic setting, although the measurement presents methodological challenges that need to be addressed. Further, the diurnal pattern of cortisol has been studied in relation to trait and state ill-being, rather than well-being. Consequently the focus of this programme of research was to explore relationships between the diurnal pattern of cortisol secretion and measures of both state and trait well-being and to examine the impact of electronically determined participant adherence to protocol within the domestic setting. In the first instance healthy, psychopathology-free young females were investigated but the work was extended to investigate the impact of aging on associations between cortisol secretion and well-being in healthy older females.

Data from healthy female participants demonstrates for the first time that moderate delays (on average 8 minutes) between awakening and the start of saliva sampling (previously considered tolerable) result in erroneous over-estimation of CAR magnitude and earlier timing of the CAR peak. This minimal level of non-adherence was not detected by self-reported awakening time, suggesting that electronic monitoring of awakening is essential in CAR research. The effects of moderate delays on the CAR measurement were explored in a detailed study, the first to sample salivary cortisol secretion at five minute intervals in the immediate post-awakening period. Over-estimation of the CAR magnitude and earlier peak were attributed to an observed approximate ten minute time lag between awakening and the start of the cortisol rise. In contrast non-adherence to the sampling protocol across the day did not impact on measurement of the diurnal cortisol measures when measured at 3-12 hours post-awakening.

In healthy young females neither state nor trait well-being/ill-being were associated with the CAR when using data strictly monitored for non-adherence during saliva sampling in the post-awakening period. Additionally, state and trait well-being were not associated with the diurnal decline or mean levels of cortisol across the day. These null findings could be attributed to the age of the sample. Previous associations between well-being and diurnal cortisol patterns have been observed mostly in middle-aged and older adults.

The new method of cortisol assessment in hair samples provided a retrospective trait measure of cortisol secretion, without the problems of non-adherence to protocol. No associations between three months hair cortisol secretion with well-being/ill-being were observed in young healthy females, in line with the results reported above using salivary cortisol. However, in the older sample associations between hair cortisol and trait well-being were evident. Higher levels of trait well-being were associated with higher hair cortisol, independently of ill-being, providing support for cortisol as an 'energiser' in healthy older female participants. Together these findings provide evidence for the neurotoxicity hypothesis of cortisol secretion; well-being did not exert effects on cortisol secretion in early adulthood but effects were evident in late adulthood in healthy psychopathology-free female samples.

The unique contribution of this programme of research lies in its consideration of methodological issues in the measurement of cortisol and well-being and its focus on positive psychology rather than the traditional psychopathology in relation to cortisol.

Declaration

The work presented in this thesis is the work of the author. Matilde Bianchin completed an internship at the University of Westminster as part of her MSc and assisted with the data collection process of study III. Hair samples obtained for study III were sent to the Technical University in Dresden for determination of cortisol.

Contents

List of Abbreviations

ACTH	Adrenocorticotropin
AUC_g	Area under the curve with respect to ground
AUC_I	Area under the curve with respect to increase
CES-D	Centre for epidemologic studies depression scale.
CAR	Cortisol awakeing response
CRH	Corticotrophin-releasing hormone
EM	Environmental mastery
HCC	Hair cortisol concentrations
HPA	Hypothalamic-pituitary-adrenocortical
HTA	Human Tissue Act
MIL-P	Meaning in life – presence
MIL-S	Meaning in life – search
MnInc	Mean increase
NA	Negative affect
PA	Positive affect
PG	Personal growth
PIL	Purpose in life
PVN	Paraventricular nuclei
PSS	Perceived stress scale
PWB	Psycholgical well-being
S1	Sample 1
SA	Self-acceptance
SAM	Sympathetic adrenomedullary system
SCN	Suprachiasmatic nucleus
SEM	Standard Error Mean
SHS	Subjective happiness scale
SQRT	Square root
SWB	Subjective well-being
SWLS	Satisfaction with life scale

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List of Publications and Presentations

Peer-reviewed papers:

Smyth, N., Clow, A., Thorn, L., Hucklebridge, F., & Evans, P. (2013). Delays of 5-15min between awakening and the start of saliva sampling matter in assessment of the cortisol awakening response. Psychoneuroendocrinology, 38(9), 1476-1483.

Smyth, N., Hucklebridge, F., Thorn, L., Evans, P., Clow, A. (in press). Salivary cortisol as a biomarker in social science research. Social and Personality Psychology Compass 1–21, 10.1111/spc3.12057

Conference Oral Presentations:

Smyth N, Thorn L, Evans P, Hucklebridge F, Clow A. Patterns of Salivary Cortisol Secretion in High and Low Well-being Students. Psychobiology British Psychological Society Annual Conference, Lake District, Sept 2011.

Smyth N, Evans P, Thorn L, Hucklebridge F, Clow A. Impact of saliva sampling delay on assessment of the cortisol awakening response (CAR) in a healthy student sample. Psychobiology British Psychological Society Annual Conference, Lake District, Sept 2012.

Smyth, N., Clow, A., Thorn, L., Hucklebridge, F., Evans, P. The CAR: impact of short delays and associations with trait and state well-being. Cortisol Network meeting, Imperial College, January 2013.

Evans P, **Smyth, N,** Thorn L, Hucklebridge F, Clow, A. Delays of 5-15 minutes between awakening and the start of saliva sampling matter in assessment of the cortisol awakening response. American Psychosomatic Society Annual Conference, Miami, March 2013.

Conference Poster Presentations:

Smyth N, Thorn L, Evans P, Hucklebridge F, Clow A. Discrepancies between self-reported and objective sampling delay for the cortisol awakening response. American Psychosomatic Society Annual Conference, Athens, March 2012.

Smyth N, Evans P, Thorn L Hucklebridge F, Clow A. State Well-being or Ill-being does not predict cortisol patterns in a young healthy sample. American Psychosomatic Society Annual Conference, Miami, March 2013.

Smyth N, Evans P, Thorn L Hucklebridge F, Clow A. Trait Hedonic and Eudemonic Well-being and Diurnal Cortisol Patterns. American Psychosomatic Society Annual Conference, Miami, March 2013.

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Chapter 1 Introduction I

Cortisol, ill-being and well-being

1.1 Overview

This chapter begins with a brief introduction to the area of psychoneuroendocrinology. It examines the role of the stress response systems in linking the mind/brain and health and briefly discuses the neuroendocrinological systems in mediating a stress response. A detailed examination of a major response system, the hypothalamic-pituitary-adrenal (HPA) axis is provided. Activation of the HPA axis results in the secretion of the steroid hormone cortisol, a major human glucocorticoid. Next, the production of cortisol in the adrenal cortex, the regulatory mechanisms involved in the termination of physiological stress responses and the major physiological actions of cortisol are described. As well as being a stress responsive hormone, in healthy individual's cortisol secretory activity shows a marked circadian rhythm, this is discussed in relation to the regulatory mechanisms involved and implications in illness and disease.

Traditionally psychology has focused on negative affective states and psychopathology; as such the physiological pathways involved are relatively well understood. In recent years, the benefits of well-being and positive functioning are becoming evident. However, the physiological pathways mediating this association are less well understood. Ill-being and well-being are thought to be independent dimensions, thus the way in which well-being affects health and the physiological pathways involved may differ. In the positive psychological literature two aspects of well-being are conceptualised hedonic (e.g. happiness and pleasure in life) and eudemonic (e.g. growth and meaning in life) well-being. The way these two aspects of well-being may relate to health and the physiological pathways mediating this association are also explored.

1.2 Psychoneuroendocrinology

The studies within this thesis fall under the area of psychoneuroendocrinology, which incorporates the interrelated disciplines of psychology, neurobiology, endocrinology, immunology, neurology, psychiatry and medicine to understand the links between the mind/brain and illness/health. It attempts to understand the physiological pathways underpinning the mind/brain and health/illness links. Since the time of the Ancient Greeks, negative psychosocial factors have been thought to play a role in the etiology of illness and disease. Since then, a compelling body of evidence documents associations between ill-being and adverse health-outcomes. For example, several reviews of epidemiological studies conclude that negative affective states, such as depression and anxiety are putative risk factors for coronary heart disease, even after controlling for known risk factors such as cholesterol, blood pressure, and smoking (for reviews, see Hemingway & Marmot, 1999; Kubzansky & Kawachi, 2000; Rugulies, 2002). A meta-analysis of 25 prospective studies of follow-up periods ranging from 2-16 years showed that subclinical and clinical depression were associated with increased risk of mortality (Cuijpers & Smit, 2002). The Whitehall studies provided compelling evidence for the links between psychosocial factors and health outcomes. These cross-sectional and longitudinal studies of British civil servants investigated the impact of negative affective states and social disadvantage on ill-health and disease. High job strain, effort-reward imbalance, low decision making and injustice at work all independently predicted incidence of coronary heart disease (Kivimaki et al., 2005; Kuper & Marmot, 2003). Lower social-economic-status was associated with poorer health and morbidity (Marmot, Ryff, Bumpass, Shipley, & Marks, 1997).

Such work has been influential in investigating the physiological pathways that underpin the mind and body link. Chronic stress activates the autonomic nervous system and neuroendocrine systems which have influences on multiple body systems (e.g. the immune system, the cardiovascular and metabolic systems), and dysregulation of them can lead to ill-health and disease

(Kristenson, Eriksen, Sluiter, Starke, & Ursin, 2004; McEwen, 2000; Padgett & Glaser, 2003). The neuroendocrine system mediates the body's physiological stress response. The neuroendocrine system is the interaction of the nervous system and the endocrine system. Together the endocrine and nervous systems provide mechanisms for communication between cells and organs. The endocrine system secrets biologically active substances (hormones) into the blood circulatory system. The primary link between the nervous system and the endocrine system is via the hypothalamus of the brain and the glandular (endocrine), anterior pituitary.

The body's physiological response to an event or stimuli perceived as stressful activates two primary neuroendocrine systems, the sympathetic adrenomedullary (SAM) system and the hypothalamic-pituitary-adrenocortical (HPA) axis. Activation of these systems result in major physiological effects on the body's tissues and can lead to changes in immune function (Sapolsky, Romero, & Munck, 2000). Activation of both these systems is via the hypothalamus but the stress response patterns differ. The SAM response to stress is immediate, the adrenal medulla is activated and within seconds catecholamines (epinephrine and norepinephrine) are released resulting in changes in blood pressure, heart rate, sweating within a few minutes. This stress response is the so-called fight-or-flight phenomenon. Whilst activation of the HPA axis is slower, with cortisol being released from the adrenal cortex and peaks approximately 20-30 minutes after the onset of the stressor (Dickerson & Kemeny, 2004). Most of the physiological actions of cortisol, however, are not exerted until about an hour after stressor onset (Sapolsky et al., 2000). The HPA axis and its physiologically important end product cortisol are at the centre of the studies presented in this thesis and are discussed at length below.

1.3 The hypothalamic-pituitary-adrenocortical axis

The hypothalamic-pituitary-adrenocortical (HPA) axis comprises the hypothalamus, anterior pituitary gland and adrenal cortex forming a neuroendocrine system. The hormone cortisol is the main end product following activation of the HPA axis. Triggers for activation of the HPA axis include immune system activation, pain, smoking, food intake, vigorous exercise, marked changes in cardiovascular tone, respiratory distress, awakening and psychological stressors. Experiencing a psychological stressor is probably the most prominent source of activation. Tasks that are uncontrollable and contain social-evaluative threat elements are the most effective in eliciting cortisol responses (e.g. Dickerson & Kemeny, 2004), for these reasons cortisol is commonly referred to as the stress response hormone.

A physiological cascade links the hypothalamus, anterior pituitary gland and the adrenal cortex (see Figure 1.1). Activation of this cascade is regulated by the nervous system, with production controlled directly by the brain. Stimulation of the neurosecretory parvocellular neurons in the paraventricular nuclei (PVN) of the hypothalamus produce corticotrophin-releasing hormone (CRH). CRH is transported to the anterior pituitary and the corticoptroph cells are stimulated to synthesis and release of adrenocorticotropin (ACTH) into the general circulation. ACTH circulates peripherally to arrive at the adrenal glands, which are situated above the kidneys. The adrenal cortex produces various steroid hormones one of which is glucocorticoids. Cortisol is the main glucocorticoid in humans, and is produced mostly by the zona fasciculate and at a lower rate by the inner zona reticularis.

Stress-induced HPA axis activation is regulated by negative feedback inhibition, this enables an individual to adapt and recover from stress. Cortisol regulates its own secretion through binding to receptor cells in the brain, which is unique to hormones whose secretion is regulated through the hypothalamus and pituitary. Increased cortisol levels are detected by cortisol receptors in multiple brain regions including the hippocampus, hypothalamus and pituitary (illustrated

in Figure 1.1). This results in the down-regulation of CRH and ACTH which in turn leads to less stimulation of the zona fasciculate of the adrenal cortex, and less cortisol secretion. In healthy individuals this negative feedback inhibition facilitates an individual's ability to adapt to and recover from stress. It maintains cortisol levels within a particular and appropriate range.

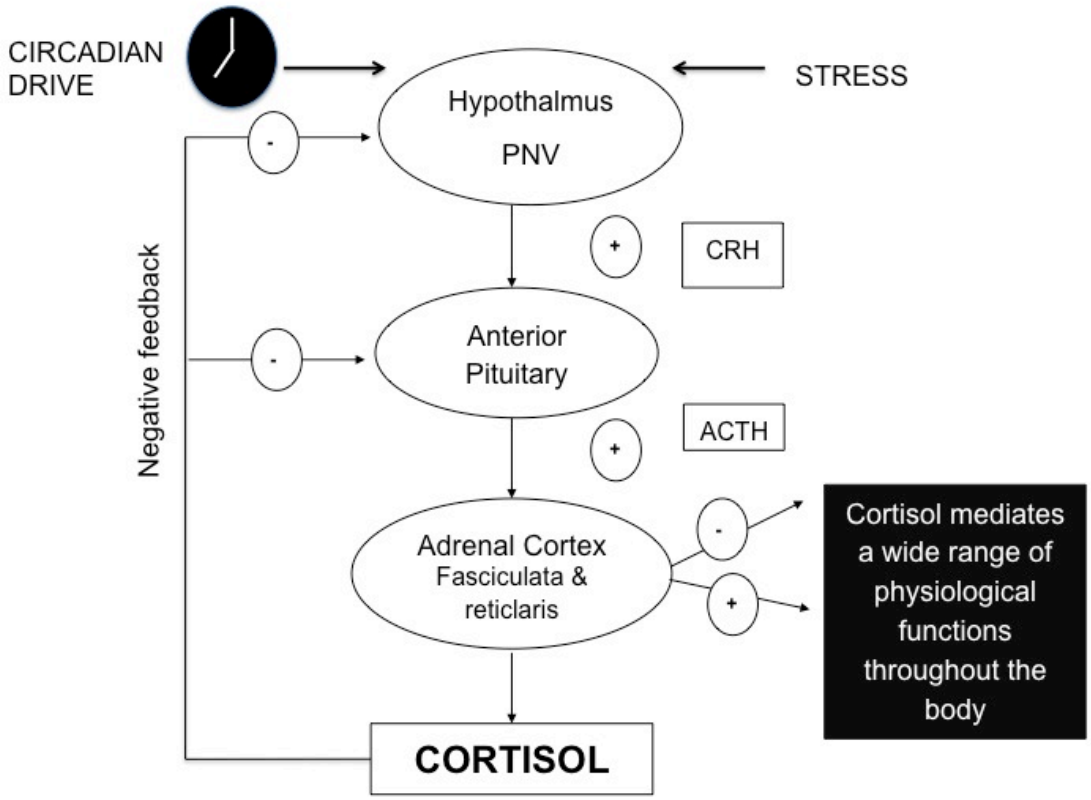

Figure 1.1 The hypothalamic-pituitary-adrenal (HPA) axis

Illustrates the physiological cascade involved in the HPA axis in humans. Corticotrophin releasing hormone (CRH) is transported to the anterior pituitary via the hypothalamic-pituitary portal blood vessels. Adrenocorticotrophic hormone (ACTH) is released into the general circulation. The '+' and '-' symbols indicate activation and inhibition respectively; cortisol exerts negative feedback as indicated to regulate the on-going cascade

1.4 Actions of cortisol

Cortisol has a wide range of physiological effects on virtually all target tissues throughout the body. It has many regulatory functions in the body for normal functioning but can also have deleterious effects at aberrant cortisol levels. One of the key roles of cortisol is facilitating the mobilization of energy resources. Cortisol increases circulating glucose concentrations through promoting breakdown of protein stored in muscles. This process is physiologically beneficial at adequate cortisol levels, but it has adverse effects of draining of the body's protein stores at excessive cortisol levels (Genuth, 1998).

Cortisol regulates blood pressure, cardiovascular function, carbohydrate metabolism, and immune function. Regulation of cardiovascular function includes enhancing vasoconstriction, maintaining normal blood pressure, fat depletion, and cardiac output (Sapolsky et al., 2000). Cortisol has long been known for its role as immunosuppressive, in the last decade it is also recognised for its role in regulating the immune system, thus cortisol is essential in the balance of immune function (see Evans, Hucklebridge, & Clow, 2000). Cortisol also has profound effects on bone, by influencing calcium absorption. However, excessive cortisol can lead to a reduction in bone mass (e.g. osteoporosis), which is caused by enhancement of bone resorption and a range of inhibitory actions on bone formation resulting in thinning of capillary walls (Genuth, 1998). Cortisol stimulates appetite and caloric intake. Excessive cortisol, for example, in Cushing syndrome, leads to obesity in the form of abnormal distribution of fat in the abdomen, trunk and face. Water and urine in the kidneys are maintained through the negative feedback actions of cortisol (Genuth, 1998).

Glucocorticoids influence neural activity, mood and behaviour. Both chronic hypercortisolism and chronic hypocortisolism observed in Cushing's disease and Addison's disease respectively present with negative psychological symptoms revealing the role of cortisol in affective processes (Porterfield, 2001).

Cortisol also regulates a variety of developmental events in the brain; high levels can have detrimental effects on brain development. Cortisol has effects on cognitive performance, but this relationship is complex and unclear. For example, administration of cortisol has been shown to enhance (e.g. Yehuda, Harvey, Buchsbaum, Tischler, & Schmeidler, 2007) and impair (Kirschbaum, Wolf, May, Wippich, & Hellhammer, 1996; Lee et al., 2007) cognitive performance, such as, memory. Table 1.1 details the actions of cortisol, each of which are synchronised around the 24-hour dark/light cycle by its circadian pattern of secretion.

Table 1.1 A summary of the actions of the hormone cortisol (adapted from Genuth, 1998)

Actions of the hormone cortisol
Support of glucose availability drawing upon protein and fat reserves (gluconeogenesis)
Inhibition of inflammatory processes and modulation of immune responses
Modulation of emotional tone
Promotion of wakefulness
Maintenance of cardiac output; increased arteriolar tone; decreased endothelial permeability
Increased glomerular filtration and free water clearance
Regulation of muscle function; decreased muscle mass
Decreased bone formation; Increased bone reabsorption

1.5 Circadian rhythm of cortisol

The actions of cortisol are more than simple stress responsive; cortisol is essential for supporting normal physiological function during normal, non-stress situations. Cortisol is one of the few hormones that is crucial for life (Baxter, Frohman, & Felig, 1995). What makes cortisol particularly interesting and important for healthy functioning is its marked circadian rhythm. The circadian pattern of cortisol was first described in a seminal study by Weitzman, Fukushima, Nogeire and colleagues (1971). In six healthy participants, with well-defined sleep-wake cycles, four distinct temporal phases of adrenocortical activity was evident: (1) a phase of minimal cortisol secretory activity from four hours pre- and two hours post-sleep; (2) followed by a preliminary secretory

episode three to five hours into sleep; (3) the main secretory phase was observed during the final three hours of sleep and one hour post-awakening and (4) phase of sporadic waking secretory activity in the subsequent diurnal period until initiation of the next circadian cycle (Weitzman et al., 1971). Subsequent work confirmed the core elements of this rhythm (e.g. Linkowski et al., 1985).

With regard to this circadian rhythm it is important to remember the pulsatile nature of cortisol secretion. It is released in secretory episodes (called the ultradian rhythm) across the 24 hour cycle at a frequency of approximately once every one to two hours (see Liu, Kazer, & Rasmussen, 1987; Weitzman et al., 1971). Although these bursts of secretion are not usually apparent when measuring cortisol in blood or saliva, pulsatility could potentially influence variability in data. An average secretory episode produces approximately 2.5 nmol/l of salivary cortisol (Wust et al., 2000). Changes in amplitude rather than frequency of these pulses predominantly account for differing cortisol concentrations across the circadian rhythm (Haus, 2007; Windle, Wood, Lightman, & Ingram, 1998). As would be expected CRH and ACTH show similar ultradian and circadian pattern (e.g. Haus, 2007).

Although the regulatory mechanisms underlying this circadian rhythm are not fully understood, it is thought to be responsible for regulating physiological function around the 24 hour sleep-wake or the light/dark cycle. One of the most important triggers for patterns of cortisol secretion is the sleep/wake or light/dark cycle, information about which is relayed to the HPA axis via the body clock, a small region of the hypothalamus called the suprachiasmatic nucleus (SCN). The SCN is located immediately above the optic chiasma and it receives information about light from the retina through the retinohypothalmic tract. This in turn results in stimulation of the PVN of the hypothalamus to release CRH, resulting in activation of the HPA axis and secretion of cortisol. This regulates cortisol secretion during the day and inhibits secretion at night (Benarroch, 2011).

A non-HPA axis dependent pathway is also thought to be involved in the circadian regulation of cortisol secretion (Bornstein, Engeland, Ehrhart-Bornstein, & Herman, 2008; Buijs, van Eden, Goncharuk, & Kalsbeek, 2003). A direct neural pathway from the SCN to the adrenal cortex via the splanchnic nerve enhances adrenal 'zona fasciculata' sensitivity to ACTH. This is thought to be associated with awakening, thereby maximising the rate of increase in cortisol secretion in the immediate post awakening period (see Clow, Hucklebridge, Stalder, Evans, & Thorn, 2010).

In this way as well as being stress responsive, in healthy individuals cortisol exhibits a marked circadian rhythm characterised by peak levels following morning awakening (known as the cortisol awakening response: CAR) and declining levels thereafter, reaching lowest levels in early sleep (Edwards, Clow, Evans, & Hucklebridge, 2001). This circadian rhythm has the role of providing the chemical signal to downstream physiological processes (that have no direct access to information about light from the SCN) about time of day and is essential for harmonious synchronisation across multiple physiological systems. The actions of cortisol listed in Table 1.1 are synchronised around the 24 hour light/dark cycle by its circadian pattern of secretion.

Short-term stress activation of the HPA axis is adaptive and essential for supporting normal daily functioning in everyday life (Baxter et al., 1995). However, both high and low levels of cortisol as well as disrupted circadian rhythms are implicated in physical and psychological disorders. For example, overall high levels are associated with major depression (Holsboer, 2000) whilst chronic fatigue syndrome is associated with lower overall cortisol levels (Crofford et al., 2004; Demitrack et al., 1991; Huber, Issa, Schik, & Wolf, 2006). Flattened cortisol circadian rhythms (either with abnormally high or low levels) are associated with a wide range of disorders, for example major depression (e.g. Huber et al., 2006) and cancer prognosis (Abercrombie et al., 2004; Sephton, Sapolsky, Kraemer, & Spiegel, 2000). Crucially, dysregulation of the HPA axis, other than that attributed to severe endocrine pathology is attributed to the cumulative effect of chronic stress, characterised by hyper- or hypo-secretory patterns depending upon the timing, nature and duration of stress

exposure. In such circumstances the dual roles of cortisol as essential circadian 'housekeeper' and stress response hormone collide with the deleterious consequences for health.

1.6 Positive Psychology

The work of Martin Seligman, founder of the positive psychology movement brought the significance of positive characteristics and human flourishing to the focus of psychology. The field of positive psychology is the study of positive emotions and character traits on well-being (Seligman & Csikszentmihalyi, 2000; Seligman, Steen, Park, & Peterson, 2005). This is an exciting new area of research; it attempts to understand, test, and enhance the potentially protective and beneficial effects of well-being on physical and psychological health. Positive psychology represents a transition from focusing on the negative aspect of human functioning to thinking about the positive aspect of human functioning, for example, positive emotional states and the influence on behaviour and health.

Research in the area of positive psychology is beginning to highlight the protective effects of well-being on health-related processes. The following two studies provide striking examples of how positive characteristics, such as, happiness and positive emotions influence good health and longevity. Longitudinal analysis of a sample of Catholic nuns revealed that the positive emotional content of writings in young adulthood (average 22 years old) was associated with longevity during a 60-year period (Danner, Snowdon, & Friesen, 2001). In a recent study of American baseball players, smile intensity predicted longevity. Smiles on photographs were used as a proxy for positive emotions and happiness. Players with smiles were less likely to die than players with no smiles (Abel & Kruger, 2010). Further, longitudinal studies demonstrate the impact of positive affective states and well-being on health. In a review of prospective and empirical studies of older adults, positive affect was associated with lower mortality, reduced morbidity and pain, and better self-reported health (Pressman & Cohen, 2005). In another study, well-being was associated with

substantially lower risk of future coronary heart disease, this effect remained significant when controlling for known influences such as demographic and medical factors (Kubzansky & Thurston, 2007). In a recent review of prospective, observational and cohort studies trait and state well-being was associated with reduced mortality in healthy and disease population studies. Further, in studies that controlled for negative affect the protective effects of well-being were showed to be independent of negative affective states (Chida & Steptoe, 2008).

Health behaviours and lifestyle may indirectly mediate the relationship between well-being and health. Well-being may be associated with health-enhancing and protective behaviours, which in turn reduces the risk of long-term risk of illness. There is compelling evidence that risky health behaviours, such as, smoking, lack of physical exercise and heavy alcohol consumption are associated with stress and negative emotions such as depression and anxiety (Biddle, Gorely, & Stensel, 2004; Kassel, Stroud, & Paronis, 2003; Regier et al., 1990). More recently, life satisfaction was associated with health enhancing behaviours such as, not smoking, healthy diet, using sun protection and physical exercise (Grant, Wardle, & Steptoe, 2009). Well-being may have an effect on future health by directly influencing psychobiological processes, including promoting healthy cortisol rhythms, faster recovery from cardiovascular stress recovery, reduced inflammation, and resilience to infection (see Dockray & Steptoe, 2010 for a review).

1.7 Structure of ill-being and well-being

The field of positive psychology raises the question whether well-being is merely the opposite of ill-being or are well-being and ill-being separate and independent dimensions. The former view assumes that well-being and ill-being are opposite ends of a bipolar continuum and that knowledge about the causes, consequences and treatments of psychological maladjustment and disorders also informs understanding of well-being, this is commonly known as the ‘mirrored’ or ‘bipolarity’ hypothesis. Typically, well-being was thought of as

merely the opposite of ill-being, those with high levels of ill-being would be expected to show low levels of well-being and vice versa.

Previously, measures of well-being tended to focus upon the presence or absence of ill-being (e.g. negative symptoms such as stress, anxiety or depression). However, more recently accumulating evidence suggests that well-being is more than the mere absence of ill-being. While it is likely that well-being will be low when ill-being is high, there is growing evidence that well-being and ill-being tend to function relatively independently. This is commonly known as the 'independence' hypothesis. The idea that well-being and ill-being represent independent dimensions of human functioning will mean that our understanding of ill-being will not necessarily be reflected in the opposite way for well-being. Testing the 'mirrored' or 'bipolarity' versus the 'independence' hypotheses deserve careful consideration in order to clarify the extent to which well-being is uniquely related to health and the physiological pathways involved.

Accumulating evidence continues to favour the 'independence' hypothesis. Early work testing these hypotheses indicated that positive and negative affect were largely independent (Bradburn, 1969). Further psychometric exploration of well-being and ill-being shows that the two domains are inversely correlated, but associations are weak, suggesting that positive affect is not simply the absence of negative feelings and experience, or vice versa. Rather, some individuals show high levels of ill-being and well-being, whist others show low levels of ill-being but also lack well-being (Diener & Emmons, 1984; Diener, Smith, & Fujita, 1995; Keyes, 2002).

Further support for the 'independence' hypothesis is evident from the distinct physiological correlates of well-being and ill-being. It is a well-replicated finding that ill-being (e.g. depression, anxiety) is associated with increased cortisol and norepinephrine (Brown, Varghese, & McEwen, 2004; Hughes, Watkins, Blumenthal, Kuhn, & Sherwood, 2004), as well as increased cardiovascular risk (Ahlberg et al., 2002; Barefoot et al., 1998). Although there is some evidence for links between well-being and physiology, few studies have examined this. Psychological well-being (e.g. purposeful life engagement) has been associated

with decreased cortisol levels, cardiovascular risk and inflammatory markers (Lindfors & Lundberg, 2002; Ryff, Singer, & Love, 2004). Furthermore, most studies have assessed either ill-being or well-being in relation to physiological biomarkers, but not both. Ryff et al. (2006) investigated the physiological correlates of both well-being and ill-being in a sample of aging women. Overall the findings from this study provided support for the 'independence' hypothesis. For the majority of neuroendocirine and cardiovascular markers that were associated with well-being they were not associated with ill-being (and vice versa). However, there was some evidence for the 'mirrored' or 'bipolarity' hypothesis, weight and glycosylated haemoglobin was negatively associated with both well-being and ill-being (Ryff et al., 2006).

Furthermore, the two domains were associated differently with health variables. The absence of well-being was more predictive of seven year follow up mortality than the presence of ill-being (Huppert & Whittington, 2003; Watson, Clark, & Carey, 1988). Compared with the presence of psychological symptoms or ill-being, the absence of well-being was a stronger predictor of mortality (Whittington & Huppert, 1998). Well-being was considered to be a better predictor of current of future health status, such as, mortality, mobility, stroke, and onset of disability than ill-being (Blazer & Hybels, 2004; Ostir, Markides, Black, & Goodwin, 2000; Ostir, Markides, Peek, & Goodwin, 2001). Currently there is evidence for both hypotheses, given the evidence for the 'independence' hypothesis it is important to investigate ill-being alongside well-being in order to explore if well-being is uniquely related to physiology or psychosocial and health variables.

1.8 Hedonic and eudemonic well-being

The meaning and structure of well-being has puzzled the mind of philosophers for centuries. It is important to understand what is meant by well-being in order to understand relationships between cortisol and well-being. Within the well-being literature, a distinction between hedonic and eudemonic well-being exists (Diener, Suh, Lucas, & Smith, 1999; Keyes, Shmotkin, & Ryff, 2002; Ryan &

Deci, 2001; Waterman, 1993). The following sections define each term and discuss the on-going debate in the literature regarding the distinction between these two aspects.

The hedonic approach is rooted in the ideas of human functioning being pleasurable, happy and satisfying and this dates back to the work of the Ancient Greeks (e.g. Epicurus), and philosophers such as Hobbes and Bentham (Ryan & Deci, 2001). The scientific study of hedonic well-being is concerned with what makes experiences and life pleasant and unpleasant. It falls under the umbrella term subjective well-being (SWB) and describes positive feelings such as happiness, pleasure and life satisfaction (Diener & Lucas, 1999). It is typically defined in terms of the presence of positive affect, absence of negative affect and satisfaction with life (Diener et al., 1999; Kahneman, Diener, & Schwarz, 2003). It is considered subjective because individuals make evaluations about themselves and the extent to which they experience a sense of wellness. Assessing the extent to which feelings and experience are positive or negative involves an affective element. However, making judgements of whether a life is satisfying involves a cognitive aspect, thus it is not strictly a hedonic aspect, but it is included in this approach amongst well-being researchers.

Research on SWB has flourished; however, other researchers have argued that well-being is more than simply being positive and satisfied (e.g. happiness). Happiness does not necessarily mean psychological wellness (Ryan & Deci, 2001). The eudemonic perspective of well-being is based on the philosophy of Aristotle, who wrote about the realization of one's true potential and living a life of contemplation and virtue (see Ryff, 1989). Recent definitions of eudemonic well-being encompass the recognition of potentials in life and striving for a fulfilling and meaningful life (Ryan & Deci, 2001). One model of psychological well-being that has integrated eudemonic concepts into large-scale population studies is the multidimensional model of psychological well-being (PWB). PWB was based on the psychological resources required for flourishing throughout the lifespan (Ryff, 1989; Ryff & Keyes, 1995). The PWB scales consists of six dimensions: individuals self-acceptance (feeling good whilst being aware of

limitations), positive relations with others (warm and trusting interpersonal relationships), environmental mastery (ability to shape own environment so as to meet personal needs and desires), autonomy (self-determination and authority), purpose in life (finding meaning in efforts and challenges) and personal growth (making the most of talents and capacities).

Eudemonic well-being is criticised for its poor definition and measurement, this may be due to less focus on eudemonic well-being and this is evident in the relatively few psychological papers published explicitly on the topic. Our understanding of hedonic or subjective well-being is clearer, therefore its definition and measurement is more rigorous (Waterman, Schwartz, & Conti, 2008). It is suggested that hedonic well-being is most likely the result of eudemonic pursuits of happiness (Ryan & Deci, 2001). However, there is no clarity on the direction of this association; research is correlational so the causal direction is often assumed. It may be likely that hedonic well-being causes eudemonic well-being. Evidence for this comes from the meta-analysis providing evidence that positive feelings play a role in people securing important life outcomes, for example, career success, or satisfying marriages (Lyubomirsky, King, & Diener, 2005). It is important to acknowledge that hedonic well-being may be a causal role in eudemonia (but also vice versa).

It is suggested that there are important differences between hedonic and eudemonic well-being (e.g. Ryan & Deci, 2001). Researchers propose that these aspects are conceptually related but empirically distinct. Factor analytical studies provide support for separate aspects of well-being. In a national sample of Americans (N=3032, aged 25-74 years) SWB and PWB were highly correlated, however, the subjective and psychological components loaded separately onto distinct factors (Keyes et al., 2002). Similar findings were subsequently replicated in students and adults from the UK. Two factors of SWB and PWB were found and this was consistent across age, sex, age and ethnicity (Linley, Maltby, Wood, Osborne, & Hurling, 2009). These studies suggest that SWB and PWB are two related but distinct dimensions. Further support for this is evident from the associations with physiological pathways.

Eudemonic well-being was more strongly related with physiological correlates (Ryff et al., 2004).

Currently, the positive psychology movement regard happiness in terms of both hedonic and eudemonic well-being. Seligman proposes that happiness (well-being) is achieved through a combination of a pleasant life (e.g. enjoyment and the savouring of positive feelings); a good life (e.g. engagement in everyday pastimes); and a meaningful life (meaning, belonging and purpose). The pleasant life falls under SWB and the latter fall under PWB. As such well-being in the studies in this thesis is defined as encompassing subjective (hedonic) and psychological (eudemonic) aspects of well-being.

Chapter 2 Introduction II

Measurement of cortisol in saliva and hair

2.1 Overview

The purpose of this chapter is to discuss the measurement of cortisol secretion in saliva and hair samples. The advantages and methodological issues of each measure are discussed. The chapter begins with an overview of the advantages of salivary cortisol in relation to the more traditional methods of blood and urine. Using salivary measures facilitates accurate investigation of the diurnal pattern of cortisol, under ambulatory conditions. The diurnal pattern of cortisol secretion can be divided into two relatively discrete components: the cortisol awakening response (CAR) and the diurnal decline. Typically, the CAR and diurnal decline are measured separately, and associations with psychosocial variables and health are explored. However, previous literature is limited by inconsistent and conflicting findings. This may be due to participant non-adherence to the saliva sampling protocol, which results in misleading diurnal cortisol patterns, particularly for the CAR. Measurement of cortisol in hair samples is a new area of research. It provides a retrospective trait measure of cortisol secretion. Sampling is relatively easy and quick and non-adherence is not an issue, however, it is a blunt measure since no diurnal cortisol activity can be determined.

2.2 Salivary Cortisol

Cortisol is a steroid and as such can be measured in any body fluid because its chemical properties ensure it can pass through all membranes of the body without hindrance. The clinical norm is to measure cortisol in blood, and sometimes urine. More recently, cortisol has been measured in saliva, which has numerous advantages over blood and urine sampling. Like plasma cortisol,

salivary cortisol provides a measure of cortisol within the last few minutes, whilst urinary cortisol provides a measure of cortisol secretion over hours (e.g. 24 hrs). In the blood, only 5-10% of cortisol is biologically active ('free') the rest is bound to large proteins (cortisol binding protein and albumin). Consequently blood cortisol is usually measured as 'total' cortisol, comprising bound and unbound components. The specific analysis of the free, biologically active cortisol element in blood is both time-consuming and expensive (Vining, McGinley, Maksvytis, & Ho, 1983). In contrast salivary cortisol solely reflects the free component and so such measures are more meaningful in terms of cortisol activity. The validity of salivary cortisol measures is evidenced by high correlations between salivary and circulating free cortisol, with most investigators reporting correlation coefficients of at least 0.90 (reviewed in Kirschbaum & Hellhammer, 1989, 1994).

Saliva sampling has several additional advantages over determination of cortisol from blood and urine samples. See Table 2.1 for a detailed summary of the strengths and considerations of using salivary measures of cortisol. Unlike blood sampling, saliva sampling is convenient, non-invasive and does not need to be carried out by medically trained personnel. The potential for repeated sampling allows close scrutiny of the dynamics of cortisol secretory activity or in ambulatory settings with participants going about their normal daily routines. Furthermore, salivary sampling method is non-stressful which is essential in any investigation of basal cortisol secretory activity. As such, salivary cortisol is the ideal assessment for basal ambulatory studies.

Table 2.1 A summary of strengths and considerations when using salivary measures of cortisol

Strengths	Considerations
Collection	
Saliva is not a Class II biohazard unless it is contaminated with blood.	Human saliva should be treated as potentially infectious material and normal microbiological safe handling procedures used at all times.
Saliva samples collected by participants – medically trained persons not required. With assistance sampling in special populations (e.g. babies or children) is possible.	Inclusion requires appropriate ethical approval.
Non-invasive procedure - sampling does not affect the cortisol levels (venepuncture can significantly increase cortisol).	Some participants dislike the dry cotton swab in salivettes. Saliva can also be collected by passive drool, which is approved with other analytes.
Sampling collection is relatively quick - approx. 1 min.	
Salivary flow rate does not affect salivary cortisol levels - standardising and recording time over which the saliva is collected is not required.	
Repeated sampling (e.g. every 15-30 min) enables assessment of dynamic cortisol profiles (e.g. over the day).	
Storage	
Stable at room temperature (approx. 2-4 weeks) - removes the need for immediate freezing. Refrigeration/freezing recommended as soon as possible to prevent bacterial growth.	Researchers collecting and storing saliva samples need to be aware of health and safety regulations e.g. in the UK the HTA regulates activities concerning the removal, storage, use and disposal of human tissue. Saliva contains human cells so it is considered a tissue within the terms of the act (see HTA www.opsi.gov.uk)
Samples can be posted for assaying.	
Samples frozen at -20˚C can be stored for an extended period (i.e. years) and are not affected by thawing and re-freezing.	
Assay	
Relatively easy to assay using a range of commercial assay providers. Required equipment is typically available in biological or medical laboratories. Or samples can be posted to commercial laboratories that offer a full service (quotation, provision of labelled salivettes, packaging materials and full results). It typically costs <£10 to assay each sample, depending on the size of the batch.	The range of cortisol assays used across different laboratories means there are no standard absolute salivary cortisol values, making it difficult to compare findings across studies. Important that all samples within a study are assayed in the same batch, using the same assay protocol so that values can be compared and integrated.

2.3 Salivary cortisol in ambulatory settings

Salivary cortisol enables measurement of the basal circadian pattern of the hormone's secretion in naturalistic settings and it is typically related with psychosocial and health variables, and more recently aggregated mood. Repeated saliva sampling enables measurement of the diurnal pattern of cortisol secretion under naturalistic settings, providing an account of real life exposure to stress and may be more relevant to long-term health outcomes.

The diurnal cortisol pattern can be divided into two relatively discrete components the cortisol awakening response (CAR) and the diurnal cortisol profile across the day (see Figure 2.1). Accordingly the following sections go on to discuss the measurement approach of the CAR and diurnal cortisol profile across the day as well as examine the methodological considerations for its measurement.

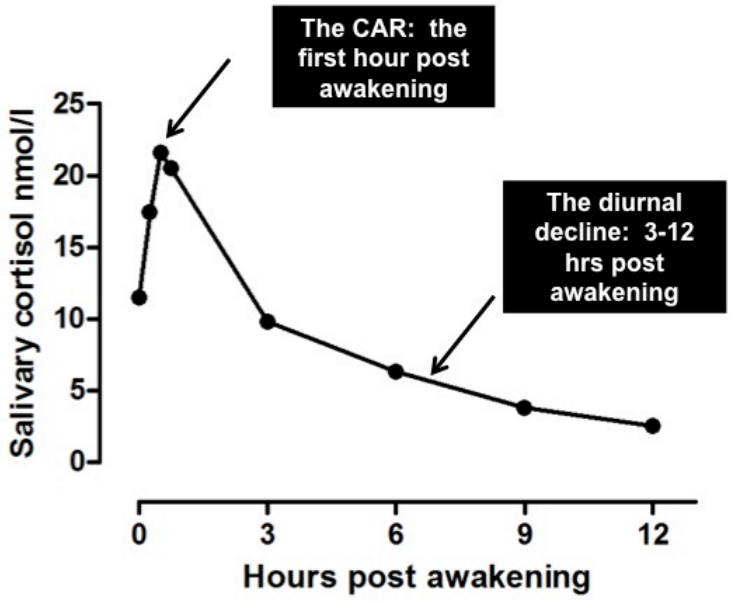

Figure 2.1 The diurnal pattern of cortisol secretion in healthy adults

Plot illustrates the cortisol awakening response (CAR) and diurnal decline. Data derived from a composite of studies conducted at the laboratory at the University of Westminster and indicate the typical values across the day

2.3.1 The cortisol awakening response (CAR)

The CAR is the 50-100% increase in cortisol levels following morning awakening, which typically peaks at 30-45 min post-awakening (see Figure 2.1). In the mid-1990s, Pruessner and colleagues were the first to bring it to the attention of social scientists by demonstrating a CAR in healthy participants (Pruessner, Kirschbaum, & Hellhammer, 1995). The same group subsequently provided a more detailed account of the CAR showing the time course and intra-individual stability of the CAR over three consecutive days and three consecutive weeks in children, younger and older adults (Pruessner et al., 1997). Pruessner and co-workers originally considered the CAR to be "a reliable biological marker for the assessment of adrenocortical activity", but the CAR is now considered a relatively distinct aspect of the circadian rhythm, initiated in response to morning awakening (Wilhelm, Born, Kudielka, Schlotz, & Wust, 2007). Although activity of the HPA axis (and consequent secretion of ACTH) is an important factor across the 24 hr period, the CAR is somewhat independent of cortisol secretion over the rest of the day (see Clow et al., 2010).

There has been considerable interest in the CAR and it promises to be a useful biomarker, perhaps more sensitive than the diurnal cortisol profile for a range of psychosocial and health outcomes (see Chida & Steptoe, 2009; Fries, Dettenborn, & Kirschbaum, 2009). However, findings regarding associations with the CAR and psychosocial or health variables have frequently been inconsistent and require further clarification (Clow, Thorn, Evans, & Hucklebridge, 2004; Fries et al., 2009). Inconsistencies are likely to be caused by methodological differences, such as, inadequate control of the saliva sampling protocol and unaccounted situational and participant variables.

When examining trait variables it is standard practice to measure the CAR on at least two consecutive weekdays. However, others recommend up to six days for accurate determination of the trait CAR (Hellhammer et al., 2007). This may be an excessive demand upon participants, to some extent negating the user-friendliness of the ambulatory approach to salivary cortisol assessment. In such cases, an awareness of possible confounding state variables may be preferable

to making routine such a demanding sampling protocol. Such state variables include awakening time, and morning light levels, both of which have been shown to impact on the CAR (Edwards, Evans, Hucklebridge, & Clow, 2001; Scheer & Buijs, 1999; Thorn, Hucklebridge, Esgate, Evans, & Clow, 2004). In addition, in healthy individuals the CAR is strongly influenced by state psychosocial variables such as, anticipation of significant workload or challenge during the day ahead, negative experiences during the previous day (e.g. Stalder, Hucklebridge, Evans, & Clow, 2009). Table 2.2 details variables that impact upon the CAR and which should be considered when conducting basal ambulatory studies.

Table 2.2 Variables to consider when conducting basal ambulatory studies

Confounding variables	
Age	• Higher cortisol levels, attenuated CAR and attenuated diurnal decline are associated with age (Evans et al., 2011; Ice, 2005).
Gender	• Women have a greater CAR magnitude compared to males (Kirschbaum, Kudielka, Gaab, Schommer, & Hellhammer, 1999; Wright & Steptoe, 2005) and males peak earlier than females (Pruessner et al., 1997).
Menstrual Cycle	• Generally no difference in the CAR between menstrual cycle (Kudielka & Kirschbaum, 2003; Wolfram, Bellingrath, & Kudielka, 2011a) except ovulation – higher CAR magnitude and earlier CAR peak (Wolfram et al., 2011a) • Females on oral contraceptives were found to have lower mean cortisol in the CAR period (Pruessner et al., 1997).
Puberty Stage/hormonal status	• Pubertal developmental changes are associated with increased daytime basal cortisol profiles and a steeper diurnal decline (Adam, 2006). Also, pre-menarche females show an earlier peak of the CAR (Oskis, Loveday, Hucklebridge, Thorn, & Clow, 2009).
Alcohol consumption	• Preliminary evidence that alcohol consumed the night before is associated with a decreased CAR the following morning (Stalder et al., 2009). More chronic consumption of alcohol is associated with an increased CAR (Adam, Hawkley, Kudielka, & Cacioppo, 2006; Badrick et al., 2008) and a reduced slope of cortisol decline (Badrick et al., 2008).

table continued on next page

Smoking	• Cortisol levels over the day (2.5 hr to 12 hr post-awakening) and the CAR are higher in smokers compared to non- or ex-smokers (Badrick, Kirschbaum, & Kumari, 2007).
Medication	• Medication use should be recorded - numerous and ever-changing range of medications impact on salivary cortisol, see Granger et al. (2009) for the pathways involved.

Determination of the CAR typically requires self-collection of saliva samples within the domestic setting at regular intervals post-awakening for 30-60 minutes (e.g. 0, 15, 30, 45 and 60 min post-awakening). Although some research indicates that unexpected awakening impacts the CAR (Born, Hansen, Marshall, Molle, & Fehm, 1999), studies have more generally shown that mode of awakening lacks influence (Pruessner et al., 1997; Wuest et al., 2000). Thus, participants are generally instructed to awake in their usual way. CAR research has traditionally focused on the CAR magnitude which is the increase in cortisol in the first 45-60 minutes of awakening (Clow et al., 2004). CAR magnitude should be measured as the mean increase [MnInc], or the near equivalent of area under the curve with respect to increase [AUCi] (see Figure 2.2 for formulae). More simply it is possible to calculate the CAR as the difference between the awakening level and a single later 'peak' measure. Although most often used in large scale epidemiological-type studies this approach is not ideal as the actual peak may be missed. This may be particularly problematic in studies of males and females. Males typically peak at ~30 min post-awakening whereas females peak later at ~45 min post-awakening (Oskis et al., 2009; Pruessner et al., 1997). Usually assessment of the CAR is limited to a 45 minute post-awakening period, rather than an hour. This is mostly for pragmatic reasons as requesting participants to refrain from normal morning activities for more than 45 minutes would be very demanding and likely limit participation. Accurate determination of the CAR crucially relies upon accurate collection of the first sample, immediately upon awakening.

The composite measures AUCg (i.e. area under the post-awakening cortisol curve with reference to ground/zero) or, equivalently, mean levels of cortisol

secretion in the post-awakening period do not provide information about the dynamic of the cortisol increase. Ideally when presenting CAR data, information about the value of the first sample and the dynamic increase (MnInc/AUCi) should both be provided. The AUGg can then be deduced from these two measures.

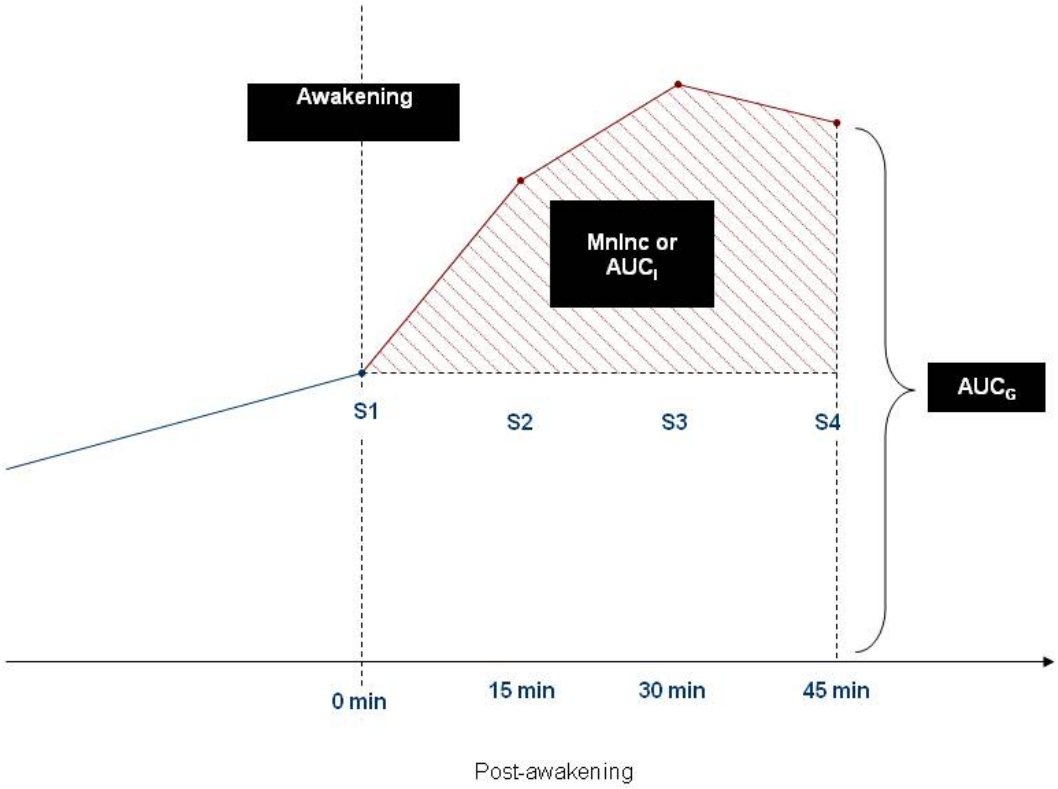

Figure 2.2 Measurement of cortisol awakening response

MnInc (mean increase) and AUC_I (area under the curve relative to increase) provides an estimate of CAR magnitude. AUCg provides an estimate of total post-awakening cortisol secretion. Formulae: MnInc = (s2 + s3 + s4)/3 – s1; AUC_I = s2 + s3 + [(s4 – s1)/2] - 2s1; AUC_G = s1 + s2 + s3 + [(s4 – s1) / 2]; or AUC_I = AUC_G - 3s1 (s1 collected immediately on awakening, s2 collected at 15 min, s3 at 30 and s4 at 45 minutes post-awakening. These formulae all assume equal time intervals, arbitrarily denoted at unity, between all samples).

2.3.2 The diurnal decline of cortisol

Cortisol secretion over the day is typically measured in terms of two key aspects: either the diurnal cortisol slope (typically a decline) or mean level over relevant sample points to provide a measure of overall cortisol secretion (mean diurnal cortisol), as shown in Figure 2.1. A steeper cortisol decline has been consistently associated with better psychosocial and physical health outcomes (Adam & Gunnar, 2001; Adam et al., 2006; Cohen et al., 2006). In line with best practice guidelines saliva sample points should always be synchronised to awakening time rather than clock time (Edwards, Clow, et al., 2001). For measurement of the cortisol slope nearly all methods use an evening measure (prior to sleep or 12 hours after awakening). However there is no consensus about the best morning time point from which to anchor the diurnal decline. As the dynamic of change in cortisol is more marked in the morning compared to the evening this is an important issue and the choice of time can substantially affect the results obtained. Various approaches have been adopted, for example, use of first awakening sample, the peak of the CAR or three hours following awakening. As the CAR is thought to have some distinct regulatory input from diurnal cortisol secretion and the CAR is known not to correlate strongly with diurnal secretion (Edwards, Clow, et al., 2001), it is recommended to keep analysis of the CAR separate from analysis of the diurnal decline (Smyth, Hucklebridge, Thorn, Evans, & Clow, 2013). Accordingly it is recommended that the diurnal decline is anchored with a morning sampling point outside of the CAR period, typically the diurnal decline is calculated as the change in secretion from 3 to 12 hours post-awakening (Smyth et al., 2013). It is good practice to collect multiple samples across the day (e.g. at 3, 6, 9 and 12 hours post awakening) and on two consecutive weekdays, which avoids distortion of data by random state factors (e.g. Thorn, Evans, Cannon, Hucklebridge, & Clow, 2011). See Table 2.2 for a summary of considerations for confounding factors when measuring the diurnal cortisol profile across the day.

Another common measure of daytime cortisol secretion is the ‘area under the daytime cortisol curve’. This provides a measure of the average cortisol

secretory activity across the day and has been associated with early life stress (Nicolson, 2004). Cortisol concentrations sampled throughout the day are averaged to give an overall measure of cortisol secretion. Again there is no consensus about whether this measure should or should not include the immediate post-awakening period of cortisol secretion. For the same reasons as outlined above it is suggested that the CAR period should be excluded from the assessment of the diurnal cortisol AUC (Smyth et al., 2013). Estimates of average daily cortisol levels complement estimates of the diurnal decline. This is necessary as a reduced slope may be associated with either hyper- or hypo-cortisolemia, so reporting the slope or levels alone is not adequate, both estimates are required. For studies which only include minimal sample points, bedtime cortisol concentrations are frequently the sole cortisol measure. Higher levels at this time can indicate a flatter diurnal cortisol slope over the day (e.g. Cohen et al., 2006; Pendry & Adam, 2007).

2.4 Non-adherence to the saliva sampling protocol in ambulatory studies

When studying the diurnal pattern of cortisol in the ambulatory setting, participants are left to their own devices to firstly remember to collect their saliva samples and secondly to collect them at the desired collection time. Researchers typically assume that participants adhere to the protocol, unless otherwise stated. Due to the dynamic and brief nature of the CAR it is essential that participants adhere to the requested saliva sampling regime, inclusion of data derived from participants non-adherent to the protocol during the immediate post-awakening period is likely to explain the discrepancies in the CAR literature since it is likely that error in the timing of saliva sampling will impact upon accurate measurement of the CAR. Cortisol measured over the day (e.g. diurnal decline and mean diurnal cortisol) may be less influenced by non-adherence to the protocol, since levels steadily decline over the day.

2.4.1 Monitoring non-adherence to the saliva sampling protocol

Researchers typically rely on participant self-reports to identify whether participants have collected samples at the desired times. The most typical of this is the diary method whereby participants report their awakening and saliva sampling times. These are used to identify non-adherence to the protocol and delayed collection of samples can be excluded from the data analysis if necessary. Using self-reports to monitor participant adherence to the protocol between 7% and 11% of participants reported a 10 minute delay in awakening and collection of sample one and this resulted in higher awakening samples and no rise from awakening to the 30 minute post-awakening sample compared to those that reported a delay of under 10 minute in sampling relative to awakening (Kunz-Ebrecht, Kirschbaum, Marmot, & Steptoe, 2004; Wright & Steptoe, 2005).

The problem with the diary method is participant's inaccurate self-reports. More objective measures of both awakening and sampling times estimated by electronic devices are recommended to monitor participant adherence to the saliva sampling protocol. The next section discusses studies that have compared self-reported and electronically monitored adherence to the protocol and the impact of non-adherence on the CAR and diurnal cortisol profile.

2.4.2 Assessment of saliva sampling times

Collection of saliva samples can be monitored electronically using track caps (e.g. Medication Event Monitoring Caps etc). These devices contain a bottle and a microelectronic cap, the cotton swabs or tubes used for saliva sampling are stored in the bottle and participants are instructed to open the bottle only when collecting the saliva sample. Each opening of the cap records the time and date and this indicates the collection time of the saliva sample. Such devices are typically used within medical settings to monitor patient adherence to medication regimens (Farmer, 1999; Schwed et al., 1999; Straka, Fish, Benson, & Suh, 1997). Prevalence of non-adherence specific to the morning period was investigated in a large sample (N = 300) of adolescents. Using track

caps, a significant number of participants that delayed saliva sampling were identified: 31% collected the awakening sample 10 minutes late, 37% collected the 30 minute post-awakening sample seven minutes early or late, and 40% collected the bedtime sample within ± 60 minutes (Halpern, Whitsel, Wagner, & Harris, 2012).

In two studies self-reported and electronically monitored saliva sampling times were compared and this revealed the inaccuracy of participant self-reports. In both studies, half of the participants were aware of the electronic estimates of sampling times. In the first study, a group of community adults (average 30 years old) collected saliva samples on one day in the morning (0 and 30 min post-awakening) and at spot samples over the day (11:00, 15:00, 20:00, 22:00 hr). Participants unaware of electronic monitoring were inaccurate in reporting their sampling times, on average self-reports were over an hour (68 minutes) later than the electronic estimates (Kudielka, Broderick, & Kirschbaum, 2003). Considering that the non-adherence window was less strict for collecting the day samples (± 60 min) 25% of participants failed to collect the samples within this time frame. Delays in saliva sampling specific to the morning period were shown to impact on the measurement of the CAR, a delay of over 10 minutes resulted in a lower rise in cortisol from awakening to 30 minutes post-awakening. Additionally the same group showed that as the delay in sampling collection increased so did the rise in cortisol. Also, the authors suggested that the magnitude of the CAR influenced the diurnal cortisol profile, in that, participants that were non-adherent had a smaller slope in cortisol over the day (Kudielka et al., 2003). In the second study females suffering from fibromyalgia and matched controls (average 50 years old) collected samples for a week; in the morning (0 and 30 min post-awakening) and over the day (16:00, 19:00, 22:00 hrs or before sleep). Unaware participants were less adherent; their self-reported sampling times were consistent with the desired sampling times. Deviations in sampling times were most prevalent for the afternoon sample (16.00 h). Sampling delay over 15 minutes during the CAR period resulted in a lower rise from awakening to 45 minutes post-awakening. Deviations (± 60 min) in samples collected over the day resulted in a flatter slope (Broderick, Arnold, Kudielka, & Kirschbaum, 2004).

One study that examined the impact of non-adherence specifically on the diurnal cortisol profile over the day (8:00 -22:00) found that deviations in saliva sampling did not result in a flatter diurnal cortisol slope or differences in average cortisol levels (Jacobs et al., 2005). The difference in results may be attributed to the measurement of the diurnal cortisol profile. The latter study specifically measured the slope of cortisol over the day, whilst the two other studies included the CAR in the slope of cortisol. The CAR was flatter due to delayed sampling and this will influence the slope of cortisol. To gain a reliable estimate of the diurnal cortisol profile the CAR should be excluded (as discussed above).

2.4.3 Assessment of awakening time

The above studies highlight the inaccuracy of participant self-reported sampling times but do not identify the delay between awakening and collection of the first sample. A measure of awakening time is needed for this, and it is particularly important for assessment of the CAR. In the domestic setting, more objective measures include electroencephalography**,** polysomnography or wrist-worn actigraph. Polysomnography records biophysiological changes that occur during sleep; it is regarded as the gold standard for determining awakening time. However, the latter, actigraphy can be used more easily within the domestic setting and allows estimation of awakening time with reference to increased activity associated with awakening. Actigraphy have been validated against polysomnography (Lichstein et al., 2006) and are widely used in non-clinical and clinical studies (Lauderdale et al., 2006).

In a study that explored genetic and environmental influences on basal cortisol, a subset of the sample had a negative CAR, for these participants electronic estimates of awakening time was available (59 out of 77). In 80% of cases, electronically monitored awakening time was earlier than participant self-reports, on average 42 minutes (range 10 min – 2.15 hr) earlier, suggesting that the negative CAR was the result of delaying sampling after the electronic estimate of awakening. To directly test this, CARs were compared between those with and without discrepancies between self-reported and electronically monitored

awakening time. The negative CAR appeared to be the result of delayed sampling relative to the electronic estimate (Kupper et al., 2005).

In several studies discrepancies between self-reports and electronic estimates of awakening times have been investigated and show that discrepancies between the two measures lead to a delay in collection of the first 'awakening' sample. Dockray, Bhattacharyya, Molloy and Steptoe (2008) compared self-reported and actigraph awakening times in patients with heart problems (average 61 years old). The authors reported that discrepancies were relatively minimal; with only over five minute difference between self-reported and electronically determined awakening times in 30% of participants. On average awakening times were earlier (average 6 min) than participant self-reports. This discrepancy resulted in delays between awakening and collecting the first sample with electronic estimates detecting (average 12 min) longer delays than self-reports (average 6 min). However, in older individuals (average 74 years old), using polysomnography, Okun et al. (2010) reported little difference (which was not significant) between self-reported and electronically monitored (average 3 min difference) awakening times. However, electronically determined awakening detected 18% more cases of delays between awakening and collection of the first sample (>15 min delay).

In both of these studies delays between awakening and collection of sample 1 resulted in erroneous CAR. Dockray et al. (2008) found that participants who delayed over 15 minutes had a higher first sample and did not show a rise between 15 and 30 min, this is likely to be because cortisol in the delayed group (> 15 min) was decreasing at this point. Likewise Okun et al. (2010) found that delays (> 15 min) resulted in a higher first 'awakening' sample and a smaller CAR magnitude compared to delays less than 15 minutes, indexed by the rise between awakening and 30 min post-awakening. They also showed that as delay in collecting the first 'awakening' sample increased the rise in cortisol decreased, they report a window of less than 16 minutes acceptable for delays in the CAR period. DeSantis, Adam, Mendelsohn and Doane (2010) investigated discrepancies in awakening times in adolescents (aged 17-19 years). Self-reported awakening times were relatively accurate, the majority of

participants (75%) reported their awakening time within five minutes of the actigraph estimate. On average self-reported awakening was six minutes later than actigraph estimates. A longer delay between awakening and collection of sample one was identified with electronic estimates compared to self-reports (7 min versus 3 min respectively) and this was also the case for delay between awakening and collecting sample two (average 3 min delay). Discrepancies between awakening times resulted in collecting the saliva samples later than the desired times. Participants with discrepancies over 15 minutes between self-reported and electronically monitored awakening time had significantly lower CARs but only in participants with high neuroticism scores (DeSantis et al., 2010).

Studies investigating the impact of non-adherence to the saliva sampling protocol on diurnal cortisol patterns have either monitored awakening times or sampling times electronically, but not both, and thus rely on demonstrably unreliable participant self-reports for the alternative variable. To obtain a full estimate of non-adherence to the saliva sampling protocol estimate of collecting samples at the desired interval relative to awakening is not measurable by assessment of sampling or awakening times alone. Thus, a combination of both awakening and sampling times are necessary in order to gain the most accurate estimate of sampling times relative to awakening. Recently, a study monitored both awakening and sampling times electronically in a sample of young adults (average 27 years old) and middle-aged adults (average 59 years old) that completed sampling in a sleep laboratory and also in a student sample (average 25 years old) that completed sampling in their domestic setting (Griefahn & Robens, 2011). Delays over 15 minutes resulted in a smaller CAR magnitude. To determine if delays between 10 and 15 minutes were acceptable, the average CAR was plotted for each successive minute by which collection of sample one was delayed, delays under about 11.5 minutes were acceptable for measurement of the CAR.

This non-adherence to the saliva sampling protocol raises concerns about the extent to which previous research has been in detecting reliable and valid relationships between the diurnal pattern of cortisol and psychosocial or health

variables. Since researchers typically rely on self-reports it is very likely that samples considered non-adherent have been included in analyses, which in turn surely significantly reduces the ability to reveal existing relationship. In this way, true relationships may not have been detected or incorrect relationships have been accepted.

Strategies to increase adherence to the saliva sampling protocol are limited. Halpern et al. (2012) used incentives as a method of encouraging participants to collect saliva samples on time, however, providing incentives in the form of money failed to promote participant adherence to the post-awakening saliva sampling period. Informing participants of electronic monitoring of their adherence to the protocol seems to be the most effective strategy in increasing their adherence to the protocol and accuracy in self-reports (Broderick et al., 2004; Kudielka & Kirschbaum, 2003).

The demands of the protocol may increase participant non-adherence. For example delays in collecting saliva samples increased with the number of study days, but no differences were observed between week and weekend days (Broderick et al., 2004). There is some evidence that awakening time influences the delay between awakening and collection of the first 'awakening' sample. In middle-aged patients, delays between awakening and collection of the first sample was less in participants that awoke later, this may be explained by early risers being drowsier and thus less prompt in carrying out the sampling procedure (Dockray et al., 2008). Consistent with this finding, in healthy adolescents poor assessment of awakening time was associated with subsequent sampling delay of the first 'awakening' sample (DeSantis et al., 2010).

Non-adherence does not appear to be due to participant factors, several studies have investigated the relationship between participant characteristics and non-adherence to the saliva sampling protocol. Delays in saliva sampling collection were not associated with age, gender or smoking status (Kudielka & Kirschbaum, 2003). Health status did not influence adherence to the saliva sampling protocol, non-adherence did not differ between women suffering from

fibromyalgia and matched healthy controls (Broderick et al., 2004). Furthermore adherence to the sampling protocol was not associated with sociodemographic characteristics (Halpern et al., 2012), similarly no relationship was found between inaccuracies in self-reported sampling times and demographic variables (Hall et al., 2011). However a relationship between ethnicity and sampling delay was evident with African-Americans being more likely to delay sampling (Hall et al., 2011). Psychosocial variables such as sleep quality, depression and perceived stress does not influence non-adherence (Okun et al., 2010).

2.5 Hair cortisol

Given the limitation of non-adherence to the saliva sampling protocol, the measurement of a valid and easily obtainable measure of long-term cortisol secretion would constitute an important advancement to the field. Table 2.3 provides a summary of the strengths and considerations of measurement of cortisol in hair samples. Over the last decade or so a new method of measuring cortisol in hair has been developed. The key aspect of this measure is that hair grows on average 1cm per month, (Wennig, 2000), and is incorporated into the hair shaft (see Figure 2.3) equating to one months cortisol secretion growth rate of 1cm over one month.

Several studies have demonstrated the validity of hair cortisol concentrations (HCC) as a marker of chronic cortisol production in humans. Studies have demonstrated abnormal HCC in conditions known to be associated with increases and decreases in HPA activity. Higher HCC was evident in conditions characterized with hyper-cortislism, for example, in pregnancy during the third trimester (D'Anna-Hernandez, Ross, Natvig, & Laudenslager, 2011; Kirschbaum, Tietze, Skoluda, & Dettenborn, 2009), endurance athletes (Skoluda, Dettenborn, Stalder, & Kirschbaum), alcoholics (Stalder, Kirschbaum, et al., 2010) and in patients with Cushing syndrome (Thomson et al., 2010). Higher HCC has also been demonstrated in other stress-related conditions in humans, for instance, in infants exposed to stressful hospital procedures

(Yamada et al., 2007), chronic pain patients (Van Uum et al., 2008) and unemployed individuals (Dettenborn, Tietze, Bruckner, & Kirschbaum, 2010).

A multi-compartment model has been proposed in which there are general models of incorporation of drugs or hormones into hair (Henderson, 1993). Figure 2.3 provides an overview of these models (for a review, see Pragst & Balikova, 2006). Cortisol incorporation through passive (or active) diffusion from blood into growing cells in the hair follicle is thought to be the primary mechanism (Cone, 1996). An alternative incorporation pathway is through sweat or sebum secretion after formation of the hair shaft. Incorporation of cortisol via these mechanisms reflects the "free" or unbound fraction of the steroid based on the free hormone hypothesis (Mendel, 1989). Cortisol could be incorporated into hair through external contamination after formation of the hair shaft, for example, use of cortisol-containing creams passed onto the hair from hands when individuals touch his or her head (Thomson et al., 2010). It may be possible that there are several pathways in which substances are incorporated into hair. However, it is also possible that there are different incorporation pathways for different substances (e.g. drugs or hormones), which suggests that movement and stability may vary by the type of drug or hormone under investigation.

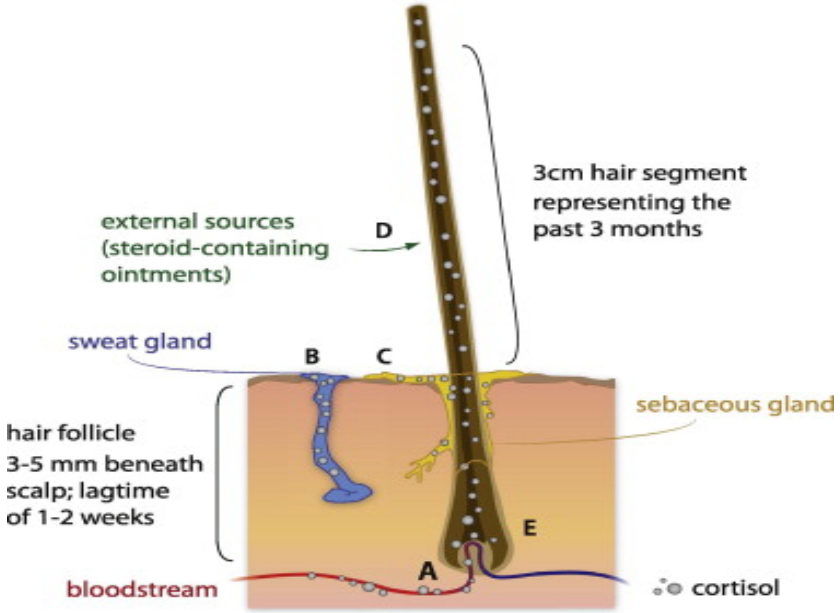

Figure 2.3 Assumed mechanisms of cortisol incorporation into hair and retrospective reflection of long-term cortisol secretion.

Cortisol may be incorporated into hair via passive diffusion from blood (A), sweat (B) and/or sebum (C) as well as from external sources (D). In addition, it has been proposed that locally produced cortisol may also contribute to hair cortisol concentrations. Taken from (Stalder & Kirschbaum, 2012).

The advantage of this method over other methods (e.g. saliva, blood and urine) is the unique feature of assessment of cortisol secretion over several months. It provides a retrospective image of cortisol secretion, providing a window into the past, which will develop research on the effects of chronic exposure over longer periods of time and after an event. A good example of the advantage of hair cortisol is the measurement of cortisol secretion over several months in patients after suffering from a heart attack. HCC was significantly higher in patients who had suffered from an myocardial infarction compared with control patients (Pereg et al., 2011).

The time period at which HCC can be reliably measured is currently debated. Some studies have reported a 'wash-out' effect, in which, HCC declines from proximal to more distal hair segments (e.g. Dettenborn, Muhtz, et al., 2012; Dettenborn et al., 2010; Kirschbaum et al., 2009; Stalder, Steudte, Alexander,

et al., 2012) while other did not find this. Similarly, evidence regarding the associations between HCC to retrospectively reflect cortisol secretion over longer time periods is questioned. Some have found that cortisol secretion closely resembled clinical conditions for more than a year (Manenschijn, van Kruysbergen, de Jong, Koper, & van Rossum, 2011; Thomson et al., 2010). Others however, have failed to find such associations, for example, HCC for detecting hyper-cortislism in the third trimester of pregnancy was only accurately reflected in hair sampled at three months but not six and nine months (Kirschbaum et al., 2009).

Studies have demonstrated that HCC is a trait measure, with high intra-individual stability in the absence of major life events (Stalder, Evans, Hucklebridge, & Clow, 2011). Also due to the retrospective nature of hair sampling and extended detection period it is relatively robust to a range of potential confounding influences, such as, natural hair colour, hair washing or dying, taking oral contraceptives, smoking status or general medication intake (Dettenborn, Tietze, Kirschbaum, & Stalder, 2012; Karlén, Ludvigsson, Frostell, Theodorsson, & Faresjö, 2011; Kirschbaum et al., 2009).

The anatomy of growing hair is rather complex (for a review, see Alonso & Fuchs, 2006; Krause & Foitzik, 2006). Hair growth originates from follicles located beneath the surface of the skin. Hair follicles grow in repeated cycles composed of three phases, including anagen (active growth), catagen (transition or regression), and telogen (resting). Hair follicles pass through each phase independently from neighbouring hair follicles. A hair follicle is in the anagen phase for the longest time, on average between 2-6 years, the duration of which determines the hair length. At any time, the majority of the scalp hairs (approx. 85-90%) are within anagen follicles. The catagen phase signals the end of the growth period and the hair follicle is prepared for the resting period. This phase lasts for a few weeks, during which time the hair follicle converts to club hair (i.e. lower end of hair follicle attaches to the hair shaft). During the resting phase, hair growth completely terminates, the hair follicle stays attached to the hair shaft until it sheds. The cycle starts again and the new hair pushes out any dead hair left. Although it is generally accepted that hair grows on

average 1-cm per month, this is an oversimplification and various factors account for the variation in hair growth. For example, growth rate is slower in males (anagen phase in women is longer than men) and older individuals (see Harkey, 1993), and hair growth is faster in Asian hair compared to African and Caucasian hair with African being the slowest (Loussouarn, El Rawadi, & Genain, 2005).

Some studies have shown that age and gender were not associated with HCC (Karlén et al., 2011; Kirschbaum et al., 2009). However, more recently, HCC was higher in younger children and in older adults, also HCC was higher in men compared to women (Dettenborn, Tietze, et al., 2012). This was the first large scale study that systematically investigated the possible confounding factors on HCC, while in previous studies these factors were investigated as secondary aims and samples were limited by small age range. Another potential influential factor is body fat, studies have demonstrated positive associations between weight and HCC (Manenschijn, Koper, Lamberts, & van Rossum, 2011; Manenschijn, van Kruysbergen, et al., 2011; Stalder, Steudte, Alexander, et al., 2012) but findings are mixed (Manenschijn, Koper, et al., 2011). Stage of hair growth may increase variability; cortisol is not incorporated into the hair shaft during the resting phase. Based on the research to date the potential factors that need to be considered in HCC studies are age, sex and BMI. It is usually that these factors are controlled for by including a small age range or excluding overweight or obese individuals. It is also important to consider the amount of times hair is washed (but only for more distal hair segments for the wash-out effect). Table 2.3 provides a summary of the strengths and considerations of measurement of cortisol in hair samples.

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Table 2.3 Strengths and considerations of measurement of cortisol in hair samples

Strengths	Considerations
Collection	
Hair sample is non-invasive Only a small amount of hair (e.g. 10mg) is required, no visible mark is left, and it is well tolerated by participants	Difficulty getting close to the scalp, especially with short hair (i.e. males). Cannot access all populations (i.e. balding, alopecia)
Storage	
Hair does not decompose like other body fluids/tissues (Balíková, 2005) and is stable for yrs at room temperature - cortisol determined in ancient hair samples (e.g. mummies) (Webb et al., 2010)	Samples should be wrapped in aluminum foil to maintain integrity and to avoid contamination and stored at room temperature
Measure of cortisol secretion	
Retrospective calendar of cortisol secretion – provides a window to the past HCC is not affected by situational characteristics e.g. no effect of smoking status, medication intake/taking oral contraceptives on HCC (Dettenborn, Tietze, et al., 2012).	Retrospective account of cortisol only valid for up to 6 months due to the 'wash out effect' (Dettenborn et al., 2010; Kirschbaum et al., 2010) Hair samples do not reflect the immediate or recent exposure of cortisol secretion – the hair follicle is embedded approximately 3-5 mm beneath the scalp No measure of acute changes in stress/diurnal pattern of cortisol
Situational or confounding factors	
Hair characteristics or treatment do not influence HCC (Karlén et al., 2011; Kirschbaum et al., 2009). No problems of non-adherence - samples are collected by the researcher	Possible confounding factors: age, gender, hair growth rate, body fat - record details and control in analyses if necessary
Determination of cortisol	
Easily sent in the post to laboratory for analysis	Still need to determine the most effective cortisol extraction method (methods of cortisol extraction differ between studies)

2.6 Aims and thesis summary

The overarching aim of this programme of research was to explore associations between cortisol secretion and well-being, given that well-being may have an effect on future health by directly influencing cortisol secretion. However, exploring these relationships under strict monitoring of adherence to protocol is crucial due to the impact of sampling delays on measurement of salivary cortisol. An overview of the studies and how they interlink is provided in Figure 2.4 and a brief outline of the aims of each study is given below. A detailed rationale for each study is provided in experimental chapters.

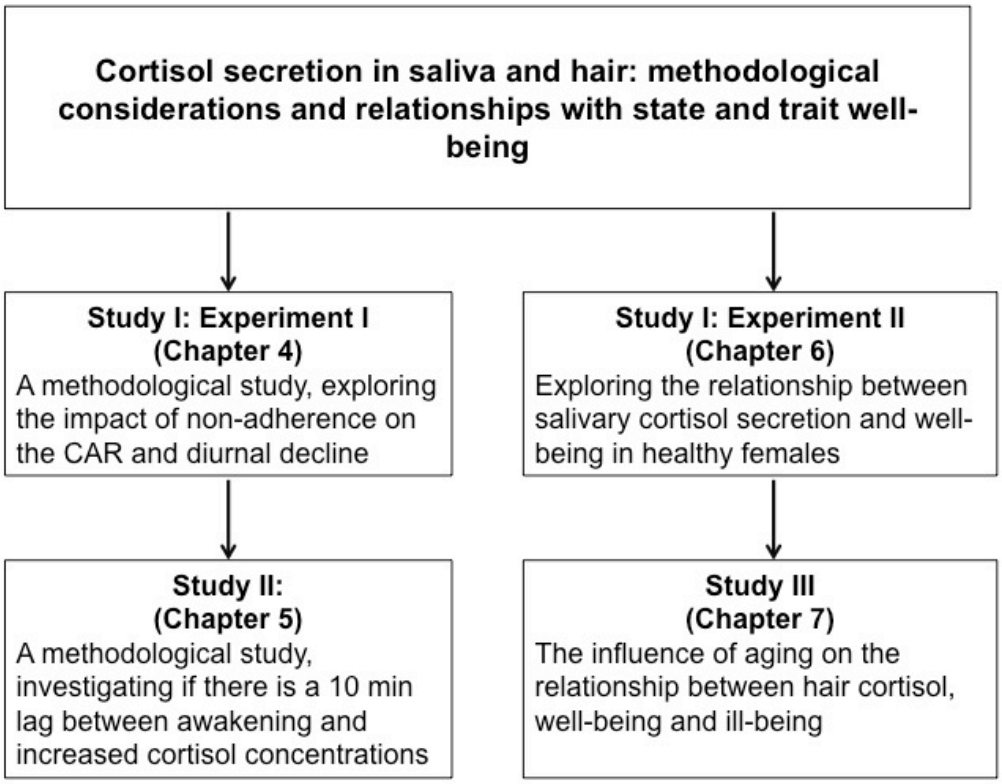

Figure 2.4 Flow diagram of the programme of research

Study I:

The main aim of this study was to explore associations between the diurnal pattern of cortisol and subjective and psychological well-being independent of ill-being in healthy young individuals in the domestic setting. Previous research demonstrates associations between lower cortisol patterns (e.g. lower cortisol in the post-awakening period and across the day) and higher levels of well-being independent of ill-being, particularly in middle-aged and older adults. However, no study to date has investigated this specifically in a younger sample. Non-adherence to the saliva sampling protocol is a key issue in this type of research. It can lead to misleading CAR estimates, which may explain the inconsistencies in the literature regarding the relationship between the CAR and well-being/ill-being. Studies have generally relied on self-report methods of monitoring adherence to the saliva sampling protocol. Some studies have used electronic estimates of awakening or saliva sampling times, but only one has utilised both. However, no study has accounted for non-adherence in the relationship between cortisol patterns and well-being using both track caps and actigraph. In this study participant adherence to protocol using both electronic estimates was monitored to examine the impact of non-adherence on cortisol patterns and to account for non-adherence in the relationship between cortisol patterns and well-being.

Study II:

The findings of Study I that moderate delay in saliva sampling lead to misleading CAR estimates provided the impetus for Study II. The aim of this study was to investigate salivary cortisol secretion within the immediate post-awakening period. No study to date has measured salivary cortisol using repeated sampling (every 5 min) within the first 30 minutes following awakening.

Study III:

The aim of this study was to explore the relationship between hair cortisol and well-being/ill-being in young and older individuals. Measurement of cortisol in hair provides an alternative method of examining relationships with well-

being/ill-being. Although salivary cortisol enables examination of the diurnal pattern of cortisol, it is limited by participant non-adherence to protocol, this is costly and time consuming to measure objectively using electronic estimates. Hair cortisol however, provides a retrospective trait measure of cortisol secretion. This enabled studying associations between cortisol and well-being/ill-being in a larger sample and comparison of young and old females without the issue of participant non-adherence

In summary the overall aims of this research were:

(1) To inform best practice methodology for the ever-increasing number of studies in this area of research

- To monitor and examine the impact of non-adherence to the saliva sampling protocol in ambulatory studies.
- Investigate the impact of moderate sampling delays on the CAR.

(2) To expand significantly the collection of published data on the association between cortisol patterns and well-being in healthy participants using well-established methodology and strict monitoring of participant adherence to protocol.

- Explore the relationship between diurnal patterns of salivary cortisol and well-being independent of ill-being in young healthy females.
- Explore the relationship between hair cortisol and well-being independent of ill-being in healthy young and older females.

Chapter 3 General Methods

3.1 Ethical Considerations

The research presented in this thesis was carried out in line with the ethical guidelines for research with human participants set out by the British Psychological Society. The guidelines set out by University of Westminster were also consulted. Ethical approval for the studies presented in this thesis was obtained from the University of Westminster. Ethical implications and the psychological consequences were considered for all aspects of the research studies. Participants read the information sheet and provided informed consent (Appendix 1-4) and asked any necessary questions. Every effort was made to create a relationship with mutual respect and confidence between the researcher and participant. All participation was voluntary and participants were encouraged to participate only if they felt comfortable to meet the requests of the study protocol. The nature of the three studies did not require deception or withholding of information, therefore, participants were fully informed of the study aims, rationale and procedure. All reasonable steps were taken to ensure that participants understood the nature of the study; participants received both verbal and written information detailing the study and were given the opportunity to ask any questions. All participants were provided with contact details of the researcher, to use if they wished to ask questions or express any concerns or issues during or following participation.

All participants were healthy adults, with no impairments either in communication or understanding, enabling them to provide informed consent. Although the participants were sometimes students of the researcher, who was therefore in a position of potential influence, participants were not pressurised to participate or remain in the study. To make participants feel at ease collecting saliva and providing hair samples they were given the opportunity to practice collecting a saliva sample or were either shown a picture of the hair sample

process or felt the amount of hair that would be cut.

Participation in the studies did not increase participants' exposure to risk or harm greater than or additional to those encountered in their normal daily routines. Some participants reported discomfort with the saliva sampling protocol. For example, some participants found the cotton swab (used for saliva sampling) distasteful. However, participants were given a practice session to ensure they were comfortable collecting saliva samples. Emotional discomfort could have potentially risen from completing the psychosocial measures pertaining to mental health. However, participants were assured that they were under no obligation to disclose information that they did not wish.

In all cases participants were made aware of their right to withdraw participation at any time, including withdrawing their data prior to publication, without explanation. Participants were made aware that if they agreed to be contacted about future research they were under no obligation to participate in it. Participants were made aware that the information they provided would be confidential. Data used for publication would be aggregated and no individual would be identifiable. The researcher was responsible for the confidentiality of the information acquired. All steps were taken in order to prevent the loss of data-security, participant names were separately coded rendering written and computerised records identifiable by only the researcher. All hard-copy data generated from the research was stored in locked cabinets in secure offices in the Regent Street campus of the University of Westminster. Consent forms and any other potentially identifiable information were kept in separate and secure cabinets. Electronic data was stored in files on password-protected computers accessible only to the researcher. Anonymous computer data files were viewable only by the supervisory team, all of whom were aware of confidentiality issues. Participants consented to saliva and hair samples being tested for the hormone cortisol. Hair samples were stored in secure cabinets in Regent Street campus and saliva samples were stored in the PSRG laboratory in the New Cavendish campus of the University of Westminster. Storage and use of saliva samples were in line with the Human Tissue Act (HTA; 2004); samples stored were recorded on the HTA tracking form. Both hair and cortisol samples were

labeled with participant code, and destroyed following analysis in line with the HTA.

Responses to the psychosocial data were completed via online questionnaires. The online questionnaires were set up by systems designed to create questionnaires, these included Mircrosoft Office Frontpage and Qualtrics and were provided by the University of Westminster. Participants accessed the questionnaires via a link, and were firstly presented with information sheets and were advised to save or print a copy for their records. Before viewing the questionnaire they were required to provide consent; the system did not allow them to go to the next stage until this was provided. Participants could only see the questionnaire items and select their responses, and were not able to see their responses once submitted. To ensure that participants completed all questions and sections of the questionnaire, participants could not submit their responses until each item was assigned a response. For each question there was an option to select 'I do not wish to answer' or 'not applicable' to enable participants to leave a question if they not wish to answer it.

On completion of each study, participants were given the opportunity to discuss with the researcher their experiences of partaking in the study, during which participants were encouraged to give their feedback regarding their experience of partaking in the research. This allowed the researcher to monitor any unforeseen negative outcomes or misconceptions. Participants were informed that they could contact the investigator via email if they wished to be informed of the research findings.

3.2 Participants and Researcher

The programme of research was conducted in the UK, with a process of on-going study design and recruitment from October 2010 to March 2013. The author of the thesis (NS: white female aged 24-26) completed all aspects of each study including the study design, data collection, analysis of biological samples (apart from hair), and statistical analysis. An MSc student completing

an internship with the PSRG at the University of Westminster (MB: white, female aged 26) assisted with data collection in study III.

For the salivary cortisol studies pregnant females were excluded since changes in the HPA-axis take place during pregnancy, specifically, higher cortisol levels during the third trimester of pregnancy (Obel et al., 2005). Participants were recruited on the basis that they were not taking prescribed medications and had not suffered from serious medical or psychiatric illnesses in the last two years. Participants providing hair samples were excluded if they were suffering from any condition of adrenocortical dysfunction or currently taking corticosteroid medication during the year before the study.

Student volunteers were drawn from the psychology department at the University of Westminster via the research participation scheme (RPS). This scheme is organised by the psychology department with the aim of engaging undergraduate psychology students in research, either as a participant or as an assistant for a period of three hours in their first year of study. Students were under no obligation to participate in any research; alternatively they could write a short essay detailing the ethical considerations for conducting research. Recruitment of student participants for studies I and III were conducted in two stages. The first stage involved completion of an online questionnaire, adverts were sent to students via email or the RPS website as well as leaflets posted around the psychology department (Appendix 5-6). Interested students were instructed to follow a link to access the questionnaire, slips with the web link were provided for student's convenience. Wider recruitment was undertaken through word of mouth, as well as leaflets and articles posted in the psychology department and the university's newspaper (Appendix 7). Recruitment for participating in the second phases (e.g. saliva or hair sampling) was through invitation via email.

Researchers within the psychology department and friends of the researcher familiar to the saliva sampling protocol volunteered to partake in study II. They did not receive any financial incentives for their participation. For study III, older female volunteers were recruited from among the University of the Third Age

and Women's Institute. Personal letters to individuals from University of the Third Age (of whom previously participated in research conducted by the PRSG) were sent inviting them to take part in a new study (Appendix 8-9). Leaflets advertising the study were also sent to the University of the Third Age and Women's Institute. They were offered a small momentary token of a £10 high street voucher for expenses.

3.3 Salivary Cortisol

3.3.1 Saliva Sampling Protocol

Salivary cortisol enables repeated sampling within participants' domestic setting and measurement of the diurnal pattern of cortisol secretion. Measurement of the CAR and diurnal decline requires repeated sampling in the post-awakening period and across the day (respectively). Determination of the CAR typically requires repeated sampling at regular intervals during the post-awakening period as cortisol levels change rapidly during this period. In study I sampling included the following time points 0, 15, 30 and 45 min post-awakening (samples 1-4), which are typically used to capture the rise in cortisol (i.e. the CAR magnitude). The diurnal cortisol profile was limited to two samples, collected at 3 and 12 hours post-awakening (samples 5-6), representing the change in cortisol from 3 to 12 hours post-awakening. In study II, to capture the cortisol activity within the immediate post-awakening period, saliva samples were collected every 5 min for the first 30 min post-awakening (samples 1-7).

Participants were instructed to awake in their usual way since mode of awakening does not influence the CAR (Pruessner et al., 1997; Wuest et al., 2000). The CAR and diurnal decline were measured on four study days to avoid distortion of data by state variables. Study days included, two weekdays and two weekend days. In study II, the saliva sampling protocol was quite intensive, to reduce the demand on participants, saliva sampling was limited to two weekdays.

Participants were asked to take nil-by-mouth (except water) and no smoking or brushing teeth during the CAR collection period and also for at least 30 min prior to saliva sampling over the reminder of the day to avoid HPA axis stimulation. For example, smoking and caffeine have been associated with increased cortisol levels (Badrick et al., 2007; Lovallo et al., 2005). For up to seven days, cortisol remains stable at room temperature (and for nine months at -20 degrees centigrade, Aardal & Holm, 1995). Participants were, however, requested to store their samples in their home freezer or fridge as soon as possible after collection of saliva. The use of salivettes (see below) allowed for hygienic storage of the samples in participants domestic freezer/fridge. Participants were provided with insulated bags in which to return their samples to the investigator upon which they were stored at -20°C in a laboratory freezer at the University of Westminster until they were assayed.

Saliva samples were collected using the salivette sampling device, which is an optimal method for obtaining full saliva samples, manufactured and sold by Sarstedt© (Sarstedt Ltd, Leicester, England). Figure 3.1 displays a diagram of a salivette adapted from the Sarstedt website (www.sarstedt.com). The device contains a cotton swab placed within a suspended insert in a capped plastic tube. Participants remove the swab from the suspended inserted tube and place it into their mouth and gently chew to stimulate saliva flow for a period of one to two minutes. It usually takes approximately one minute to obtain up to 0.5-1 ml of saliva. Once saturated with saliva the swab is placed back in the suspended insert of the tube and firmly capped. Saliva is released from the cotton swab by centrifuging the salivette and clear saliva is released into the centrifuge vessel via a hole in the suspended insert.

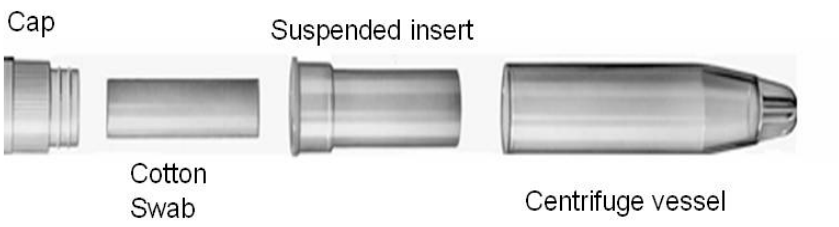

Figure 3.1 The salivette saliva sampling device (Sarstedt Ltd)

Participants were encouraged to practice collecting a saliva sample using the salivette device to ensure that they were comfortable collecting samples. Participants were provided with a salivette for each sampling point per sampling day (with the cotton swabs removed, see below). Salivettes were divided into ziploc bags for each sampling day and were labelled with participant code, sample tube, sampling day and sampling time. The ziploc bags were labelled with participant code and sampling day. To minimize collection mistakes the labels were colour-coded to symbolise the different collection times and days. As well as verbal instructions, detailed written instructions were provided regarding the sampling protocol and the correct use of the salivettes.

3.3.2 Adherence to the saliva sampling protocol in the domestic setting

Assessment of diurnal cortisol patterns in the domestic setting means that participants are left to their own devices to adhere to the protocol. As mentioned in chapter 2, one of the main aims of this thesis was to take every possible measure to maximise participant adherence to the protocol and to put measures in place to control for potential violations. Non-adherence to the protocol may arise from little or misunderstanding information about the research protocol and/or intentional non-adherence due to little motivation of the participant or underestimating the accuracy needed. Some incidences of non-adherence can be prevented through engaging the participant in the research protocol.

Participants attended one-to-one research sessions with the researcher (NS) with the aim of providing clear instructions about every aspect of the protocol, and engaging participants in the protocol. During the research session participants were briefed comprehensively; the whole procedure was explained in detail, using graphical illustrations of the protocol. They received a study pack, which included all the necessary materials for saliva sampling and the electronic equipment used for monitoring awakening and sampling times.

To aid participants in carrying out the protocol in their domestic settings they were provided with study guidelines detailing every aspect of the study protocol (Appendix 10) and were also given the researchers contact information to use if they encountered any problems or concerns during the study. Finally, participants were asked to relay the study protocol back to the researcher to ensure that they understood each aspect of the study. It was emphasized that they should only participate if they felt confident and happy to adhere to the protocol. To ensure that participants returned study materials participants could return them at times and places convenient for them.

3.3.3 Monitoring adherence to the saliva sampling protocol

3.3.3.1 Self-report measures

Participants provided self-reported awakening and sampling times on each study day using diaries (Appendix 11-12). After awakening and collection of sample 1, participants calculated their sampling times for the day relative to their awakening time. They were also asked to record the time of actual collection of each saliva sample in order to identify any deviations from the desired sampling time. During the one-to-one research session, participants were asked to practice calculating their salvia sampling collection times to ensure they understood how the sampling points related to their awakening times.

For each sample, they were also asked to record any problems with sampling (e.g. failed to remain nil-by-mouth, smoking or brushing their teeth in the 30 min prior to saliva sampling). In study I, participants also rated their sleep quality on a five-point Likert scale, ranging from much better than usual to much worse than usual, how they awoke (e.g. alarm clock, somebody they asked to wake them, noises or spontaneously) and their ease of awakening on a five point Likert scale, ranging from very difficult to very easy on mornings of each study day.

3.3.3.2 Objective proxy of awakening and saliva sampling times

It was argued in chapter 2 that participants' self-reported awakening and saliva sampling times are inaccurate, comparison with electronic estimates show that awakening times are typically earlier and sampling times are typically later than self-reports. Electronic estimates of awakening and sampling times provide a proxy of an objective measure, and together they provide real-saliva sampling times (i.e. sample collation time relative to awakening time).

Sampling on awakening is crucial for accurate estimates of the CAR; delay in sampling on awakening will also delay subsequent sample points. To assess awakening time, participants wore an activity device on their wrist (see Figure 3.2) the night prior to each study day. The actiwatch-score device (Actiwatch-Score, Cambridge Neurotechnology, Cambridge, UK) is a piezoelectric motion sensor recording physical activity.

Figure 3.2 The actiwatch-score device

Awakening times were estimated using the actigraph software; it distinguished sleep and awakening periods by reduced and increased activity respectively. Figure 3.3 displays an example recording of awakening time from the actiwatch-score software. Actigraphy has been validated against the gold standard polysomnography (Lichstein et al., 2006) and is widely used in non-clinical and clinical studies (Lauderdale et al., 2006).

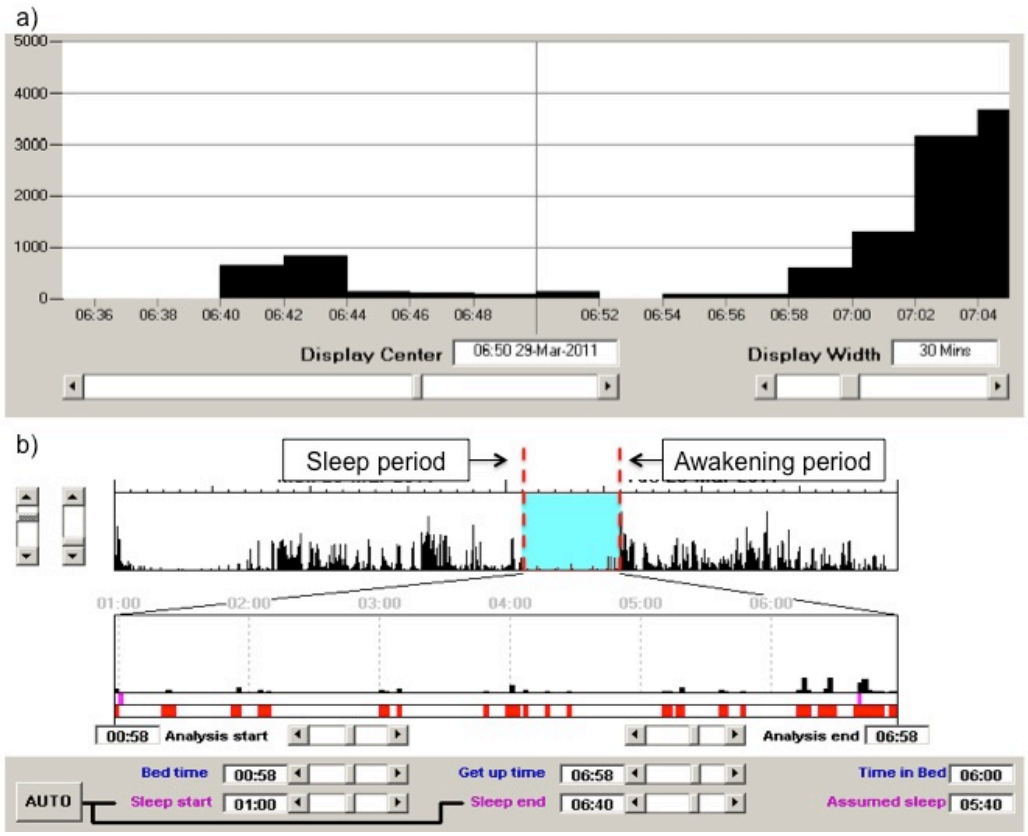

Figure 3.3 Actigraph analysis software
(a) Actogram showing the awakening period (b) actigraph software used to determine the point of awakening

Sampling times were determined by track caps (e.g. Medication Event Monitoring: MEM Caps), the cotton swabs normally in the salivettes and used for saliva sampling were stored in the track caps (Figure 3.4) — participants were instructed to open this device only at sampling times. Following saliva collection, swabs were returned to the correctly labelled salivette for storage. Opening of each cap recorded the date and time and these timings were used as a proxy measure of participants' saliva sampling times.

Figure 3.4 Track cap device: Medication event monitoring (MEM) cap

Both electronic devices were used to electronically determine actual saliva sampling collection times and determine non-adherent delays to sampling. Desired saliva sampling times of each sampling point were calculated for each participant based on his or her actigraph estimated awakening time (i.e. 0, 15, 30 and 45 min, and 3, 12 hr post-awakening). Actual sampling time was determined by subtraction of MEMs-determined sampling time from the desired sampling time.

Participants were made aware of the importance of their adherence to the protocol in particular collecting samples at the desired times, and they were informed that the electronic devices would verify their awakening and sampling times. This has previously been shown to increase adherence to the saliva sampling protocol (Broderick et al., 2004; Kudielka et al., 2003).

3.3.3.3 Short Messaging Service (SMS) Protocol

Practical advice was provided to participants on preparing for their upcoming study days (e.g. prepare and place study materials next to their bed the night prior to their study day to prevent delays in the mornings due to having to search for the materials). As well as this reminders were used to ensure that participants remembered to carry out certain tasks.

Text messaging is a useful and welcomed method of communicating with participants. It was crucial that participants remembered to wear the actiwatch-score to bed the night prior to each study day, and during each sampling day. Repeated saliva sampling across the day can be difficult for participants to remember to collect the samples. Text messages were used to remind participants to wear the actiwatch-score to bed and during the day, prepare study materials the evenings prior to each study day and to collect the day saliva samples (i.e. sample 5 & 6). SMS-messages were easily and securely sent using a cost-effective automated text messaging service (TextAnywhere). Participants had access to a mobile phone and this was exploited in order to remind participants about upcoming study days. Participants were sent a text message the evening prior to each study day reminding them to wear the actiwatch-score to bed and to place sampling packs next to their bed. Text messages were used to remind participants to complete the morning diary. Participants were reminded to collect the 3 and 12 hr post-awakening samples by text messages. Use of text messages are a cost-effective method of increasing adherence to a range of health behaviours and completion of diary entries (e.g. Anhoj & Moldrup, 2004), and have also been used in salivary cortisol studies to prompt participants to collect saliva samples (Oskis et al., 2009).

3.3.4 Determination of cortisol in saliva samples

Saliva samples were assayed by the author (NS) in the PSRG laboratory at the University of Westminster. After saliva samples were thawed, clear saliva was released from the cotton swabs into the centrifuge vessel through centrifuging samples at 3500 rpm for 10 min. Samples were assayed using the Cortisol Enzyme Linked Immuno-Sorbent Assay (ELISA) developed by Salimetrics LLC (USA). All of the Salimetrics assay kits are commercially available. This assay exploits immunological processes in the measurement of cortisol and is designed specifically to quantify the measurement of salivary cortisol. The assay is highly sensitive to low values of cortisol, with detection of cortisol as low as 0.16 nmol/l. Salimetrics report that the salivary cortisol assay correlates highly with those from a serum cortisol assay (r = 0.91, p < 0.0001, n = 47

samples, see www.salimetrics.com). The amount of saliva required for the assay is just 25μl of saliva per test.

The assay kit contains a microtitre plate with 96 wells coated with monoclonal antibodies to cortisol; cortisol standards, which represent known cortisol concentrations (values in nmol/l: 82.77, 27.59, 9.19, 3.06, 1.02, 0.33). The standards and unknowns (saliva samples from participants) were pipetted into the wells in the microtitre plate, and an enzyme conjugate (cortisol labelled with horseradish peroxidase) was added. The test principle is that cortisol in standards and unknowns compete with the enzyme conjugate for antibody binding sites in the wells on the microtitre plate. Following incubation for one hour unbound components are washed away using a phosphate buffered solution containing detergents. Bound cortisol peroxidase is measured by reaction with tetramethylbenzidine (TMB) solution, which produces a blue colour following 30 min incubation in the dark. The reaction is terminated by added sulphuric acid, which is a stop solution producing a change in the colour (blue to yellow). Within 10 min of adding the stop solution the optical density was read on plate reader with a 450nm filter. The amount of cortisol peroxidase present is inversely proportional to the amount of cortisol present, which visually follows that the more yellow the solution in each well, the less cortisol.

The researcher carefully followed the procedure provided by Salimetrics for all the salivary cortisol assays performed. In addition to this samples from one participant were assayed on the same day and each sample was assayed in duplicate. On the rare occasion that the percentage variation between the duplicate samples was greater than 10% the sample was re-assayed on another plate. In this way intra-assay variation was less than 10% for all studies. Other known concentrations of cortisol, the high and low controls, (27.6 nmol/l and 2.76 nmol/l, respectively) were treated like unknowns and used to determine inter-assay variability, which was below 10% for all studies. On average both intra and inter assay coefficients of variation were comfortably below 10%.

3.4 Hair Cortisol

3.4.1 Hair sampling protocol

Hair samples were cut with clean fine scissors as close as possible to the scalp from the vertex posterior of the head. This area has the least variation (e.g. 16%) as compared to other areas (e.g. 31%) (Sauve, Koren, Walsh, Tokmakejian, & Van Uum, 2007). Further, it has the largest percentage of follicles in the anagen phase (85%) and the fastest growth rate of up to 1.12 mm per day (Harkey, 1993). The hair follicle is not included in the analysis as they have previously been shown to produce cortisol responses to corticotropin-releasing hormone stimulation (Ito et al., 2005). Before cutting the hair participants were given the opportunity to feel or look at the amount of hair to be cut. The number of hair strands obtained varied in accordance with participant's permission to cut more or less hair. Hair samples were wrapped in aluminium foil and stored in a dry and dark place. Figure 3.5 shows the process of collecting hair samples. It is generally accepted that hair grows on average 1cm per month (Wennig, 2000), therefore the most proximal 1cm of hair represents the previous month's cortisol exposure. Based on this, a 3cm segment of hair nearest to the scalp was examined. This sample was assumed to represent the cumulative cortisol secretion over the previous three months.

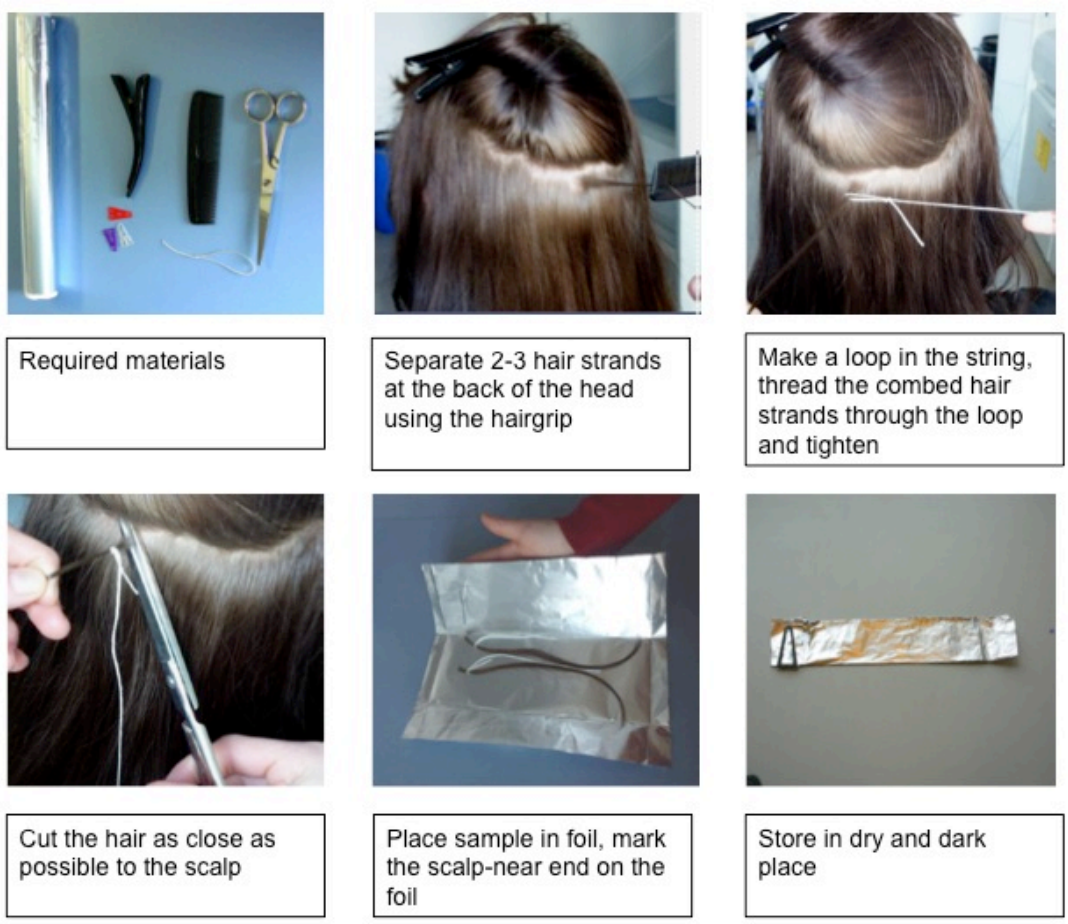

Figure 3.5 Hair sampling process

3.4.2 Determination of cortisol in hair

In the laboratory, hair strands were lined up and the first 3 cm scalp near hair strands were cut. Samples were analysed at the Technical University in Dresden. The wash procedure followed the laboratory protocol in (Kirschbaum et al., 2009). The hair segment was washed with 2.5 ml of isopropanol and gently mixed on an overhead rotator for 3 min. The wash cycle was repeated twice and samples were left to dry for at least 12 hr. The extraction of cortisol followed the laboratory protocol in (Stalder, Steudte, Miller, et al., 2012). After weighing, 10mg of whole, non-pulverised hair were transferred into 2 ml cryo vial (Eppendorf, Hamburg, Germany) 1.800 ml of pure methanol was added and the virals were slowly rotated over 18h at 45° C for steroid extraction. Samples were spun in a microcentrifuge (at 10.000 rpm) for 2 min. Next, 1 ml of the

clear supernatant were transferred into a new 2 ml cryo vial and the alcohol was left to evaporate at 60°C under a constant stream of nitrogen until the sample is completely dry (takes approximately 20 min). Finally, samples were reconstituted; 0.4 of phosphate buffer was added (CAL A, IBL-Hamburg, Germany) and to mix the viral it was vortexed for 15 sec. For cortisol determination, 20 microliters from the vials were removed and assayed with a commercially available immunoassay with chemiluminescence detection (CLIA, IBL-Hamburg, Germany).

3.5 Ecological momentary assessment method

In line with the Ecological Momentary Assessment (EMA) method (Stone & Shiffman, 1994) positive and negative mood was rated across the day. The EMA method enables assessment of mood in participants' natural setting and during their normal routines enabling assessment in changes in mood within and between days.

Between 10.00 and 21.00 hr participants rated their mood at five random points. They rated the extent to which they were experiencing positive and negative mood states. On a five point Likert scale, ranging from (1) 'not at all' to (5) 'very much'. Participants rated the extent to which they felt happy, pleased, stressed, down, frustrated, goal-directed, busy and in control. This method has been used previously in salivary cortisol studies (e.g. Steptoe, Gibson, Hamer, & Wardle, 2007; Steptoe, O'Donnell, Badrick, Kumari, & Marmot, 2008).

3.5.1 Electronic recording of EMA mood ratings

To ensure mood ratings were completed in real-time and not retrospectively, the mood ratings were recorded using an electronic device - the actiwatch-score. Prior to the study days, the researcher (NS) programmed the device to beep at five random times on each of the study days. Participants were told that the watch would provide a series of letters representing each of the mood adjectives and they were instructed to use the watch to rate their mood. To aid

participants in completion of the mood ratings, participants were provided with a mini key ring attached to the watch detailing the letter code for the mood adjectives (e.g. P = pleased, H = happy, S = stressed, D = down, F = frustrated, G = goal-directed, B = busy, C = in control). If participants missed the beep on the watch, they were asked to record the date and time and their mood ratings on paper.

During the one-to-one research sessions, participants were shown how to enter their mood ratings using the actiwatch-score device and were asked to practice this with the researcher to ensure they were comfortable using it on their own. They also received written instructions for using the device detailed in the study guidelines.

3.6 Measures: demographic, hair, and psychosocial variables

3.6.1 Demographic Variables

Demographic questions (Appendix 13) included age, sex, ethnicity, relationship status, smoking status, subjective health and perceived social status. Smoking status was assessed on a five-point Likert scale (current, occasional, ex-smoker, never smoked) and subjective health status was assessed on a single item scale ranging from poor (1) to excellent (5). The ladder measure (Goodman et al., 2001) was used to measure participants' perceived social status. This involved participants placing themselves on a ten-step ladder, which represented where individuals stood in society. The upper end represented individuals that are the best off (individuals with the most money, most education and the best jobs), and the lower end represented individuals who are the worst off (individuals with the least money, least education and the worst jobs or no job). Participants marked the step they felt represented their social standing.

3.6.2 Hair characteristics

Hair characteristic questions (Appendix 14) included participants natural hair colour, type of hair (i.e. curly, straight or wavy), number of washes per week, hair treatments (e.g. colours, perms etc), and use of hair products (including hair stimulators).

3.6.3 Subjective Happiness Scale

The Subjective Happiness Scale (SHS; Appendix 15) was designed to measure global subjective happiness; it determines if an individual is happy or unhappy based on the individual's perspective (Lyubomirsky & Lepper, 1999). For two items, respondents characterise themselves using absolute ratings (e.g. in general, I consider myself (1) not a very happy person to (7) a very happy person). The remaining items were rated relative to other peers, participants rate the extent to which brief descriptions of individuals happiness and unhappiness apply to them on a seven point scale ranging from (1) not at all to (7) a great deal. Scores are obtained by reversing the negatively worded item (item 4) and averaging responses to the four items. Possible scores range from 1 to 7, higher scores indicate greater happiness.

The authors of the scale tested its psychometric properties in fourteen studies compromising of US students and community samples. The studies make up a large (N=2,732) demographically diverse sample. In four of these studies internal consistency (α = 0.70 to 0.94 M=0.86) and adequate test-retest reliability (r = .90 for four weeks) was demonstrated. In another study the one factor structure was demonstrated, the four items loaded strongly onto one factor. In longitudinal studies, good test-retest reliability was demonstrated, time period ranging from three weeks to one year (rs ranging from .55 to .90, average r = .72). In three college student samples and a retired adult sample, the SHS correlated highly with other happiness and well-being measures (rs ranging from 52 to .72, average r = .62). The SHS also correlated moderately with several dispositional constructs that are theoretically and empirically related to happiness, for example optimism, positive or negative emotionality,

extraversion and neuroticism (rs ranging from.36 to 0.60, average r = 0.51), demonstrating convergent validity (Lyubomirsky & Lepper, 1999).

3.6.4 Positive and Negative Affect Schedule

The Positive and Negative Affect Schedule (PANAS; Appendix 16), is a 20-item scale that assesses positive affect (10 items) and negative affect (10 items). Feelings of alertness, activation, enthusiasm and, more generally, pleasurable engagement with the environment is typical of high positive affect whilst lethargy and sadness are typical of low positive affect. Feelings of anger, tension, distress, and more generally, unpleasant engagement with the environment is typical of high negative affect and absence of such feelings is typical of low negative affect (Watson et al., 1988). Participants rated the extent to which they felt each emotion on a five-point Likert scale ranging from (1) very slightly to (5) extremely. The PANAS can be presented in different time frames, and does not affect the psychometric properties (Watson et al., 1988). Scores for the positive and negative scale are calculated by summing the separate scores on the two dimensions. For each dimension scores range between 10 and 50.

The scale exhibits good psychometric properties. Watson et al. (1988) reported Cronbach's alpha coefficients for various time frames (α = .86-.90 for positive affect and .84-.87 for negative affect). The scale exhibited good test-retest reliability over an eight week period (α = .47 to .68 for positive affect and .39 to .71 for negative affect). The authors also reported evidence for the validity of the PANAS, it was highly correlated in the expected direction for each dimension with general distress and dysfunction, depression, and state anxiety are more. Further Crawford and Henry (2004)demonstrated that PA and NA are two distinct dimensions, but are moderately negatively correlated in a large general adult sample.

3.6.5 Satisfaction with Life Scale

The Satisfaction with Life Scale (SWLS; Appendix 17) is a short five-item tool measuring individual's global cognitive judgements of their satisfaction with life (Diener, Emmons, Larsen, & Griffin, 1985). Items are designed to measure respondents overall judgments of their life. Judgments are based on the individual's own standards of evaluation. Although they are not designed to assess satisfaction of individual's life domains (e.g. health, finances, or relationships) respondents can integrate their satisfaction with such domains in formulating their responses. All items are positively stated (e.g. in most ways my life is close to my ideal), focusing on the positive or pleasant (and not the negative) side of individual's emotions. Respondents rate the extent to which they agree with each statement on a seven-point Likert scale ranging from strongly disagree (1) to strongly agree (7). Possible scores range from 5 to 35, with higher scores indicating greater life satisfaction.

The authors demonstrated good psychometric properties of the scale in US student and elderly samples (Diener et al., 1985). In the student sample the scale exhibited good test-retest reliability (α = .87 for two months) and factor analysis indicated one factor, which accounted for 66% of the variance. The scale correlated moderately in the expected direction with other measures of subjective well-being, such as happiness and positive affect, and psychopathology, such as neuroticism. Correlations indicate that individuals satisfied with their lives are generally experiencing well-being and free from psychopathology. In the elderly sample the scale exhibited good internal consistency, it correlated moderately with interviews and other measures assessing an individual's life satisfaction (rs ranging from .43 to .46), item total correlations were moderate (rs ranging from .61 to .81). Other studies have demonstrated good psychometric properties for this scale (see Pavot & Diener, 1993).

3.6.6 Meaning in Life Scale

The Meaning in Life scale (MIL; Appendix 18) is a 10-item tool assessing two dimensions of meaning in life (Steger, Frazier, Oishi, & Kaler, 2006). It included two sub scales, including the presence of meaning (MLQ-P) and search for meaning (MLQ-S) in life. The MLQ-P consists of five-items assessing the level of meaning or purpose in one's life (e.g. I understand my life's meaning). The MLQ-S consists of five-items assessing the extent to which respondents seek to find meaning and understanding in one's life (e.g. I am searching for a meaningful life). Respondents rate the extent to which each statement describes themselves on a seven-point Likert scale ranging from absolutely untrue (1) to absolutely true (7). One item (item 9) is negatively worded and reversed scored prior to summing items on each sub-scale. Scores for each subscale range from 7 to 35, higher scores indicate greater meaning in life for the presence subscale and greater searching for a meaningful life for the search subscale.

Steger et al. (2006) tested the psychometric properties of the scale in an undergraduate student sample. Exploratory factor analysis showed a two factor structure in two independent studies, the first taps into having meaning or purpose in life, measuring the subjective judgments of a meaningful life. The second taps into the search for meaning in life, measuring drive and orientation toward finding a life with meaning. Good internal consistency was exhibited for both subscales (αs range from .86 to .88). Convergent validity was demonstrated through the presence subscale moderately positively correlating with other measures of well-being (e.g. life satisfaction) and negatively with measures of ill-being (e.g. depression) (rs ranging from .61-.74) and the search sub-scale being unrelated to the well-being measures but positively with the ill-being measures. Discriminant validity was demonstrated for both sub-scales being distinct from conceptually different measures. Steger et al. (2006) provides evidence for the independence of the presence and search components. Further evidence is provided and shows that searching for a meaningful life can be particularly positive when the search being sought is for deeper meaning from an already meaningful life.

3.6.7 Ryff's Scales of Well-being

Ryffs' scales of psychological well-being (RPWB; Appendix 19) is a multidimensional measure assessing the psychological aspect of wellbeing (Ryff, 1989). It assesses many aspects of well-being, which include self-acceptance; establishing good relations with others; a sense of autonomous thinking and acting; the ability to manage complex environments in line with personal needs and values; pursuing meaningful goals and a sense of purpose in life; continued growth and development as a person. These dimensions are theory driven and the development of the dimensions is outlined in Ryff et al. (1989). The original RPWB consists of 84 items; each dimension consists of 14 items. The studies presented in this thesis used the mid-length version, consisting of 54 items (9 per dimension). Respondents rate the extent to which they agree with statements on a six-point (1-6) Likert scales ranging from strongly disagree to strongly agree. Responses are summed for each of the six dimensions (about half of the items are negatively word, items are reverse scored), possible scores range between 6 and 64. A score for each dimension is obtained; a high score indicates that an individual has mastered that area of his or her life. A low score indicates that the individual struggles to feel comfortable with that area of his or her life. Table 3.1 provides further information of the characteristics of a high or low score for the six dimensions.

Table 3.1 Definitions of theory-guided dimension of well-being with example items for each dimension

Self-acceptance:
Refers to the positive evaluations an individual makes about his or her self and past life (e.g. I like most aspects of my personality). High scorer possesses a positive attitude toward the self; acknowledges and accepts multiple aspects of self, including good and bad qualities; feels positive about past life. Low scorer feels dissatisfied with self; is disappointed with what has occurred with past life; is troubled about certain personal qualities; wishes to be different than what he or she is.
Positive relations with other:
Refers to an individual having good quality relationships with others (e.g. most people see me as loving and affectionate). High scorer has warm, satisfying, trusting relationships with others; is concerned about the welfare of others; capable of strong empathy, affection, and intimacy; understands give and take of human relationships. Low scorer has few close, trusting relationships with others; finds it difficult to be warm, open, and concerned about others; is isolated and frustrated in interpersonal relationships; not willing to make compromises to sustain important ties with others.
Autonomy:
Refers to an individual; with a sense of self-determination (e.g. I have confidence in my opinions even if they are contrary to the general consensus). High scorer is self-determining and independent; able to resist social pressures to think and act in certain ways; regulates behavior from within; evaluates self by personal standards. Low scorer is concerned about the expectations and evaluations of others; relies on judgments of others to make important decisions; conforms to social pressures to think and act in certain ways.
Environmental Mastery:
Refers to an individual mastering and controlling his or her environment (e.g. I am quite good in managing the many responsibilities of my daily life). High scorer has a sense of mastery and competence in managing the environment; controls complex array of external activities; makes effective use of surrounding opportunities; able to choose or create contexts suitable to personal needs and values. Low scorer has difficulty managing everyday affairs; feels unable to change or improve surrounding context; is unaware of surrounding opportunities; lacks sense of control over external world.
Purpose in life:
Refers to an individual's goals in life and belief in his or her life having purpose and meaning (e.g. some people wander aimlessly through life, but I am not one of them). High scorer has goals in life and a sense of directedness; feels there is meaning to present and past life; holds beliefs that give life purpose; has aims and objectives for living. Low scorer lacks a sense of meaning in life; has few goals or aims, lacks sense of direction; does not see purpose of past life; has no outlook or beliefs that give life meaning.
Personal growth:
Refers to an individual's sense of continued development and growth in his or her life (e.g. I think it is important to have new experiences that challenge how you think about yourself and the world). High scorer has a feeling of continued development; sees self as growing and expanding; is open to new experiences;

has sense of realizing his or her potential; sees improvement in self and behavior over time; is changing in ways that reflect more self-knowledge and effectiveness. Low scorer has a sense of personal stagnation; lacks sense of improvement or expansion over time; feels bored and uninterested with life; feels unable to develop new attitudes or behaviors.

Table adapted from (Ryff, 1989, p. 1072).

Despite the extensive use and interest in the theoretical structure of the RPWB scale and the evidence of a six-factor structure through factor analytical studies carried out by Ryff and colleagues (Phillapa, Clarke, Carol, & Wheaton, 2001; Ryff & Keyes, 1995) the psychometric properties of the full scale has been questioned. In a sample of females in a UK cohort (n = 1179) the factorial validity and structure of the scale (42-item version) were tested. Two of the dimensions (autonomy and positive relations with others) failed to explain the general well-being factor, suggesting that psychological well-being may not consist of these dimensions (Abbott et al., 2006). The authors suggested that a more parsimonious model is needed if the six dimensions are to be retained. Thus, due to the questioning of these two dimensions they were excluded from the studies presented in this thesis.

3.6.8 Perceived Stress Scale

The Perceived Stress Scale (PSS: Appendix 20) is a measure of generalised perceptions of stress; it is designed to measure the degree to which situations in an individual's life are perceived as stressful (Cohen, Kamarck, & Mermelstein, 1983). It is designed for use with community samples with at least a high school education. The authors state that the items are quite general in nature and thus relatively free of content specific to any sub population group. The items tap into the unpredictability, uncontrollability and how overloaded an individual's life is and some items ask about individuals current level of stress in their life. The full scale PSS, comprises of 14 items referring to individuals thoughts and feelings of events that occurred over the last month. Seven items are positively worded (e.g. "In the last month, how often have you felt that you were effectively coping with important changes occurring in your life") and

seven are negatively worded (e.g. “In the last month, how often have you been upset because of something that happened unexpectedly”). Responses are made on a five point (0-4) Likert scale (never, almost never, sometimes, fairly often, very often). Scores are obtained by reverse scoring the items on the seven positive items and then summing across all 14 items. Possible scores range from 0 to 56, with a high score indicating higher perceived stress. The PSS is not considered to be a diagnostic instrument, thus, no specific categories are proposed by the authors for “high”, “medium” or “low” stress scores.

The authors tested the psychometric properties of the scale in two student and one community sample (Cohen et al., 1983). The scale exhibited good internal consistency (rs ranging from .84 to .86). Good test-retest reliability over short time intervals (over 2 days r = .85, after 6 weeks r = .55). It correlates highly with indices of depressive symptomatology (rs ranging from .65 to .76) and moderately with number of life events (rs ranging from .17-.35). The PSS is reported to be a better predictor of health outcomes (e.g. physical symptomatology rs ranging from .52 to .65 and social anxiety rs ranging from .37 and .48) than measures of life events since the focus is on appraisal of events rather than simply number of events. In clinical samples the scale exhibited two components; perceived distress and perceived coping (Hewitt, Flett, & Mosher, 1992). The factor structure has not been tested in non-clinical samples (Hewitt et al., 1992). The authors propose that the PSS can be viewed as a tool assessing a state that identifies people at risk of a clinical psychiatric disorder.

Shorter versions of the scale are also available, the 10-item version (PSS-10) consists of four positively worded items and six negatively worded items (Cole, 1999). The four positively worded items are reverse coded and items are summed across all the items, possible scores range between 0 and 45. The four-item version (PSS-4) consists of four items (items 2, 4, 5 and 10 from the PSS-10). Two items (items 4 & 5) are positively stated and reversed scored prior to summing across items, possible scores range from 0 and 16. Both

versions use the same scale as the full version; a higher score indicates higher perceived stress.

The PSS-10 exhibited a better factor structure and internal reliability than the PSS-14 and correlations between the PSS-10 and other measures of stress were evident showing construct validity. The PSS-4 exhibited less reliability but its factor structure and predictive validity were good. Thus, authors recommend use of the PSS-10 and use of PSS-4 for situations that require brief measures of stress perceptions. Studies in this thesis used the 10-item and 4-item versions.

3.6.9 Centre for Epidemologic Studies Depression Scale

The Centre for Epidemologic Studies Depression Scale (CES-D; Appendix 21) developed by Radloff (1977) measures levels of depressive symptoms in the general population. It is a self-report tool measuring the frequency of depressive feelings and behaviours. The items are based on symptoms associated with a clinical diagnosis of depression. It consists of 20 items compromising six scales reflecting depressed mood, feelings of guilt and worthlessness, feelings of helplessness and hopelessness, psychomotor retardation, loss of appetite and sleep disturbance. Respondents are presented with statements about the way they may have felt or behaved in the last week. Statements are worded positively (e.g. I felt as good as other people) and negatively (e.g. I felt that everything was an effort). Respondents rate the frequency they experienced each statement on a four-point Likert scale (0 = rarely or none of the time to 3 = most or all of the time). The positively worded items (items 4, 8, 12, 16) are reversed-coded prior to summing the score on each item to obtain a total score. Possible scores range from 0 to 60 with higher scores indicating greater presence of depression symptomatology, a score set at 16 is the cut-off point for high depressive symptoms (Radloff, 1977) but others suggest a higher cut off point to be used (Boyd, Weissman, Thompson, & Myers, 1982; Zich, Attkisson, & Greenfield, 1990).

Radloff (1977) demonstrated good psychometric properties of the CES-D in American healthy adults and clinical samples. The high inter-item and item-scale correlations were weaker in the general population than the clinical sample. Internal consistency was high in the general population (α = .84 to .85) and higher for the patient sample (α = .85 to 92). Test-retest reliability was highest for shorter intervals of 2 to 8 weeks (r = .51 to .67), and weaker for longer intervals of 3 to 12 months (r = .32 to .54). The CES-D discriminates between clinical and general samples; scores were significantly higher in the clinical sample compared to the CES-D average scores of the general population samples. The scale correlated highly with other depression measures, indicating concurrent validity and correlated weakly with measures measuring different constructs (e.g. aggression, social functioning). The strong correlations between the CES-D and life events demonstrated the measures sensitivity to current mood state. The CES-D has been demonstrated to assess symptoms of depression across different racial, gender, education level, and age samples (Knight, Williams, McGee, & Olaman, 1997; Radloff, 1977; Roberts, Vernon, & Rhoades, 1989). Also good factorial validity has been demonstrated for the scale, correlations with the CES-D items were higher for the scales total score than with related but distinct constructs, for example, with self-esteem, and state or trait anxiety (Orme, Reis, & Herz, 1986).

3.6.10 The Depression Anxiety Stress Scales

The Depression Anxiety and Stress Scales (DASS; Appendix 22) was designed to measure three related negative emotional states of depression, anxiety and stress (Lovibond & Lovibond, 1995). The original scale consists of 42 items; each of the three dimensions consists of 14 items. The depression dimension measures dysphoria, hopelessness, devaluation of life, self-deprecation, lack of interest/involvement, anhedonia, and inertia (e.g. I felt that life was meaningless). The anxiety dimension measures autonomic arousal, skeletal muscle effects, situational anxiety, and subjective experience of anxious affect (e.g. I felt scared without any good reason). The stress dimension is sensitive to levels of chronic non-specific arousal, assessing difficulty relaxing, nervous

arousal, and being easily upset/agitated, irritable/over-reactive and impatient (e.g. I found myself getting agitated). Table 3.2. provides further information of the characteristics of a high score for the dimensions.

On a 4-point Likert scale (0-4) respondents rate the severity or frequency to rate the extent to which they have experienced (did not apply to me, applied to me to some degree, or some of the time, applied to me to a considerable degree, or a good part of time, applied to me very much, or most of the time) each statement. Scores are obtained for Depression, Anxiety and Stress are calculated by summing the scores for the relevant items. A shorter form of the scale is available, this is the 21-item version and seven items make up each dimension (stress = 1,6,8,11,12,14,18; depression = 3,5,10,13,16,17,21; anxiety = 2,4,7,9,15,19,20). To obtain the score, responses are summed for the relevant items and multiplied, possible scores for each dimension range from 0-42. A higher score for each dimension indicates higher negative emotional states. Table 3.2 details the characteristics of a high score on the three dimensions.

Large population studies of clinical and non-clinical studies have demonstrated good psychometric properties for the 42-item version (Brown, Chorpita, Korotitsch, & Barlow, 1997; Lovibond & Lovibond, 1995). For example, in a non-clinical sample, factor analysis showed three factors representing depression, anxiety and stress. The DASS discriminated between the three dimensions but they are still moderately correlated with each other, the stress scale exhibited higher correlations with the anxiety compared to the depression scale. Convergent validity was demonstrated with high correlations between the DASS dimensions and similar measures. Further support for the 42-item and 21-item versions was demonstrated (Antony, Bieling, Cox, Enns, & Swinson, 1998), the 21-item version exhibited a better factor structure and smaller inter-factor correlations than the 42-item version. The 20-item was used in the research for this thesis.

Table 3.2 Characteristics of a high score on the DASS depression, anxiety and stress dimensions.

Depression
Self-disparaging
Dispirited, gloomy, blue
Convinced that life has no meaning or value
Pessimistic about the future
Unable to experience enjoyment or satisfaction
Unable to become interested or involved slow, lacking in initiative
Anxiety
Apprehensive, panicky
Trembling, shaking
Aware of dryness of the mouth, breathing difficulties, pounding of the heart, sweatiness of the palms
Worried about performance and possible loss of control
Stress
Over-aroused, tense
Unable to relax
Touchy, easily upset
Irritable
Easily startled
Nervy, jumpy, fidgety intolerant of interruption or delay

Table taken from http://www2.psy.unsw.edu.au/dass/over.htm

3.7 Approach to statistical analysis

The assumption of normality is required for use of parametric statistical tests, such tests are more powerful than their counterpart (non-parametric) tests in which the normality assumption is not made. Cortisol data are often positively skewed (e.g. Edwards, Evans, et al., 2001), and an appropriate transformation is required to yield normally distributed data. In all the studies presented here the raw cortisol data was positively skewed. To normalise sample distributions for inferential analyses, square root or log transformations were performed.

Measurement of the diurnal pattern of salivary cortisol included six cortisol measures to examine the CAR (measured in saliva samples collected at and sampled at 0, 15, 30 and 45 min post-awakening) and the decline in concentrations thereafter (samples collected at 3 and 12 hrs post-awakening). Analyses for the CAR and the diurnal cortisol profile were separate. Composite cortisol measures were computed for each participant for each study day. The

CAR magnitude was computed as the mean increase of cortisol from the first sample (MnInc: Mean [S2,S3,S4] – S1). In addition to measures of the CAR, starting levels of cortisol at S1 was used as an outcome measure. Two composite measures for the diurnal cortisol profile were calculated. An estimation of the decline in cortisol concentrations over the day was obtained by subtracting sample 6 from sample 5 (diurnal decline). Accompanied with the decline measure is the mean diurnal cortisol calculated as the mean of sample 5 and 6 (i.e. S5 + S6 / 2).

Typically statistical approaches to cortisol data are conducted with correlation, regression or analysis of variance (ANOVA). Such methods are limited in dealing with data patterns involving multiple repeated measures and often unbalanced data sets, leading often to needless exclusion of participants with missing data points from the analysis. Flexible and increasingly used alternatives are mixed regression modelling approaches which permits multi-level analysis of data. Such strategies are deemed most appropriate for designs incorporating multiple repeated measures over time with fixed and random parameters (Blackwell, de Leon, & Miller, 2006) and enable modelling of dynamic aspects of the diurnal cortisol cycle (Adam & Kumari, 2009). Such methods do away with crude artificial correction for sphericity violation associated with conventional general linear method 'solutions' to the problem of repeated measures. They permit full and simultaneous consideration of both within- and between-participant associations, adjusting for missing data (assuming it is 'randomly' missing) so participants are not necessarily excluded from analysis simply because some repeated-measures data are missing. Mixed regression modelling was used in all studies in this thesis when dealing with multiple repeated measures.

In repeated-measures units of measurement are nested within individuals. Mixed regression modelling was used for study I-II. The data contained a two-level hierarchical structure - Level 1 represented measures over four days which were nested within participants (level 2) The mood ratings in study 1 were a time-covarying covariate of the outcome (i.e. mood fluctuates over the four study days). Centering of such data makes the data completely within-persons,

as it eliminates the between person differences in mood on the outcome (i.e. cortisol patterns). Scores on each study day are expressed as a value above or below each participant's mean score across study days, shifting the zero point of the data to a more meaningful reference. The nested design of this study I (4 days of data nested within each participant) allowed use of mixed regression modelling to test whether the within-person relationship between well-being or ill-being and diurnal patterns of cortisol secretion varies as a function of between-person characteristics. Although hair cortisol did not contain repeated measurement, using the mixed regression modelling allowed for easier investigation of the interaction effects between well-being/ill-being and age on hair cortisol.

Other analyses performed were ANOVAs, t-tests or Chi-square tests to examine differences in demographic, situational and psychosocial variables between groups of interest (e.g. well-being groups). Pearson's tests of correlation were conducted to explore simple zero-order relationships between cortisol measures, psychosocial variables and mood data. For all correlations, scattergrams were produced to check that any significant associations were valid and not unduly influenced by outliers etc. All given values of p are two-tailed. All statistical analyses were performed using SPSS.

Chapter 4 Study I: Experiment I

Impact of non-adherence to the saliva sampling protocol on the diurnal pattern of cortisol

4.1 Introduction

Determination of the CAR and diurnal decline typically requires self-collection of saliva samples within the domestic setting. For the CAR, samples are collected at regular intervals during the post-awakening period, between 30 and 60 minutes (e.g. 0, 15, 30, 45 and 60 min post-awakening), the peak of secretion characteristically occurs between 30 and 45 minutes post-awakening (Oskis et al., 2009; Pruessner et al., 1997). For the diurnal cortisol profile, samples collected across the day are synchronised to awakening (e.g. 3, 6, 9 and 12 hr post-awakening).

Studies measuring the diurnal pattern of cortisol in the domestic setting usually rely upon unsupervised participant adherence to saliva sampling protocols. More objective measures include electronic monitoring of sampling and awakening times using track caps and actigraph (respectively), and these have been shown to be more accurate than participant self-reports. Participant's self-reported saliva sampling times are typically earlier than the track caps, which suggests that participants report their sampling times in line with the researchers desired sampling time (Broderick et al., 2004; Kudielka et al., 2003). Actigraph estimates of awakening time are typically earlier than participant self-reports. The discrepancies in awakening time leads to a delay in collecting the first awakening saliva sample (DeSantis et al., 2010; Dockray et al., 2008; Kupper et al., 2005) which is crucial for measurement of the CAR. Informing participants of electronic monitoring of sampling times can be an effective strategy to maximising participant motivation in collecting saliva samples at the

desired times and accuracy for reporting sampling times (Broderick et al., 2004; Kudielka et al., 2003). Earlier awakening and poor assessment of awakening is associated with greater delay between awakening and collection of the 'awakening' sample, this may be due to sleep inertia in the immediate post-awakening period and highlights the problems of self-assessment of awakening time. Non-adherence to the saliva sampling protocol does not appear to be associated with participant characteristics such as demographic or psychosocial characteristics suggesting that sampling delay is a random factor.

The dynamic and brief nature of the CAR makes it likely that errors in the timing of saliva sampling will impact upon its accurate measurement. The immediate post-awakening period (i.e. the CAR period) is associated with the presence of sleep inertia which is a state of reduced cognitive and motor performance (Tassi & Muzet, 2000) and is likely to increase the difficulty of adhering to protocol requested timings, even in well-intentioned participants. Until recently, quantification of the CAR has almost exclusively focused upon its magnitude (e.g. mean increase [MnInc] measures, or the near equivalent of areas under the curve with respect to increase [AUCi]. More economically, some have taken simple differences between awakening level and a single later measure, (typically at 30 min). However the *timing* of the CAR peak is also an important variable. For example, it has been shown to be related to gender, with males peaking earlier than females (Pruessner et al., 1997); menstrual cycle, with females in the ovulation phase peaking later (Wolfram et al., 2011a); hormonal status, with pre-menarche females peaking earlier than menarche females (Oskis et al., 2009); and individuals with better cognitive function peaking earlier (Evans, Hucklebridge, Loveday, & Clow, 2012). This often overlooked CAR characteristic is likely to be increasingly studied and adds to the CAR's value as a biomarker. Although in recent years participant non-adherence to protocol has received increasing attention with respect to CAR magnitude, there has been no investigation of this issue with respect to the timing of the CAR peak, and even with respect to CAR magnitude there have been no comparative studies of delayed sampling on the different commonly used quantifications mentioned above.

Sampling delay in the post-awakening period results in attenuated CAR magnitude. For instance, studies that have estimated awakening time by actigraph or polysomnography have reported that delays over 15 minutes lead to higher awakening samples and flatter CARs of smaller magnitude (DeSantis et al., 2010; Dockray et al., 2008; Okun et al., 2010). These studies did not electronically monitor sampling times, but more recently awakening sampling times were electronically monitored and delays of 11.5 minutes resulted in attenuated CAR (Griefahn & Robens, 2011).

Pooling the evidence, it seems to be broadly accepted that non-adherence of more than 15 minutes leads to under-estimation of CAR magnitude, for instance, apparently higher first samples and reduced CARs. By default these studies imply that sampling delays of up to 10-15 minutes are 'safe' and acceptable in this line of research. However, studies have not directly tested the impact of virtually no delay compared to moderate delays (i.e. < 10-15 min) on measurement of the CAR and this needs to be directly investigated. It is a convenient finding that delays less than 10-15 min do not affect the CAR magnitude since it simplifies the data collection process, in that delays of this nature can be included in analyses meaning that less data is wasted; however, it is theoretically challenging. It is understood that the moment of awakening initiates the CAR (see Clow et al., 2010; Wilhelm et al., 2007) and it is assumed that the rise in cortisol secretion is linear from awakening to 15-30 min later. If this is the case, then moderate delays (< 10-15 min) would result in attenuated CAR magnitude since the 'awakening' sample would be higher compared to those that collected their awakening sample without any delays, thus, there would be less of a rise from awakening since a proportion of the CAR will have happened before sampling and not visible to researcher and therefore show an attenuated CAR magnitude. The conundrum may be resolved, however, if there is a time lag of 5-15 minutes between awakening and increased cortisol concentrations in saliva. This would mitigate any reduction in the CAR magnitude due to such moderate sampling delays. However an implication of this scenario may be that CAR magnitude is over-estimated and the timing of the CAR peak would occur earlier than if the first sample was actually collected at the moment of awakening. In view of a burgeoning literature addressing

associations between the CAR and psychosocial and health domains, what is or is not ‘tolerable’ measurement error is something in need of urgent investigation.

Previous studies have investigated if sampling delay influences measurement of the diurnal cortisol profile, but in these studies saliva samples were collected according to clock time and not awakening time. One study showed that sampling delay did not influence the diurnal cortisol profile (Jacobs et al., 2005). On the other hand, two other studies showed that sampling delay resulted in a flatter diurnal decline, however in these studies the diurnal decline was anchored from the CAR, which was attenuated from sampling delay in the post-awakening period. Due to the steady decline in cortisol over the day it is likely to be less influenced by sampling delay when anchored outside the CAR period.

4.1.1 Aims

In the current study participant adherence to the saliva sampling protocol was electronically monitored. Awakening times were verified by actigraphy and saliva sampling times were verified by track caps, participants were made aware that the electronic estimates would be used to verify their self-reported awakening and sampling times. The study sought to investigate the impact of previously thought tolerable delays of up to 15 min on the CAR magnitude and timing of the CAR peak. Further, it was also possible to investigate if non-adherence to saliva sampling across the day impacted on the diurnal cortisol profile. The specific aims and objectives of the current study are displayed below (see Table 4.1).

Table 4.1 Specific aims and objectives

Aims	Objectives
Monitor adherence to the saliva sampling protocol	Electronically monitor awakening and saliva sampling times using actigraph and track caps, along with participants self-reports
Investigate the prevalence and type of non-adherence to the saliva sampling period	Determine sampling delay relative to awakening time Compare sampling delay determined by electronic estimates and participants self-reports
Investigate the impact of moderate delays on measurement of the CAR	Compare the CAR magnitude on days in which saliva sampling relative to awakening was delayed with days when delay was minimal. Investigate the impact of sampling delay relative to awakening on the timing of the CAR peak on delayed and minimal delay days.
Investigate the impact of non-adherence to measurement of the diurnal cortisol profile	Compare the diurnal cortisol profile on non-adherent days and adherent days

4.1.2 Hypotheses

Although participants were aware of electronic estimates of adherence to the saliva sampling protocol, it was hypothesised that participants would delay sampling. It was also hypothesised that sampling delay would be attributable to delay between awakening and collection of the first ('awakening') sample and participants self-reported sampling times would be in line with the electronic devices. Further, it was hypothesised that there would be differences in the CAR magnitude on non-adherent and adherent study days; specifically moderate non-adherence (delays of 5-15 min between awakening and commencement of saliva sampling) in comparison to adherent days (defined as less than five min delay) would be associated with an over-estimated CAR magnitude.

4.2 Method

4.2.1 Participants

Fifty female psychology students (21.0±4.4 years) were recruited on the basis that they were healthy, not taking prescribed medication or suffering from any medical or psychiatric illness (ascertained by self-report). The majority of participants were White (60%) or Asian (27%). Subjective social status was self-assessed with the 1-10 'ladder' measure (Goodman et al., 2001) and on average was 5.6±1.4. The majority of participants did not smoke (63%) or were occasional smokers (16%). Participants received no financial incentive to take part in the study but did receive course credits. For one participant, samples on one of the study days were outside the range of the cortisol assay detection range and a further four participants did not return their samples for two of the study days. All five were excluded from the analyses. The University of Westminster ethics committee approved the protocol. All participants provided signed informed consent.

4.2.2 Materials

Participants were provided with a study pack which included full standardised written instructions, a saliva sampling kit containing four colour-coded Ziploc bags labelled day 1-4, each containing six colour-coded Salivettes (saliva sampling devices, Sarstedt Ltd., Leicester, England), labelled tube 1-6 with their cotton swabs removed (see below). Participants were provided with a record sheet to record their awakening and saliva sampling times. Participants were also provided with electronic devices, which monitored their awakening times (wrist-worn actiwatch-score device) and saliva sampling times (MEM Cap), which contained the swabs from the salivettes.

4.2.3 Procedure

Participants individually attended a research study induction session at the University of Westminster with the lead researcher (NS). During this session (duration 15-25 min) participants provided informed consent, received full verbal and written instructions on procedures, and practised the techniques for collecting and recording times of saliva samples. Participants were informed about the need to adhere to the strict sampling regime relative to awakening time and were informed that the electronic devices would be used to check on the accuracy of their saliva sampling. Prior to each study day, participants were reminded to prepare for the study day (e.g. place samples next to their bed and wear the actiwatch-score device to bed) via automated text messages. Participants were instructed to collect saliva samples immediately on awakening (S1) and 15 (S2), 30 (S3), and 45 (S4) min and 3 (S5) and 12 (S6) hr later. Participants were instructed to awake in their usual way and this was either spontaneous or by an alarm clock. During the saliva collection period participants were instructed to refrain from smoking, brushing their teeth, exercising and take nil by mouth except water. Samples were initially stored in a domestic freezer until they were returned to the laboratory to be stored at -20ºC until assayed. Participants were asked to fill in a record sheet on each day entering their awakening times, their protocol-required saliva sampling times based on their awakening time that day, and their actual saliva sampling times. To remind participants to collect the saliva samples throughout the day, text messages were sent near to their sampling times. The study materials and the saliva samples were returned to the researcher at the end of the study.

4.2.4 Electronic monitoring of adherence to saliva sampling protocol

As well as the usual self-report diary method of recording awaking and sampling collection times, electronic devices were used to monitor awakening and saliva sampling times electronically. To assess awakening, participants wore an activity device (Actiwatch-Score, Cambridge Neurotechnology, Cambridge, UK) on their wrist; this is a piezoelectric motion sensor recording physical activity.

Awakening times were estimated using the actiwatch-score software that distinguishes sleep and awakening periods by reduced and increased activity respectively. To monitor saliva sampling collection times the cotton swabs removed from the salivettes and used for saliva sampling were stored in the MEM cap — participants were instructed to open this device only at sampling times. Following saliva collection, swabs were returned to the correctly labelled salivette for storage. Openings of the MEM cap were a proxy for the collection timing of the saliva samples. Both electronic devices were used to electronically monitor actual saliva sampling collection times and determine non-adherent delays to sampling: desired saliva sampling times were calculated for each participant based on the actigraph estimated awakening time (i.e. 0, 15, 30 and 45 min and 3 and 12 hr post-awakening) and actual sampling was determined by subtraction of MEMs-determined sampling times from time of awakening for each saliva sample.

4.2.5 Cortisol assessment and assay

Cortisol assays were carried out at the University of Westminster. Samples were thawed and centrifuged for 10 minutes at 3,500 rpm. Cortisol concentrations were determined by Enzyme Linked Immuno-Sorbent Assay developed by Salimetrics LLC (USA). The lower limit of sensitivity is <0.01638 nmol/l. The standard range in assay is 0.513 – 8.468 nmol/l. Correlation of assay with serum: $r(47) = 0.91$, $p < 0.0001$. Intra and inter-assay variations were both below 10%.

4.2.6 Statistical analysis

Cortisol concentrations ranged between .004 and 55 nmol/l and values were moderately positively skewed. To normalise sample distributions for inferential analyses, square root transformations were performed.

Electronic estimates of saliva sampling times relative to awakening (derived from actigraph estimated awakening and MEMs-determined sampling times)

were determined for each participant on each day. Days with delays over 15 minutes were excluded from analyses as the purpose of the study was to assess the impact of shorter more moderate periods of sampling delay. Saliva sampling in the CAR period was categorised as essentially 'adherent' when the delay on that day between awakening and provision of first saliva sample was less than 5 minutes. 'Non-adherent' days were categorised as those having a delay between 5-15 minutes over this period. The effects of delay condition on the CAR were investigated in respect of four outcome measures. The first was the mean increase of cortisol from the first sample (MnInc: Mean [S2,S3,S4] – S1), which, together with its near equivalent of Area Under the Curve with respect to increase from S1 (AUCi) constitutes the typical composite measure of CAR magnitude in studies which collect multiple post-awakening saliva sample points. The three other CAR outcome measures were the increases in cortisol level from S1 to each subsequent sample points i.e. S2-S1, S3-S1, and S4-S1. These three measures are the defining components of the composite MnInc measure. It should be noted that the S3-S1 component (assuming accurate timing) represents the simple rise in cortisol in the first 30 minutes following awakening, and has often been used in larger studies where only two samples are collected to assess the CAR. In addition to measures of the CAR, differences in starting levels of cortisol at S1 were examined as a function of delay. The possible effects of delay on tendency to earlier peaking was investigated by polynomial trend analysis for all sample points with the expectation that quadratic coefficients, indicating a slowing or reversal of cortisol increase over time, would be greater in the delayed condition.

Days in which samples 5 and 6 were collected one hour earlier than desired sampling time were excluded from analysis since there was only a small number (S5 = 4 days, S6 = 2 days) of days. Saliva sampling across the day was categorised as essentially adherent if samples 5 and 6 were collected within one hour of the desired time (i.e. ± 59 min). Non-adherent days were categorized as samples collected over one hour of the desired time (> 60 min). The effects of delay condition on the diurnal cortisol period were investigated in respect of two outcome measures. The first was the diurnal decline measured as the slope from 3 to 12 hrs post-awakening (diurnal decline: 3 hr – 12 hr) and

average cortisol secretory over the day (mean of cortisol at 3 and 12 hrs post-awakening: S5 + S6/ 2)

The effects of delay conditions on CAR and diurnal cortisol outcome measures were investigated using mixed regression modelling. The question asked of the data was whether participants' CARs on days on which they delayed collecting the first sample as defined above were significantly different from their CARs on days with non-existent or minimal (< 5 min) delay. Secondly the question asked of the data was whether participants' diurnal cortisol activity on days in which they delayed collecting samples, as defined above, were significantly different from their diurnal cortisol activity on days categorised as adherent. The mixed regression dimension used in modelling CAR or diurnal cortisol outcome measures included participant identity (subject variable) and order of study day (repeated effect) as random effects and delay condition and intercept as fixed effects parameters. A simple auto-regressive (AR1) co-variance structure was specified for the repeated measures effect of study days. The assumption of an AR1 co-variance structure for the repeated measures in the mixed regression models was supported as optimal in all cases except for the CAR component of S4-S1, where a compound-symmetry (CS) assumption provided a marginally better fit. However differences between Schwarz's Bayesian Criterion (BIC) were trivial (<2) as were estimates of fixed effects parameters, thus for simplicity AR1 solutions are presented in all cases. More complex models were initially run for all dependent variables to check that any emerging within-subjects effects of delay were not confounded with any effects of the covariate of clock time of awakening and there were no interactions with day of study, which might have suggested delay effects were significantly restricted to a sub-set of the overall data. In all cases, no significant additional main or interactive effects emerged, and results from final models involving the single predictor of delay are presented.

4.3 Results

4.3.1 General descriptives for delay data

All delay data were highly positively skewed due to a small tail of outlying long delays. Median, mode, and inter-quartile range most appropriately summarize the data. Median delay between awakening and collecting the first sample (S1) was 4 min, mode was 2 min, and inter-quartile range 2-9 min. MEMs monitoring of saliva sampling times revealed that thereafter participants collected saliva samples (S2-S4) very closely in accordance with protocol, around a median and modal average of 15 min for each interval (i.e. precisely the protocol requirement), and an inter-quartile range of 15-17 min between S1 and S2 samples, 14-16 min between S2 and S3 samples, and 15-16 min between S3 and S4. Non-adherence in the CAR period was thus largely attributable to inaccurate assessment of awakening time. Saliva samples collected across the day (i.e. S5 and S6) deviated from the desired sampling time in the form of collecting saliva samples earlier or later. The general desciptives for this type of non-adherence to collecting samples 5-6 are presented in Table 4.2 and show that generally participants collected these samples close to the desired sampling times.

Table 4.2 General descriptives for non-adherence for the saliva sampling protocol over the day

Saliva sample	Median (min)	Mode (min)
Sample 5 (3 hr post-awakening)		
Delayed	0:10	0:02
Early	-0:14	-0:01
Sample 6 (12 hr post-awakening)		
Delayed	0:14	0:00
Early	-0:03	-0:01

There were no differences between participants' MEMs-determined sampling times and participants' self-reports. The high degree of adherence in respect of

intervals between samples is consistent with expectation for participants who are informed that their timings are being objectively checked.

4.3.2 Impact of non-adherence on the CAR

Full data including actigraph estimates of awakening times, MEMs estimates of sampling-times, and four post-awakening cortisol values were available for 149 days. Analyses were undertaken only on days in which the delay between awakening and collection of S1 was less than 15 min. Furthermore, in order to ensure maximum accuracy of the analyzed data-base, days in which samples 2-4 were collected 7.5 min earlier or later than the required 15 min sampling intervals were excluded; in other words all included sample points were closer to their protocol required time than an earlier or later point. These further restrictions resulted in a final data-base of 115 days, comprising 65 days with essentially accurate (delay < 5 min.) data, and 50 days where delay was between 5 and 15 min.

Data within adherent and non-adherent categories was not significantly skewed and Table 4.3 displays the mean electronically estimated delay for adherent, and non-adherent days for the CAR period. Self-reported awakening time is also shown; it was significantly later than actigraph estimated awakening time for both delay conditions.

Table 4.3 Mean (±SD) delay between actigraph awakening, collection of the 1st ('awakening') sample, and self-reported awakening for delay groups

	Difference in Time Mean (SD) Min:S	
Delay Conditions	Actigraph awakening & S1 collection	Actigraph & self-reported awakening
<5 min (N=65 days)	01:47 (01:24)*	02:55 (05:28)*
5-15 min (N=50 days)	08:26 (02:48)*	07:55 (03:34)*

*p <.001. N.B. degree of difference between unsynchronised self-report times and synchronised actigraph values for different delay groups are meaningful, absolute differences are not.

Moderate delay of 5-15 min in providing the first awakening sample was associated with a significantly greater CAR as measured by MnInc compared to adherent data (< 5 min delay). When MnInc was decomposed into its component parts, the same significant effects were evident when CAR was defined as cortisol rise between S1 and S2 and S1 and S3 but not between S1 and S4. Table 4.4 shows the mean (SEM) difference between the delayed and adherent data for each CAR measure.

Table 4.4 Mean (±SEM) difference in CAR magnitude between delayed and adherent data and statistics

CAR measure	Difference in CAR magnitude between delayed and adherent data	Adherent data	Non-adherent data	Statistics
	Mean (±SEM)	Mean		
MnInc	2.27 (1.05)	2.93	5.20	F = 4.68; df = 1,109.21; p = .033
S2-S1	2.16 (0.99)	1.71	3.87	F= 4.76; df = 1,111.52; p = .031
S3-S1	3.17 (1.39)	2.87	6.04	F = 5.18; df = 1,108.68; p = .025
S4-S1	1.59 (1.20)	4.07	5.66	F = 1.74; df= 1.94.23; p =.191

Analysis of levels of S1 cortisol showed very similar starting-point values of 6.78 and 6.02 nmols/l for non-delayed and delayed conditions respectively which in line with expectation were not significantly different (F = 1.45; df = 1,109; p = .230). Also in line with expectation, on average cortisol peaked earlier (S3) in the delayed condition than in the non-delay condition (S4). Polynomial trend analysis was used to examine this in more detail. Typically and on average, as we have discussed, cortisol rises to its peak at somewhere between 30 min and 45 min after awakening. Thus, over a period of at least 45 min following awakening, data is typically characterised by a large linear rise component but also a (usually) smaller quadratic (curvilinear) component reflecting negative acceleration towards a peak and any post-peak decline. In the delayed condition, significant linear and quadratic trends were evident (F = 45.57, df =

1,123.42, $p < .001$, and $F = 8.599$, $df = 1,123.42$, $p = .004$ respectively). By contrast, in the adherent non-delay data, a significant linear trend was evident ($F = 33.08$, $df = 1,185.85$, $p = .020$) but the quadratic trend failed to reach conventional significance ($F = 3.43$, $df = 1, 185.85$, $p = .066$).

Figure 4.1 shows the real time pattern of cortisol secretion plotted at eight min intervals using values calculated from the data file using electronically derived assessments of awakening and sampling time. An eight min sample interval was utilised to illustrate vividly the impact of the average sampling delay (8 min) of the delayed condition in this study.

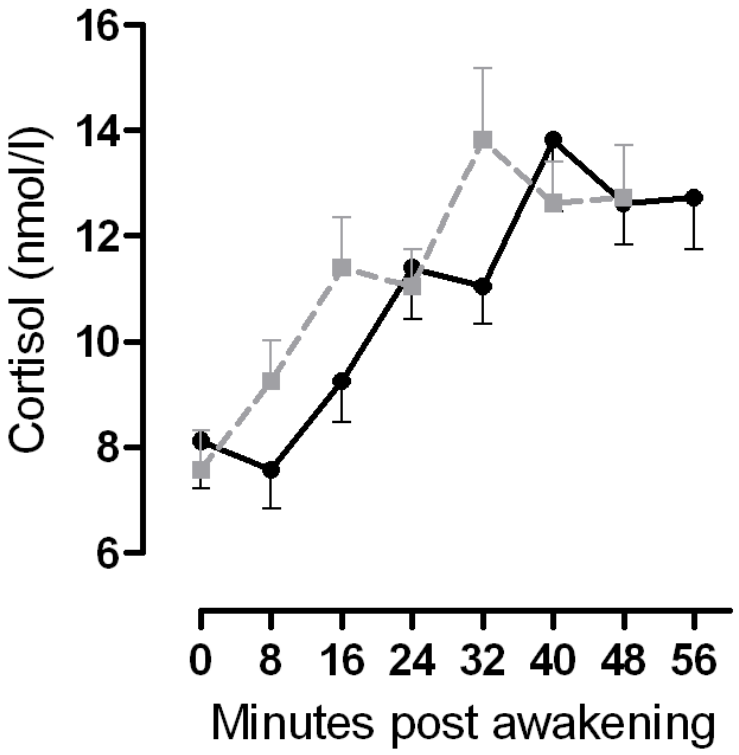

Figure 4.1 Real-time patterns of cortisol secretion

The solid black line shows the real-time pattern of cortisol secretion, plotted at 8 minute intervals. The dotted grey line illustrates the impact of a delay of 8 minutes between awakening and collection of S1: the line has been shifted 8 minutes to the left

4.3.3 Impact of non-adherence on the diurnal cortisol period

For S5 and S6 full electronically determined sampling times (determined by actigraph awakening time & MEMs estimates of sampling times) were available for 141 days. It was investigated if cortisol values differed on days in which samples were delayed compared to adherent days (e.g. ± 59 min). For S5, 15 days were categorised as delayed. Cortisol levels at 3 hour post-awakening (i.e. S5) did not significantly differ on delayed and adherent days (F = 2.098, df = 1, 118.780, p = .150). For S6, 12 days were categorised as delayed. Cortisol levels at 12 hour post-awakening (i.e. S6) were not significantly different form delayed days compared to adherent days (F = .256, df = 1, 121.335, p = .613).

Next the impact of sampling delay was explored for the diurnal decline and mean diurnal cortisol. Days were categorized as adherent if both samples were collected within 1 hour (± 59 min) of the desired time (N = 98). Days categorized as non-adherent if either sample were collected one hour later than the desired sample time (N = 26). Again days in which samples were collected over one hour earlier than the desired sample time were excluded (N = 4 days). Sampling delay had no effect on the diurnal decline (*F* = 1.927, *df* = 1, 117.918, *p* = .168) or mean diurnal cortisol (*F* = .456, *df* = 1, 113.807, *p* = .501).

4.4 Discussion

This study has shown for the first time that moderate delays between awakening and collection of the first saliva sample (between 5-15 min, average 8±3 min delay) that were previously thought 'tolerable' resulted in estimation of greater CAR magnitude and earlier CAR peaks. This level of non-adherence could not be detected using self-reports of time of awakening since such self-reports were on average 8±11 min later than actigraph estimates. These findings have implications for theory and best practice in accurate determination of the CAR. Sampling delay across the day did not affect measurement of diurnal decline or mean diurnal cortisol. This is the first study to examine the impact of sampling delay on measurement of the diurnal cortisol profile when

saliva samples are synchronised to awakening time, and the diurnal cortisol profile is anchored outside the CAR period.

The healthy young female participants were aware that their adherence to protocol was being monitored electronically, and their self-reported saliva sampling times were in line with the MEM Cap times and broadly collected at the desired 15 minute time intervals for the CAR period. On the majority of days samples collected during the day (S5 & S6) were close to the desired sampling time. This is in line with previous studies showing that informing participants that saliva sampling times were being electronically monitored increases their adherence to the protocol (Broderick et al., 2004; Kudielka et al., 2003). However, as noted, participants' self-reported awakening times were significantly later than actigraph estimates, resulting in a delay between awakening and collection of the first sample. Collection of subsequent samples (samples 2-4) were synchronised to the first sample. Although it was not directly tested in this study, the initial delay between awakening and collection of sample 1 was likely to be related to co-incident sleep inertia. This is a state of reduced cognitive and motor performance following awakening (Tassi & Muzet, 2000). This is likely to increase the difficulty of adhering to protocol requested timings, even in well-intentioned participants. Thus, these findings emphasis the need for electronic measures of awakening in CAR research as self-reported estimates at this time are not sufficiently reliable.

The average delay between actigraph estimated awakening time and collection of the first sample on the non-adherent days was on average 8±3 minutes. Sampling delay was around 10 minutes for collection of S5 and S6. These sampling delays could not be explained by participant characteristics, as the participants could be either adherent or non-adherent on different study days. Neither were differences between CAR profiles nor diurnal cortisol profiles on adherent and non-adherent study days associated with particular study days or day differences in awakening time. These findings are consistent with previous reports of no relationship between psychosocial factors and saliva sampling delay (e.g. Hall et al., 2011; Okun et al., 2010).

The novel findings in this study are that relatively minimal sampling delays of 8±3 minutes were associated with greater CAR magnitude when data was analysed either using the MnInc from 0-45 min or the frequently used change in cortisol concentration from sample one to 30 min post-awakening. The same minimal level of non-adherence in this sample was also associated with an apparent average CAR peak at 30 min post-awakening compared to the more typical female peak at 45 min post-awakening (Oskis et al., 2009). These findings raise a query over the validity of post-awakening sampling delays previously assumed to be acceptable, at least in women. However, it cannot be assumed that these findings extend to males since timing of peak is typically earlier (Pruessner et al., 1997).

Consequently the CAR apparently peaks earlier than if the first sample was actually collected at the moment of awakening. As the timing of the CAR peak has been linked to measures of gender, work overload, hormonal status and cognitive function (Evans et al., 2012; Kudielka & Kirschbaum, 2003; Oskis et al., 2009; Pruessner et al., 1997; Schulz, Kirschbaum, Pruessner, & Hellhammer, 1998; Wolfram, Bellingrath, & Kudielka, 2011b) this finding has implications for theory and practice and definitely merits further investigation. Even if such associations with timings of CAR peaks were to prove robust to control for accuracy of awakening time, there might still be a need for re-assessment of parameter estimates of such effects. A similar point could be made in regard to the provision of accurate incidence estimates of so-called 'negative CARs' in normal populations when timing of samples is carefully assessed. In the present study, a little less than a quarter of days showed no evidence of a post-awakening rise, and this proportion was comparable in both delay and non-delay conditions, suggesting that biases caused by delay influence the magnitude and timing of a CAR if it occurs, but not whether it occurs or not.

As expected the measurement of diurnal cortisol activity across the day was not influenced by sampling delay. This contrasts with previous studies showing that sampling delay results in a flatter decline (Broderick et al., 2004; Kudielka et al., 2003), but in these studies the decline in cortisol was anchored from the CAR,

which was attenuated due to sampling delay in the morning period. The current findings are however consistent with Jacobs et al. (2005) who also found no influence of delay on diurnal decline of cortisol.

This study adds to the body of literature that highlights the necessity of electronic monitoring of participant adherence to requested saliva sampling protocols in CAR research: even moderate non-adherence impacts on the measurement of the CAR. Adoption of these electronic devices provides a more accurate measure of the timing of saliva sampling than self-report in a domestic setting. In this study, using a combination of these devices enabled detailed real-time analyses of the impact of non-adherence previously not investigated, for instance between 5-15 minutes delay between awakening and collection of S1. Previous studies that have monitored adherence (either electronically or by self-report) have focused upon the impact of delays greater than 10-15 min post-awakening. The data presented here shows that accurate determination of the CAR (at least in females) requires more attention to detail to participant adherence than previously thought necessary. Although the implications of this study are inconvenient in terms of the practicalities for accurate estimation of the CAR within the domestic setting it may shed light on why there is so much inconsistent evidence for the association between the CAR and a range of psychosocial and health variables.

In conclusion moderate non-adherence (average delays of 8±3 min between awakening and collection of sample one) has been shown to impact on the accurate measurement of the CAR magnitude and peak time in a healthy young female sample. Future research that seeks to use the CAR magnitude and timing of the CAR peak as a biomarker should take careful electronic measures of adherence to protocol in all respects. Accurate knowledge of sampling delay could be used as a covariate in determination of the CAR, ensuring that data is not wasted but adjusted accordingly (e.g. mapping cortisol samples to real timings). Collecting saliva samples in the domestic setting during the challenging post-awakening period, when participants are likely to be in a state of sleep inertia, substantially increases the risk that misleading results will arise. Conveniently, non-adherence to the sampling protocol across the day did not

impact on measurement of the diurnal cortisol measures when measured at 3 to 12 hr post-awakening.

Chapter 5 Study II

A 10 min time lag between awakening and rise in cortisol levels

5.1 Introduction

Wilhelm et al. (2007) compared pre- and post-awakening measurements of cortisol and ACTH in both blood and saliva. They revealed significantly steeper increases in cortisol and ACTH after awakening reflecting processes specific to the sleep/wake transition, indicating conclusively that the increased secretory activity of these hormones after morning awakening is, in part, caused by the process of awakening itself. In other words the CAR is a genuine response to awakening. In this study, as with other studies, the CAR is represented as a linear response to awakening. However, blood and saliva were sampled at 15 minute intervals and the period immediately following awakening was not scrutinised.

Findings from Study I (Experiment I) are suggestive of a time lag in salivary cortisol secretion between awakening and the start of increased cortisol secretion, which characterises the CAR. In other words awakening sets off a chain of events, which leads to, increased cortisol secretion, which takes 5-15 minutes to manifest as increased cortisol concentrations in saliva. This proposed time lag would explain the finding that awakening cortisol levels were not different between the adherent and moderately non-adherent day data in Study I (Experiment I). A moderate delay (i.e. 5-15 min) in collection of S1 would lead to the assumed 'awakening' sample being collected during the time lag period when cortisol levels are relatively stable (i.e. no dynamic increase during this time). The over-estimated CAR magnitude found in Study I (Experiment I) would be a consequence of the real-time CAR-assessment period being shifted just sufficiently along the time axis to maximise the average

level of cortisol measured in subsequent samples relative to the first sample but not being shifted sufficiently for that average to be influenced unduly by significantly lower post-peak values (unlike for longer delays > 15 min). The peak of cortisol secretion would occur up to 15 minutes earlier than if the first sample was actually collected at the moment of awakening, which explains why the timing of the peak is earlier.

In summary, studies typically investigate the CAR in saliva samples collected at 15 minute intervals with the assumption that cortisol rises linearly between sample points; however to the knowledge of the author this assumption has not been investigated directly. Smaller intervals between post-awakening saliva samples are required to explore directly whether there is a predicted time lag between awakening and the post-awakening rise in cortisol secretion.

5.1.1 Aims

In order to understand the findings of Study I (Experiment I) that moderate delays impact on the CAR, the current study explored directly whether there was a predicted time lag between awakening and the post-awakening rise in salivary cortisol secretion in an intensive study utilising five min intervals between post-awakening samples. The specific aims and objectives of the current study are displayed below (see Table 5.1).

Table 5.1 Specific aims and objectives

Aims	Objectives
Monitor adherence to the saliva sampling protocol	Electronically monitor awakening and saliva sampling times using actigraph and track caps, along with participants self-reports Determine sampling delay relative to awakening time
Investigate cortisol secretion in the immediate period following awakening	Investigate cortisol secretion in five minute intervals for the first 30 minutes following awakening

5.1.2 Hypotheses

In light of the findings of Study I (Experiment I), it was hypothesised that cortisol in saliva would not increase linearly from awakening and that a 10-15 minute time lag between awakening and significant increase in cortisol levels would be observed thereafter.

5.2 Method

5.2.1 Participants

Ten male and female participants (M = 32, SD = 13.6 years) were recruited on the basis that they were familiar with the saliva sampling protocol. All were laboratory research personnel; they were healthy, not on medication or pregnant. Participants did not receive any incentives to take part in the study. Three participants collected saliva samples on one study day only, the remainder collected samples on two days.

5.2.2 Procedure

Participants were instructed to collect saliva samples on awakening and every five minutes for the first 30 minutes post-awakening. As well as the self-report method, awakening and saliva sampling times were monitored electronically by actigraph and MEM caps respectively. As in Study I, the same materials were provided and participants received the same instructions for saliva sampling. Cortisol concentrations were determined by Enzyme Linked Immuno-Sorbent Assay (see chapter 3 for details).

5.2.3 Statistical analysis

Cortisol concentrations ranged between 2.29 and 25.65 nmol/l and values were moderately positively skewed. To normalise sample distributions square root transformations were performed. Real sampling times was determined from electronically determined saliva sampling times relative to electronically

determined awakening time. Sample timings were mostly highly accurate but four of 17 days of data available showed some small delay (06:45±02:13 min) and were excluded. The mixed regression dimension used in final models of the data included participant identity as random subject variable, fixed effects parameters of sample point and study day, and intercept as both fixed and random parameters. The dependent variable was cortisol concentration.

5.3 Results

Initial analysis of all data revealed a typical CAR profile of substantial linear increase in cortisol over the 30 minute post-awakening period (F = 114.35; df = 1, 78.98; p < .001). Given sampling was confined to the first 30 minutes where few participants are likely to have peaked, the quadratic term just eluded significance (F = 3.69; df = 1, 78.98; p = .058). In addition, cortisol levels were significantly lower on the second day of the study (F = 26.08; df = 1,82.02; p < .001). There was however no significant interaction term.

Given the significant main effect of study day across all sample points, it was modelled as an additional fixed parameter in comparisons of differences between cortisol immediately post-awakening (S1) and points thereafter. Cortisol level at awakening was compared sequentially with each subsequent sample (5, 10, 15, 20 etc. min post-awakening) to investigate the point at which cortisol levels rose significantly. In line with expectation, there were no significant increases in cortisol levels before the 15 minute point following awakening. Thereafter, cortisol levels were significantly higher than at awakening. Table 5.2 displays the mean (±SEM) differences from awakening and inferential statistics for all sampling points, while Figure 5.1 provides a plot of estimated marginal means for the whole sampling period, which clearly shows the distinct latency of the CAR for the first 10 minutes of the period.

Table 5.2 Estimated marginal means for sample points 0 to 30 min post-awakening sampling points

Cortisol sampling points	Difference between Means (nmols/l) (±SEM. = 1.02)	F Statistic, p value
0 - 5 min	-0.12	$F = 0.01$, $p = .932$
0 - 10 min	0.81	$F = 2.13$, $p = .164$
0 - 15 min	2.02	$F = 5.92$, $p = .028$
0 - 20 min	3.02	$F = 13.65$, $p = .002$
0 - 25 min	5.02	$F = 44.41$, $p < .001$
0 - 30 min	6.28	$F = 32.71$, $p < .001$

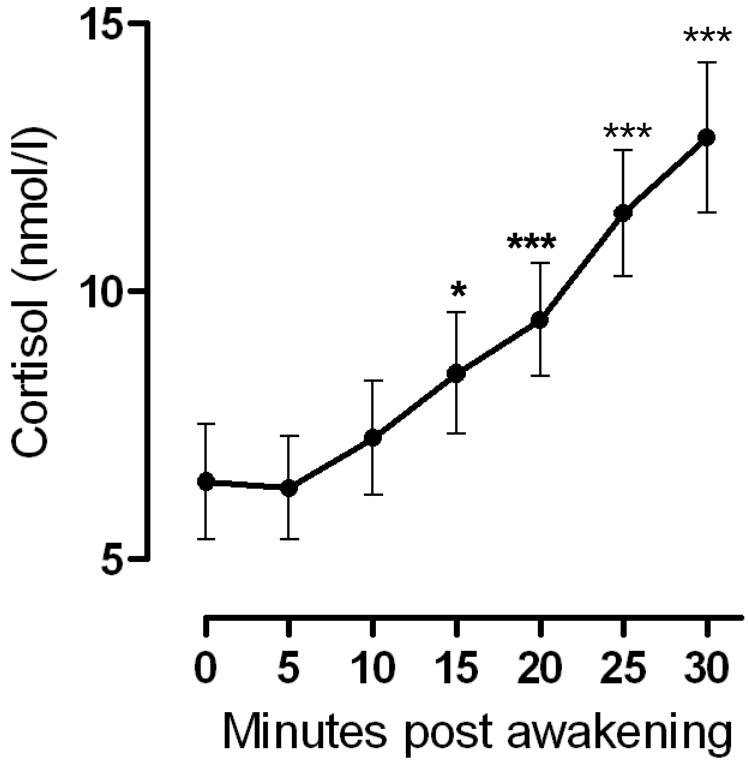

Figure 5.1 Time lag in cortisol secretion following awakening Cortisol samples 0 to 30 minutes post-awakening
***p < .05 *** p < .001 (S1 to subsequent sample specified axis)**

5.4 Discussion

In this study the time lag hypothesis was tested in a small but intensive study of laboratory research personnel that were monitored for adherence and volunteered to collect saliva samples at five minute intervals for the first 30 minutes post-awakening. As predicted there was no significant change in cortisol from awakening until the 15 minutes post-awakening point. This provides further support to the theoretical explanation for the observed effects in Study I (Experiment I).

There are also theoretical implications in regard to understanding the underlying physiology of the CAR, something which has possibly been somewhat neglected in the collective effort to establish reliable psychosocial correlates. While it is widely understood that secretion of cortisol is pulsatile, the assumption of linear continuity between saliva sampling points, referred to earlier, has doubtless relied on the further assumption that individuals' pulsatile profiles should average out to support the assumption of linearity in aggregated data. An aspect of the findings presented here is that they suggest individual patterns of secretion may not vary randomly immediately after awakening but are synchronised by the processes of awakening (a tipping point) initiating a similar process in all participants that results in a more or less uniform increase in cortisol secretion some 10 minutes later. This synchrony would be lost later in the day with individual variation in the time lag between bursts of cortisol secretion (i.e. the ultradian rhythm). So the data presented here is consistent with the evidence that awakening *per se* is the trigger for the CAR (Wilhelm et al., 2007).

The time lag in this study may be explained by the patterns of secretion of ACTH and cortisol in the post-awakening period. ACTH is the required secretogue for cortisol. In males an interval between release of ACTH and cortisol following awakening has been demonstrated (Wilhelm et al., 2007). On average ACTH peaked 15 minutes after awakening whereas cortisol peaked at 30 minutes after awakening, although in this study samples were collected at 15 minute intervals only, so it could be that peaks of cortisol and ACTH were

earlier or later than observed. Furthermore, cortisol secretion has been demonstrated to begin at about 10 minutes after initiation of ACTH secretion in a case study employing blood sampling every 5 minutes from 01:00 to 09:00 hours (Gallagher et al., 1973). Awakening is stimulatory to ACTH and the time lag then could be due to the complex sequential conversion of cholesterol to cortisol, in which ACTH stimulation is only the first step.

Results should be interpreted cautiously; one of the limitations of this study is the small sample size. Future research should test the time lag in a larger sample, this would enable testing of individual differences in cortisol secretory activity in the first 15 minutes following awakening. The finding of a time lag would have methodological implications for CAR research. Great care should be taken with the determination of awakening time as per the findings of Study I (Experiment I). Awakening is the trigger to the CAR and must be determined in an accurate way (i.e. electronic estimations rather than self-report).

In conclusion, as predicted a time lag between awakening and initiation of increased cortisol secretory activity was observed for the first time. The previous assumption that cortisol increased in a linear manner was not supported by the findings of this study. This finding explains the findings of Study I (Experiment I) that moderate delays in collection of the awakening sample result in over-estimated CAR magnitude in the first 45 minutes and earlier peak. This ‘time lag’ could be explained in terms of the ultradian pulsatile pattern of HPA activity and the conversion of cholesterol to cortisol following stimulation of ACTH at the adrenal cortex.

Chapter 6 Study I: Experiment II

Diurnal patterns of cortisol; relationships with trait and state well-being in young healthy females

6.1 Introduction

There is accumulating evidence suggesting the beneficial effects of well-being on future health outcomes (Pressman & Cohen, 2005) even after controlling for known covariates (Chida & Steptoe, 2008; Kubzansky & Thurston, 2007). There are several possible pathways through which well-being may confer favourable health outcomes (for a review, see Dockray & Steptoe, 2010). One possible pathway is via the neuroendocrine system. Both the CAR and diurnal cortisol profile have been associated with well-being. However, the literature is limited by inconsistent findings, particularly for the CAR. Variation in findings may be due to differences in the dimension of well-being measured (i.e. subjective or psychological, state or trait), the time of day of cortisol measurement, aspect of the circadian rhythm measured, and age or gender of the sample. Further non-adherence to the saliva sampling protocol is problematic for CAR studies and may contribute to the mixed findings present in the literature.

The subjective aspect of well-being is typically investigated in relation to cortisol. Lower levels of positive affect and less variation of positive affect in the afternoon was associated with higher evening cortisol in older adults (Simpson et al., 2008). In middle-aged adults (N = 227, average 45-59 years old) lower cortisol over the day (measured at 2 hr intervals between 08:00-23:30) was related with higher aggregated happiness ratings. The happiest individuals exhibited 32-34% less cortisol on work and leisure days after controlling for

psychological distress and other known covariates (Steptoe, Wardle, & Marmot, 2005). These results were replicated in a three year follow-up of the same individuals (Steptoe & Wardle, 2005). The effect of well-being being independent of ill-being demonstrates the unique relationship between well-being and cortisol. Others however have failed to find associations between well-being and diurnal cortisol patterns. In healthy young females (N = 556, M = 27, 18-61 years) aggregated positive affect was not related with lower cortisol output over the day, but aggregated negative affect was related with higher cortisol (Jacobs et al., 2007). The differences in findings between the studies may be the age of the participants (i.e. young vs middle-aged). It is possible that the association between cortisol and well-being vary with age; differences in cortisol output over the day in relation to well-being may evolve with increasing age.

Recently more recognition has been given to the psychological aspect of well-being; evidence suggests that it may be more strongly related to cortisol. In an older female sample, subjective well-being (measured by the PANAS) was not associated with cortisol over the day but higher levels of psychological well-being (e.g. personal growth and purpose in life) was associated with lower cortisol (Ryff et al., 2004). This finding suggests that both aspects of well-being should be measured in relation to cortisol. This effect was more pronounced for those over 75 years of age (Ryff et al., 2004), which supports the notion that such relationships may vary across the lifespan. In the same study ill-being (e.g. anxiety, anger) was not associated with cortisol secretion over the day (Ryff et al., 2006). This provides support for the 'independence view' of ill-being and well-being and warrants the need for investigating well-being and ill-being at least in relation to cortisol secretion.

Cortisol has a marked circadian rhythm and it is best practice to measure cortisol secretion according to awakening time and not clock time (Edwards, Clow, et al., 2001). Further, the CAR and diurnal decline should be investigated separately since the CAR involves different regulatory processes (Clow et al., 2004). Associations between well-being and the CAR or diurnal cortisol profile vary across studies. In 72 middle-aged males (M = 34, SD = 9 years) higher

aggregated positive affect was related with lower cortisol in the first 60 minutes following awakening and a smaller CAR magnitude, after controlling for negative affect, age, and BMI. However, no relationship with cortisol measures and the PANAS were observed (Steptoe et al., 2007). The lack of association with the PANAS suggests that aggregated ratings of positive affect may be more strongly related with well-being. Positive affect measured by the PANAS shows a distinctive circadian rhythm, positive affect on awakening is low and increases during the morning reaching peak levels during the afternoon and gradually declining in the evening (e.g. Simpson et al., 2008). The EMA method captures changes in mood over the day, which may be a more robust measure of capturing mood and thus more strongly related with cortisol patterns. The same group failed to replicate the CAR findings in a sample of older adults (N = 2873, 50-74 years), aggregated positive affect was not related with the CAR magnitude but was associated with cortisol output over the day (Steptoe et al., 2008). Likewise, Lai et al. (2005) did not find the CAR to be associated with positive affect in a sample of 80 young Chinese adults (average 27 years old), but higher levels of positive affect was related with lower cortisol over the day (assessed at spot time points).

Only one of the studies above electronically monitored saliva sampling times and this was for samples collected across the day which has not been shown to influence measurement of the diurnal cortisol profile (Jacobs et al., 2007). The other studies relied on participant self-reported adherence to the saliva sampling protocol, which is largely inaccurate and results in researchers failing to identify delays in sampling, which is particularly problematic for accurate measurement of the CAR (see chapter 4). In a smaller intensive study of 50 healthy older adults (average 74 years olds) sampling times were electronically monitored using track caps. A combination of ill-being and well-being was associated with lower cortisol in the first 45 minutes of awakening. Individuals exhibiting the 'best' levels of well-being (e.g. a combination of high levels of well-being and low levels of ill-being) exhibited less cortisol in the first 45 minutes of awakening. But no associations were found with the CAR (MnInc) or diurnal decline (3-12 hr post-awakening). The combination of well-being and ill-being suggests that they have to co-exist to have an impact on well-being,

therefore supporting the view that both well-being and ill-being need to be measured in relation to cortisol secretion (Evans et al., 2007). However, this study did not electronically monitor awakening times, and as revealed from Study I (Experiment I) electronic estimates of awakening time are essential to identify moderate delays in the post-awakening period which impact on CAR measurement.

In a meta-analysis inconsistent relationships between the CAR and psychosocial variables were evident. For instance, ill-being (e.g. depression, stress) was associated with an increased and decreased CAR. Positive psychological traits (such as well-being, happiness, optimism, vitality, lively/energetic feelings, self-esteem, and self-efficacy) were associated with a reduced CAR, which is consistent with positive characteristics having biological correlates that are the opposite of negative affective responses (for a review, see Chida & Steptoe, 2009). However, to further complicate the issue the CAR is suggested to be an adaptive response that helps individuals to meet anticipated demands of the day ahead (Adam et al., 2006). Evidence to support this comes from larger CARs evident in healthy individuals (Kudielka & Kirschbaum, 2003) and a larger CAR on work compared to week days (Kunz-Ebrecht et al., 2004; Schlotz, Hellhammer, Schulz, & Stone, 2004). Further the CAR was associated with study-day anticipations of the level of obligations; days anticipated to be busier were associated with an elevated CAR (Stalder, Evans, Hucklebridge, & Clow, 2010; Stalder et al., 2011). Thus it is difficult to predict the direction of the association between the CAR and well-being.

Gender differences may exist in the associations between cortisol and well-being, in healthy adults (N = 334, age M = 29, 18-54 years) males with low aggregated positive affect exhibited a high flat cortisol rhythm, and females with high aggregated positive affect exhibited a low flat cortisol rhythm. In males no decline in cortisol over the afternoon was observed, whilst for females, cortisol in the morning was low and remained low over the day (Polk, Cohen, Doyle, Skoner, & Kirschbaum, 2005). Furthermore, the CAR magnitude and cortisol peak differ for males and females; women exhibited larger CARs (Kunz-Ebrecht et al., 2004; Wright & Steptoe, 2005) and peak later (Oskis et al., 2009;

Pruessner et al., 1997).

The notion of age difference in the relationship between well-being and ill-being is already apparent in the studies discussed above. Evidence that the circadian rhythm of cortisol becomes disrupted in older age (Sapolsky, 1999), as well as higher cortisol levels, attenuated CAR and attenuated diurnal decline are associated with older age (Evans et al., 2011; Ice, 2005) further supports this notion. Additionally, mood changes are observed with older age; negative mood is generally shown to decrease with age and increases in the very old (e.g. Kessler & Staudinger, 2009). Findings for positive mood are less clear, studies show a decrease or increase in positive mood with old age (e.g. Mackinnon et al., 1999; e.g. Mroczek & Kolarz, 1998) or no change (e.g. Carstensen, Pasupathi, Mayr, & Nesselroade, 2000).

Few studies have investigated associations between well-being and cortisol specifically in younger individuals. In young adults (19-26 years old) depression was related with higher cortisol levels (Van Honk et al., 2003). Studies specifically investigating the CAR or diurnal cortisol profile have been on samples of mixed ages or middle-aged and older individuals. To the knowledge of the author, no study has directly explored well-being in relation to the CAR and the diurnal cortisol profile in younger individuals only (i.e. age 20-30 years). There is growing interest in how stress affects the HPA axis across the life span (Lupien, McEwen, Gunnar, & Heim, 2009) and thus it is important to understand if the effects of well-being on favourable health are only relevant among the aging population or if they extend to younger adulthood. An understanding of this is important in developing effective interventions aimed at preventing and treating chronic stress or disease.

6.1.1 Aims

The aims of this study were to examine the structure of the trait well-being and ill-being measures using factor analysis, to investigate the components from the factor analysis in relation to diurnal cortisol patterns, and to explore associations between the diurnal pattern of cortisol (CAR and diurnal decline) and trait/state

well-being independently of ill-being in young healthy females. A further aim was to monitor participant adherence to protocol electronically enabling identification of non-adherent samples, which could be removed, or statistically controlled in analyses examining patterns of cortisol and well-being. The specific aims and objectives of the current study are displayed below (see Table 6.1).

Table 6.1 Specific aims and objectives

Aims	Objectives
Explore the structure of trait ill-being and subjective/psychological well-being	Conduct factor analysis to explore the structure of the trait ill-being and subjective/psychological well-being measures
Categorise state well-being and ill-being based on the mood ratings	Explore relationships between the daily mood ratings
Account for non-adherence to the saliva sampling protocol in associations between diurnal cortisol patterns and well-being/ill-being	Control for electronically determined (e.g. actigraph and track caps timings) non-adherence in analyses exploring associations between diurnal cortisol patterns and well-being/ill-being
Explore relationships between diurnal cortisol patterns and trait/state well-being independent of ill-being	Measure the CAR and diurnal decline across four days Explore associations between diurnal cortisol patterns and wellbeing and ill-being For any significant effects of well-being on diurnal cortisol patterns control for ill-being

6.1.2 Hypotheses

It was hypothesized that there would be a relationship between well-being and the CAR across the four study days, however, given the mixed findings for associations between psychosocial variables and the CAR the direction of the relationship was not specified. It was predicted that there would be a positive association between the diurnal decline and well-being; people with higher well-being would have a steeper slope from the three to twelve hours post-awakening across the four days. Further, the mean diurnal cortisol was predicted to be negatively associated with well-being; mean cortisol from three

to twelve hours post-awakening across the four days would be lower in those with higher levels of well-being. It was also hypothesised that well-being would be uniquely related with cortisol; associations between cortisol patterns would be independent of ill-being.

6.2 Method

Recruitment of participants was in two phases; phase I involved completion of an online questionnaire of well-being and ill-being measures. Based on participants' scores, participants were recruited to take part in the second phase, which involved the salivary sampling and mood ratings over four days.

6.2.1 Participants

Male and female students were recruited from the psychology department (N = 240) at the University of Westminster. Participants were on average 21 (SD = 4.6, range 18-50) years old. On average participants rated their perceived social status in the middle of the ladder (5.3 ± 1.6). The majority was White (43%) or Asian (32%). Participants completed an online questionnaire consisting of demographic questions and measures of trait well-being and ill-being.

6.2.2 Psychosocial Measures

A full description of the demographic questions and psychosocial measures used in this study is provided in chapter 3, as a reminder to the reader an overview of the measures is provided here. Demographic questions included age, sex, ethnicity, and marital status. Participants also reported their smoking status (e.g. current, occasional, ex-smoker, never smoked), health status (e.g. 1 = poor, 5 = excellent), and subjective social status using the 'ladder' measure (Goodman et al., 2001). Hedonic well-being was assessed using the 4-item subjective happiness scale (SHS), the 5-item satisfaction with life scale (SWLS) and positive affect (PA) from the positive and negative affect schedule (PANAS). Higher scores indicated greater happiness, life satisfaction and positive affect

respectively. Psychological well-being was measured using the meaning in life scale, which consists of meaning in life (MIL-P; higher scores indicated greater meaning in life) and searching for a meaningful life (MIL-S; higher scores indicated greater searching for a meaningful life); and Ryff scales of psychological well-being (RPWB), only four dimensions were used (EM = environmental mastery, PIL = purpose in life, PG = personal growth, SA = self-acceptance), higher scores indicated greater psychological well-being. Ill-being, was measured using the 10-item perceived stress scale (PSS), the 20-item centre for epidemiologic studies depression scale (CES-D), and negative affect (NA) from the positive and negative affect schedule (PANAS). Higher scores indicated greater perceived stress, depression and negative affect respectively.

6.2.3 Statistical analysis

All measures were scored in accordance with the recommended guidelines (see chapter 3 for details). Mean scores (based on individual responses to individual measures) were computed for missing responses. Factor analysis was conducted to explore the structure of the well-being and ill-being measures. The measures of well-being and ill-being were analysed by means of principle component analysis, with varimax rotation. The various indicators of factorability indicated good factorability and the residuals indicated a good solution. For example, the KMO and Bartletts test was significant (statistic = .901, $p < .001$) indicating that there was a good amount of variance within the data explained by the factors. The anti-image covariance values were small, which is indicative of good factor structure and the correlations showed that the KMO statistic for each variable included in factor analysis were all above .8 indicating that it was not necessary to drop any of the measures. Identification of participants exhibiting high or low well-being were based on scores on the well-being component.

6.3 Results: Phase I

6.3.1 General descriptives for demographic, health and psychosocial variables

The majority of participants were female (N = 197, 82%). All of the measures exhibited good reliability statistics (see Table 6.2). Pearson's correlations among the well-being and ill-being indicators correlated in the expected direction (see Table 6.3). The SHS, PA, SWLS, MIL-P EM, PG, PIL were positively correlated with each other. The NA, PSS and CES-D were positively correlated with each other.

Table 6.2 Reliability & descriptive statistics for psychosocial measures

Scale	α	Mean (±SD)	Min-max
SHS	.822	4.90 (1.17)	1-7
SWLS	.839	21.32 (6.49)	5-35
PA	.802	33.05 (7.78)	11-48
NA	.770	21.66 (7.63)	10-46
CES-D	.898	17.28 (10.69)	2-52
MIL-P	.871	24.39 (6.51)	6-35
MIL-S	.892	21.37 (7.89)	5-35
PSS	.853	19.06 (6.61)	3-34
EM	.807	36.20 (7.45)	13-53
PG	.719	41.39 (6.23)	29-54
PIL	.693	38.96 (6.28)	22-52
SA	.852	36.59 (8.49)	10-54

SHS = subjective happiness scale; PA = positive affect; SWLS = satisfaction with life scale; MIL-P = meaning in life – presence; MIL-S= meaning in life – search; EM = environmental mastery; PG = personal growth; PIL = purpose in life; SA = self-acceptance; NA = negative affect; PSS = perceived stress scale; CES-D = centre for epidemologic studies depression scale.

The well-being measures correlated negatively with the ill-being measures except for the searching for a meaningful life measure, which was not related with MIL-P, PA, or PA (see Table 6.3).

Table 6.3 Pearson's correlations for the well-being and ill-being indicators

	PA	SWLS	MIL-P	MIL-S	EM	PG	PIL	SA	NA	PSS	CES-D
SHS	.532***	.601***	.469***	-.155**	.588***	.359***	.466***	.628***	-.374***	-.508***	-.578***
PA	-	.478***	.381***	-.024	.524***	.405***	.514***	.504***	-.226***	-.429***	-.477***
SWLS		-	.528***	-.180***	657***	.358***	.551***	.741***	-.362***	-.540***	-.540***
MIL -P			-	-.229	.490***	.325***	.572***	.564***	-.208***	-.300***	-.356***
MIL-S				-	-.218***	-.058	-.160**	-.204***	.240***	.255***	.274***
EM					-	.538***	.650***	.727***	-.483***	-.701***	-.619***
PG						-	.681***	.514***	-.269***	-.466***	-.395***
PIL							-	.714***	-.393***	-.543***	-.601***
SA								-	-.431***	-.621***	-.659***
NA									-	.601***	.711***
PSS										-	.718***

*** $p < .0001$, ** $p < .01$ SHS = subjective happiness scale; PA = positive affect; SWLS = satisfaction with life scale; MIL-P = meaning in life – presence; MIL-S= meaning in life – search; EM = environmental mastery; PG = personal growth; PIL = purpose in life; SA = self-acceptance ; NA = negative affect; PSS = perceived stress scale; CES-D = centre for epidemologic studies depression scale

6.3.2 Structure of well-being and ill-being indicators

The associations between the well-being and ill-being measures suggested shared variance among them. To explicitly test the structure of the well-being and ill-being measures exploratory factor analysis was conducted. Principal component analysis revealed two components with an eigenvalue greater than 1.0 for the well-being and ill-being measures. The scree plot also indicated two components (see Figure 6.1). The two components accounted for 62% of total variance explained by the solution to the factor analysis, component 1 accounted for 41% and component 2 accounted for 21%. The component matrix (Table 6.4) shows the component loadings prior to rotation. It shows that the majority of measures correlated with component 1. The MIL-S correlated more strongly with component 2.

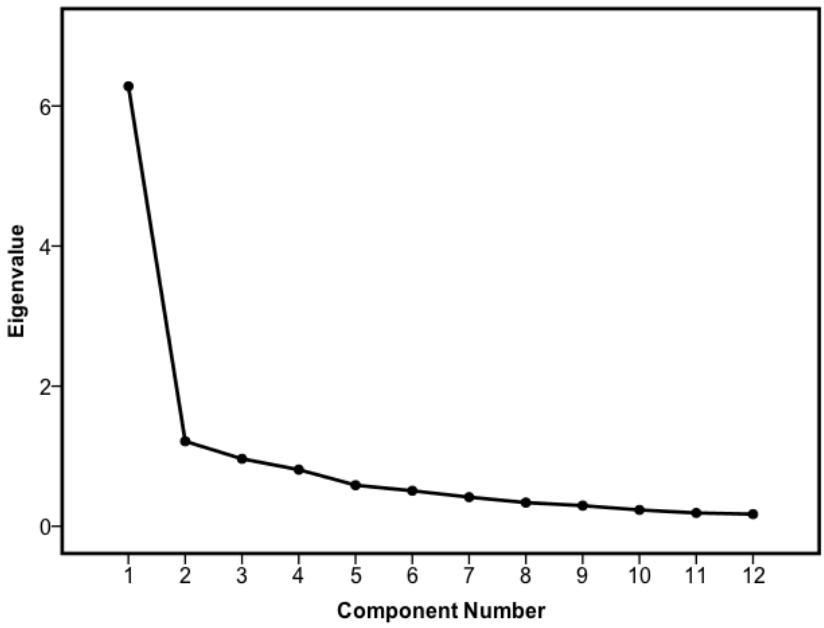

Figure 6.1 Scree plot showing two eigenvalues (> 1)

The rotated component matrix (Table 6.4) shows the factor loadings after rotation. It shows the unique relationship between each component and each measure, relationships exclude overlap between components. SHS, PA, MLQ-P, SWLS, EM, PG, PIL and SA are related with component 1. NA, depression, MLQ-S and PSS are related with component 2

Table 6.4 Factor analysis of well-being and ill-being measures

Measure	Component Matrix		Rotated component matrix Components	
	1	2	1	2
SHS	.741	.088	.679	-.309
SWLS	.781	.124	.732	-.299
PA	.652	.338	.732	.049
NA	-.615	.575	-.229	.810
CES-D	-.817	.336	-.525	.711
MIL-P	.627	.290	.687	.077
MIL-S	-.287	.564	.046	.631
PSS	-.788	.301	-.518	.665
EM	.858	.017	.743	-.430
PG	.631	.286	.688	.082
PIL	.812	.217	.807	-.235
SA	.872	.107	.802	.360

Note. Presented is the component matrix showing the correlations between components 1 and 2 before rotation and the rotated component matrix correlation between component 1 and 2 after rotation. SWB = subjective well-being; PWB = psychological well-being; IB = ill-being; SHS = subjective happiness scale; PA = positive affect; SWLS = satisfaction with life scale; MIL-P = meaning in life – presence; MIL-S= meaning in life – search; EM = environmental mastery; PG = personal growth; PIL = purpose in life; SA = self-acceptance; NA = negative affect; PSS = perceived stress scale; CES-D = centre for epidemologic studies depression scale.

Figure 6.2 shows the component plot in rotated space. Component 1 can be seen as representing hedonic and eudemonic well-being and component 2 represents ill-being. A positive value indicates a high score and a negative value indicates a low score on the components. To identify participants experiencing high and low levels of well-being, scores on the well-being component were divided. Participants whose scores fell into the upper quartile (top 25% of scores) were categorized as high well-being. Participants whose scores fell into the lower quartile (bottom 25% scores) were categorized as low well-being.

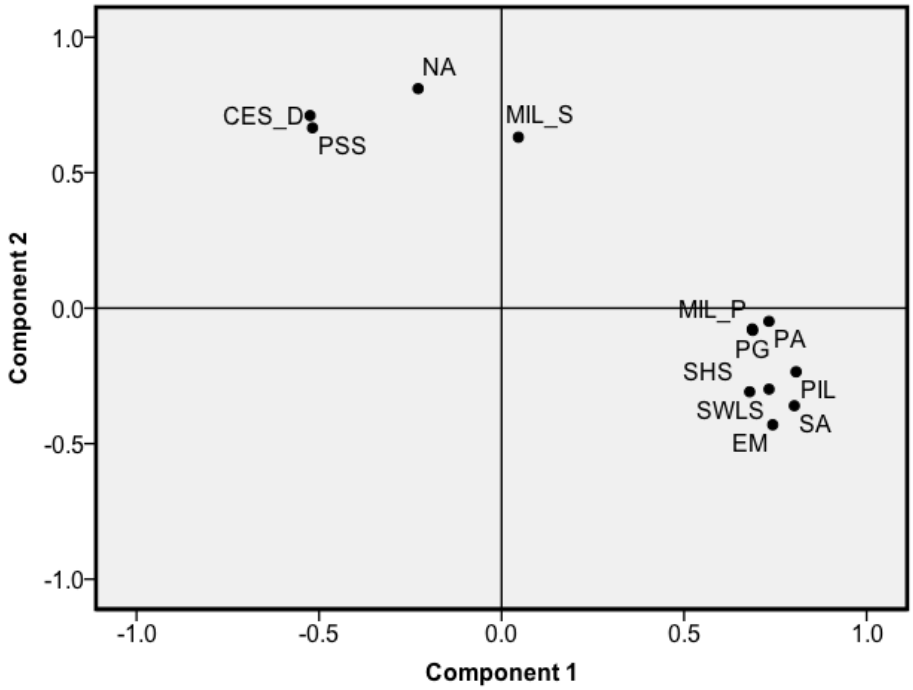

Figure 6.2 Component plot in rotated space

6.4 Method – Phase 2

The participants and saliva sampling protocol for this study are the same as described in Study I: Experiment 1 (see chapter 4). To remind the reader an overview will be provided.

6.4.1 Participants

Fifty female students that participated in phase 1 of the study were invited to take part in the second phase. First they were selected based on the well-being category they fell into (i.e. high or low well-being group) in phase 1. The high and low well-being participants were selected so cortisol profiles could be compared between participants exhibiting high and low well-being with the aim of increasing statistical power in a modest sized sample. Participants were also recruited on the basis that were not taking any prescribed medication or had suffered from any medical or psychiatric illness in the last two years. Participants received no financial incentive to take part in the study but did receive course credits. For one participant, samples on one of the study days

were outside the range of the cortisol assay detection range and a further four participants did not return their samples for two of the study days. One individual was older than the age of interest. All six participants were excluded from the analyses. The University of Westminster ethics committee approved the protocol. All participants provided written informed consent.

6.4.2 Materials

The study pack included study instructions, a saliva sampling kit (24 labelled salivettes), electronic devices used to record awakening and mood ratings, (actiwatch-score) and sampling times (MEM caps including the 24 cotton swabs for saliva sampling). The actiwatch-score was used to collect participants' mood ratings. The device was pre-programmed prior to the study to prompt participants to rate their mood at random times on each of the four days.

6.4.3 Procedure

Participants individually attended a research study induction session at the University of Westminster with the lead researcher (NS). During this session (duration 15-25 min) participants provided informed consent, received full verbal and unwritten instructions on procedures, practiced the techniques for collecting and recording times of saliva samples and practiced using the score function of the actiwatch-score. Participants were strongly encouraged to adhere to the saliva sampling protocol and were informed that their awakening and sampling times were being monitored electronically. The evening prior to the study days, participants were reminded of the upcoming study days via text messages. Participants were instructed to collect six saliva samples (samples 1-6: 0, 15, 30 and 45 min, and 3 and 12 hr post-awakening). Participants were instructed to awake in their usual way. During the saliva collection period participants were instructed to remain nil-by-mouth (except water). Samples were initially stored in a domestic freezer until they were returned to the laboratory to be stored at -20ºC until assayed. Participants were asked to complete the study diary on each day to recode awakening and sampling times. Text messages were sent to participants to remind them to collect samples 5-6. In line with the EMA method (Stone & Shiffman, 1994), throughout the day, participants rated their

mood at five random times. Participants were prompted to rate their mood via the wrist-worn actiwatch-score device. Participants rated on a scale of not at all (1) to very much (5), the extent to which they were feeling pleased, pleased, happy, stressed, down, frustrated, goal-directed, busy, and in control. The study materials and the saliva samples were returned to the researcher at the end of the study.

6.4.4 Cortisol assessment and assay

Cortisol assay was the same as in Study I: Experiment I (see chapter 4), determined by Enzyme Linked Immuno-Sorbent Assay.

6.4.5 Statistical analyses

Cortisol concentrations ranged between .004 and 55 nmol/l and values were moderately positively skewed. To normalise sample distributions for inferential analyses, square root transformations were performed.

The composite measures of cortisol were calculated for the CAR and diurnal cortisol cycle. CAR magnitude was calculated as the mean increase of cortisol from the first sample (MnInc: Mean [S2,S3,S4] – S1). In addition to the CAR magnitude, starting levels of cortisol at awakening (S1) was used as an outcome measure. The diurnal cortisol measures included the diurnal decline, the mean difference from S5 to S6 and accompanied with the mean levels over the day, the diurnal mean cortisol (S3 + S6 / 2). Actual timing of sample collection was based on electronic estimates of saliva sampling times relative to awakening (derived from actigraph estimated awakening and MEMs-determined sampling times) this was determined for each participant on each day. All days in which delay in sampling in the CAR period exceeded 15 minutes were excluded for analyses involving the CAR or S1. Sample delay in the CAR period (e.g. adherent <5 min, non-adherent 5-15 min) as specified in chapter 4 was used as a covariate in analyses involving the CAR and S1. Days in which samples 5 and 6 were collected earlier than 1 hr were excluded from analyses involving the diurnal cortisol cycle.

Pearson's correlations were conducted for the positive and negative mood ratings, and based on the direction of these associations mood ratings were combined to obtain a measure of state well-being and ill-being. The well-being or ill-being data were collapsed to obtain a day-level measure. State well-being and ill-being were person-centered. The difference between an individual's day score and mean score over four days was calculated (e.g. mean WB – well-being on day 1, day 2, day 3 or day 4). Trait well-being and ill-being components (from phase 1) were grand-mean centered so that an individual's well-being or ill-being score reflected individual's deviation from the sample mean (e.g. $WB_{participant} - WB_{mean}$).

The relationships between well-being or ill-being and cortisol measures were investigated using mixed regression modelling. First the relationship between state well-being and ill-being and cortisol rhythm was investigated. The question asked of the data was how does a woman's cortisol rhythm change in responses to fluctuations of her own usual pattern of well-being or ill-being. Next the relationships between trait well-being or ill-being and cortisol rhythm were investigated. The question asked of the data was how does each participant's cortisol rhythm change in responses to fluctuations in their own pattern of well-being or ill-being compared to the sample mean. The mixed regression dimension used in modelling cortisol outcome measures included participant identity (subject variable), and order of study day (repeated effect), and well-being or ill-being indicators (state or trait) and intercept as fixed effects parameters. A simple auto-regressive (AR1) co-variance structure was specified for the repeated measures effect of study days. The assumption of an AR1 co-variance structure for the repeated measures in the mixed regression models was supported as optimal in all cases. More complex models were initially run for all dependent variables to check that any emerging within-subjects effects of well-being or ill-being indicators were not confounded with any effects of the covariate of clock time of awakening and there were no interactions with day of study, which might have suggested well-being or ill-being effects were significantly restricted to a sub-set of the overall data. In all cases, no significant additional main or interactive effects emerged.

6.5 Results – phase 2

6.5.1 Participant characteristics for high and low well-being participants

Participants included in the analyses consisted of 24 high and 20 low well-being females. Given that participants were selected on the basis of their trait well-being score, as expected, trait well-being differed among the high and low well-being groups ($F_{(1, 43)} = 129.827$, $p < .001$), with the high well-being group exhibiting higher levels of well-being (see Figure 6.3). Significant differences in trait ill-being ($F_{(1, 43)} = 17.302$, $p < .001$) between the well-being groups were also observed, with the high well-being group exhibiting lower levels of ill-being (see Figure 6.4).

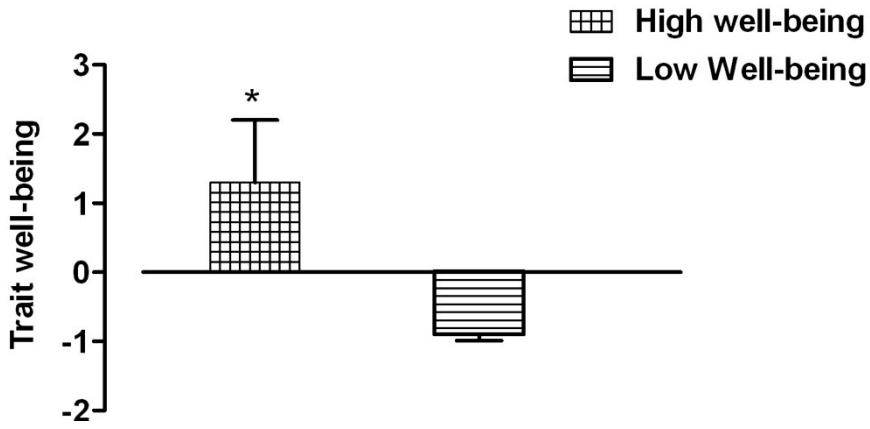

Figure 6.3 Differences in trait well-being between high and low well-being groups *p < .001

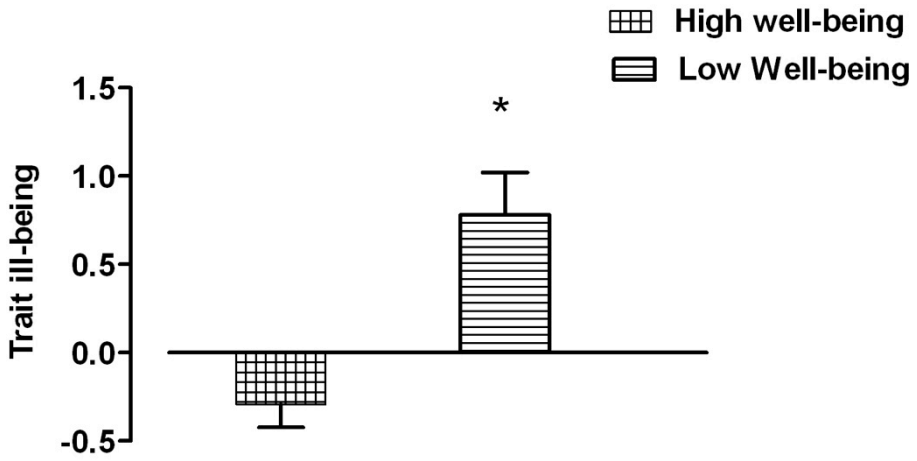

Figure 6.4 Differences in trait well-being between high and low well-being groups *p < .001

Table 6.5 presents the descriptives for the high and low well-being groups. Group comparisons on demographic and health variables were made. There were significant differences in perceived social status among the well-being groups, with the high well-being group perceiving higher social status. Significant differences emerged in self-reported health status, with the low well-being group reporting poorer health. The majority of individuals were non-smokers (N = 27, 61%) and there were no significant differences between smoking statuses among the well-being groups.

Table 6.5 Mean (±SD) demographics between the well-being groups

		WB Group [a] M (SD)	
	High	Low	Group differences
Age (yrs)	21.5 (3.1)	19.4 (2.8)	F = 2.739
SES	6.0 (1.3)	5.1 (0.8)	F = 5.496*
Health status	2.2 (0.79)	3.9 (0.9)	F = 29.621**
Smoker N (%)	6 (18 %)	8 (21 %)	X^2 = 3.626

*p < .05, **p < .001; [a] Well-being groups based on the trait well-being and ill-being components presented in phase 1 of this study.

6.5.2 General descriptives for state well-being and ill-being data

The participant's daily ratings of positive and negative mood made up the state well-being and ill-being variables. The mood data were based on a potential 880 observations. The majority of participants completed the full five mood ratings on each study day. Table 6.6 shows the percentages of completed mood ratings for each study day.

Table 6.6 Mood ratings N (%) completed by participants on each study day

			Mood ratings N (%)			
Study Day	0	1	2	3	4	5
1	0	2 (4.5)	2 (4.5)	5 (11.4)	12 (27.3)	23 (52.3)
2	3 (6.8)	2 (4.5)	3 (6.8)	5 (11.4)	10 (22.7)	21 (47.7)
3	2 (4.5)	2 (4.5)	6 (13.6)	6 (13.6)	6 (13.6)	22 (50.0)
4	0	2 (4.5)	5 (11.4)	4 (9.1)	11 (25.0)	22 (50.0)

Study day: 1 = Tuesday; 2 = Wednesday; 3 = Saturday; 4 = Sunday

Pearson correlations were conducted between the positive and negative mood ratings (see Table 6.7) to explore associations between the mood ratings and in turn categorise mood ratings into state well-being or ill-being. The positive mood items (happiness, pleased and goal-directed) were positively related with each other. The negative mood ratings (out of control, frustrated, stressed, and down) correlated in the expected direction. However, not all of the positive and negative mood ratings correlated with each other. For example feeling stressed was not correlated with feeling pleased or goal directed. Feeling frustrated was not correlated with feeling goal directed. All the other mood ratings correlated with each other in the expected direction. Based on the direction of these correlations state well-being and ill-being scores were computed. State well-being consisted of the positive mood ratings, including happiness, pleased and goal directed. State ill-being consisted of the negative mood ratings, including stressed, down, frustrated and out of control.

Table 6.7 Pearson correlations between the mood ratings

	Pleased	Goal Directed	Out of Control	Frustrated	Stressed	Down	Busy
Happy	.821**	.536**	-.659**	-.495**	-.451**	-.605**	.446*
Pleased		.395*	-.561**	-.328*	-.220	-.290*	.415*
Goal Directed			-.618**	-.240	-.253	-.440**	.496**
Out of Control				.670**	.466**	.560**	-.216
Frustrated					.767*	.755**	.089
Stressed						.738*	.132
Down							-.176

**$p < .0001$, *$p < .05$

Mixed regression modelling was used to investigate if the well-being groups exhibited different levels of state well-being and ill-being. As expected state well-being differed among the high and low well-being groups (F = 7.795, df = 1, 68.991, p = .007) (see Figure 6.5). The high well-being group exhibited lower levels of state ill-being over the four days compared to the low well-being group (F = 24.320, df = 1, 66.3, p < .001) (see Figure 6.6).

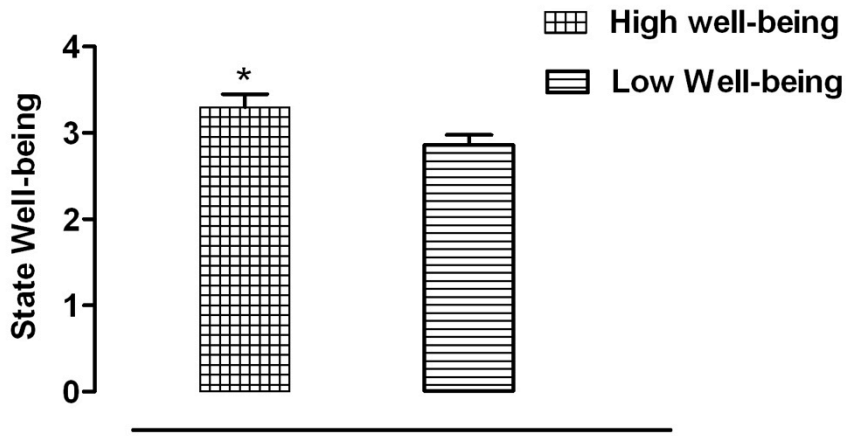

Figure 6.5 Differences in state well-being between the well-being groups
***p* < .001**

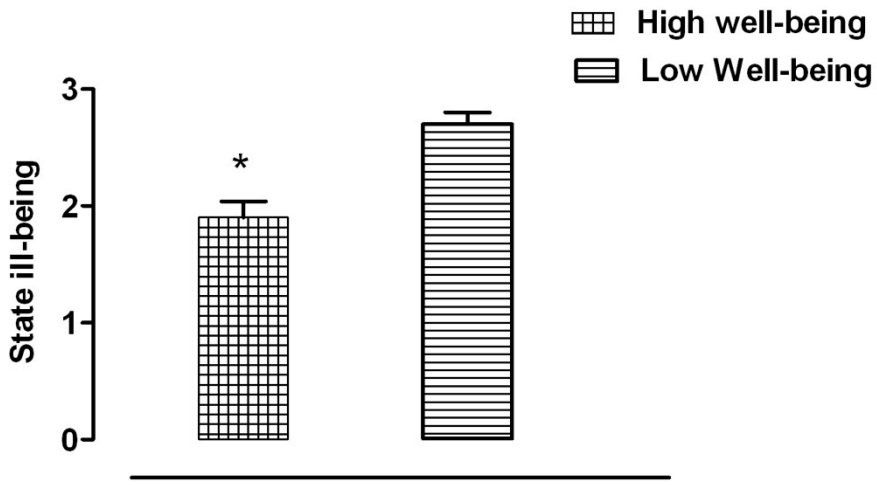

Figure 6.6 Differences in state ill-being between the well-being groups
***p* < .001**

6.5.3 The CAR: associations with well-being and ill-being

Figure 6.7 shows the mean diurnal cortisol pattern for all participants over the four days. There were no differences in the expected (mean) CAR or awakening cortisol levels over the four study days, on weekend or week days, or condition (e.g. 1st day Tuesday or Saturday). For results of this analysis see Appendix 23, Table 1. Demographic and health variables were not associated with the CAR or awakening cortisol levels (see Table 6.8).

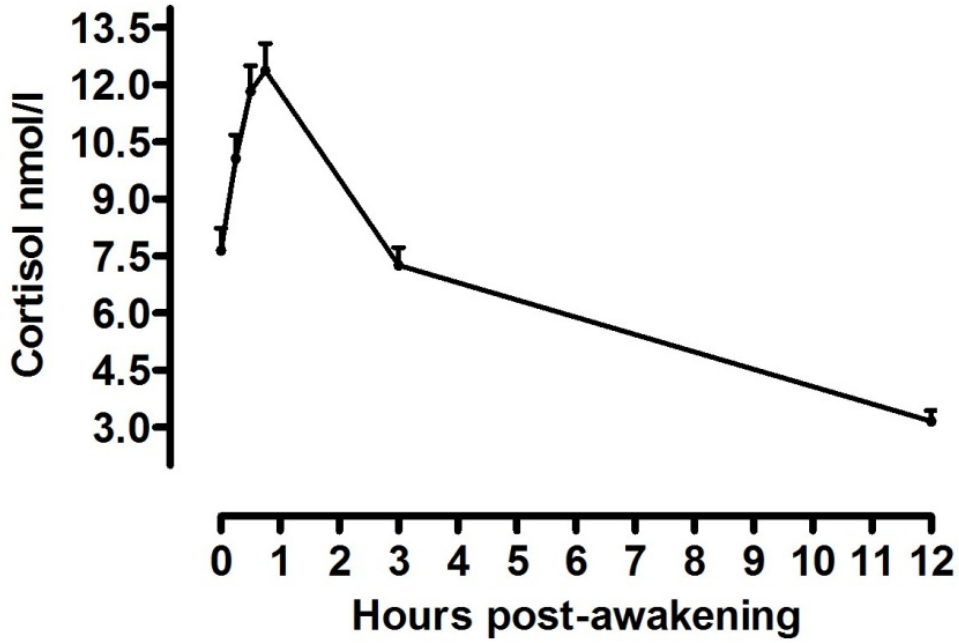

Figure 6.7 **Mean (+SEM) cortisol levels across four days. The diurnal pattern of cortisol (samples collected < 15 min delay)**

Table 6.8 Demographic and health variables and the CAR (MnInc) and awakening cortisol levels

Fixed effect	*MnInc* Coefficient (SE)	p	*S1 (SQRT)* Coefficient (SE)	p
Intercept	3.712 (1.203)	<.003	2.375 (0.236)	<.001
Non-smoker	1.993 (1.228)	.112	0.1533 (0.259)	.557
Intercept	6.953 (2.971)	.049	2.557 (0.592)	<.001
SES	-0.337 (0.508)	.510	-0.136 (0.102)	.895
Intercept	4.024 (1.678)	.021	2.829 (0.332)	<.001
Health status	0.424 (0.527)	.424	-0.119 (0.103)	.252

MnInc = mean increase; S1 SQRT = awakening cortisol levels

Results of the analysis exploring the relationship between the well-being and ill-being indicators and the CAR and awakening levels are presented in Table 6.9. The expected (mean) CAR for the overall sample on average state well-being days was 3.785, which was significantly different from zero. The estimated value of 1.417 represents how the CAR varied in response to deviations from a women's average level of state well-being. As a woman's state well-being was above the mean of their well-being for the four days their CAR was higher, but this was not significant suggesting no association between the CAR and fluctuations in a person's state well-being. The expected (mean) awakening cortisol level for the overall sample on average state well-being days was 2.536, which was significantly different from zero. The estimated value of 0.219 represents how awakening cortisol levels varied in response to deviations from a women's average level of state well-being. As a woman's state well-being was higher than their mean wellbeing over the four days their awakening cortisol levels were on average higher, however, this was not significant suggesting no association between awakening levels and fluctuations in a the women's state well-being. The expected (mean) CAR for the overall sample on average state ill-being days was 5.175, which was significantly different from zero. The estimated value of -1.038 represents how the CAR varied in response to deviations from a woman's average level of state ill-being. As a woman's daily ill-being was lower than their mean ill-being over the four days their CAR was reduced, but this was not significant suggesting no association between the CAR and fluctuations in a person's state ill-being. The expected

(mean) awakening cortisol level for the overall sample on average state ill-being days is 2.483, which is significantly different from zero. The estimated value of -0.153 represents how awakening cortisol levels vary in response to deviations from a woman's average level of state ill-being. As a woman's daily ill-being decreases from their mean ill-being over the four days their awakening cortisol levels was lower, but this was not significant suggesting no association between awakening levels and fluctuations in a person's state ill-being in the sample as a whole. The associations of trait well-being and ill-being with the CAR and awakening levels were also investigated. Neither trait well-being nor ill-being was associated with the CAR or awakening cortisol levels (see Table 6.9).

Table 6.9 The CAR and awakening levels associations with well-being or ill-being indicators

Fixed effect	MnInc Coefficient (±SEM)	*p*	S1 (SQRT) Coefficient (±SEM)	*p*
Intercept	5.174 (0.826)	<.001	2.483 (0.149)	<.001
State WB	1.417 (1.379)	.307	0.250 (0.206)	.229
Intercept	5.175 (0.828)	<.001	2.483 (0.149)	<.001
State IB	-1.038 (1.098)	.347	-0.153 (0.168)	.365
Intercept	5.203 (0.838)	<.001	2.480 (0.149)	<.001
Trait WB	-0.426 (0.473)	.929	-0.063 (0.091)	.491
Intercept	5.192 (0.839)	<.001	2.475 (0.149)	<.001
Trait IB	0.149 (0.614)	.810	0.093 (0.116)	.432

S1 SQRT = awakening cortisol levels

6.5.4 The diurnal cortisol cycle: associations with well-being/ill-being

Figure 6.7 shows the mean diurnal decline for all women over the four days. There were no differences in the expected (mean) diurnal decline or average cortisol levels over the four study days, on weekend or week days, or condition (e.g. 1st day Tuesday or Saturday). For results of this analysis see Appendix 23, Table 2. Demographic or health variables were not associated with the diurnal decline or average cortisol levels (see Table 6.10).

Table 6.10 Demographic and health variables and the diurnal decline and average cortisol levels

Fixed effect	Diurnal decline Coefficient (±SEM)	*p*	Mean diurnal cortisol Coefficient (±SEM)	*p*
Intercept	3.869 (0.624)	<.001	5.019 (0.586)	<.001
Non-smoker	0.585 (0.797)	.466	0.346 (0.747)	.645
Intercept	6.129 (1.583)	<.001	6.436 (1.675)	<.001
SES	-0.367 (0.277)	.190	-0.234 (0.293)	.430
Intercept	4.142 (1.007)	<.001	6.535 (0.927)	<.001
Health status	0.030 (0.322)	.245	-0.452 (0.296)	.134

Results of the analysis exploring the relationship between well-being and ill-being and the diurnal decline or average cortisol levels are presented in Table 6.11. The expected (mean) diurnal decline cortisol measures or average cortisol levels were not associated with fluctuations in participants' state well-being or ill-being. Nor was the diurnal decline or average cortisol levels associated with a person's deviation in trait well-being or ill-being from the sample mean.

Table 6.11 The diurnal cortisol cycle and associations with well-being or ill-being indicators

Fixed effect	Diurnal decline Coefficient (±SEM)	*p*	Mean diurnal cortisol Coefficient (±SEM)	*p*
Intercept	4.265 (0.389)	<.001	5.186(0.373)	<.001
State WB	-0.707 (0.390)	.419	-0.747 (0.576)	.198
Intercept	4.265 (0.385)	<.001	5.182 (0.382)	<.001
State IB	0.672 (0.793)	.398	0.954 (0.515)	.066
Intercept	4.227 (0.387)	<.001	5.233 (0.364)	<.001
Trait WB	-0.197 (0.307)	.523	0.089 (0.288)	.760
Intercept	4.228 (0.389)	<.001	5.232 (0.364)	<.001
Trait IB	0.100 (0.385)	.797	-0.060 (0.360)	.868

Next both well-being and ill-being indicators were included in the model to investigate if relationships were present with well-being and the diurnal decline or mean diurnal cortisol while controlling for ill-being (and vice versa). No main effects emerged. Further no consistent significant interaction effects between

well-being and ill-being indicators on the diurnal decline or average cortisol levels emerged. Since delay in collecting S5 and S6 did not influence estimate of diurnal decline or average cortisol measures they were not included in the model. To ensure that sampling delay did not influence the results sampling delay for collection of S5 and S6 were entered in the model but results were unchanged. Further, situational variables (e.g. awakening time, study day) were also entered into the model but results were unchanged.

6.6 Discussion

The main aim of this study was to investigate the relationship between the diurnal pattern of cortisol (the CAR and diurnal decline) and trait/state well-being independent of ill-being in a young healthy female sample. Well-being and ill-being was measured in two ways; firstly, a trait measure of ill-being and well-being was assessed using validated questionnaire measures. According to the factor analysis, ill-being and well-being were separate dimensions, and the subjective and psychological aspects of well-being were considered one dimension. Based on the well-being or ill-being component scores, participants categorised as high or low well-being completed the next phase of the study. Over four days in line with the EMA method positive and negative mood was assessed to provide a measure of state well-being and ill-being. On these days the CAR and diurnal cortisol profile were also assessed. To circumvent issues of non-adherence to the saliva sampling protocol, both awakening and sampling times were electronically monitored. Long delays in sampling in the CAR period (i.e. > 15 min) were excluded from analyses with the CAR. Moderate delays during the CAR period (i.e. 5-15 min) were controlled in analyses involving the CAR. Non-adherence for the diurnal decline was not an issue in this sample; however, to ensure accuracy, deviations in sampling (± 60 min) were excluded from analyses involving the diurnal decline or mean diurnal cortisol. In these healthy young females categorised as high or low well-being, trait/state measures of well-being/ill-being were not associated with diurnal patterns of cortisol, including, awakening cortisol levels, the CAR, diurnal decline or mean diurnal cortisol.

The null findings of the current study are difficult to interpret with respect to previous literature, which is limited by mixed findings. The current study failed to find significant associations between trait well-being and diurnal decline or mean diurnal cortisol. Based on the factor analysis the well-being component included measures of both subjective and psychological well-being. Others have found associations for lower cortisol and psychological well-being but not subjective well-being (Ryff et al., 2004). To ensure that using the well-being component did not miss relationships associations between cortisol patterns and the individual measures of well-being were also explored; however, no relationships were found. Trait well-being was not a stronger predictor of lower mean diurnal cortisol than trait ill-being. This differs from another study that showed well-being but not ill-being was related with lower cortisol across the day (Ryff et al., 2006; Ryff et al., 2004). Previous studies that assessed trait ill-being typically include negative affect or depression, and these measures were separately explored in relation to cortisol patterns. In the current study the ill-being component was investigated in relation to cortisol patterns included several measures of ill-being (e.g. negative affect, depression and search for a meaningful life), again associations with these individual measures and cortisol patterns were explored to ensure no relationships were missed. Other studies have found associations with morning cortisol levels and a combination of trait well-being/ill-being (Evans et al., 2007). A combination of well-being/ill-being in the current study and cortisol patterns was also explored however no associations were observed.

Relationships have been observed between higher aggregated positive affect or happiness and lower cortisol over the day (Steptoe & Wardle, 2005; Steptoe et al., 2005). However, cortisol was measured at spot samples over the day according to clock time. In the current study cortisol over the day was measured according to awakening time (i.e 3 and 12 hr post-awakening). The lack of associations with trait well-being and the CAR are consistent with (Steptoe et al., 2007) who also failed to find an association. However, did find a relationship with aggregated ratings of happiness and a smaller CAR in males. In the current study mood was also assessed by aggregated mood ratings over

the day, using this measure of well-being but no associations were observed with the CAR. However, the sample was female, previously CAR profiles have been shown to differ between males and females (e.g. Kunz-Ebrecht et al., 2004; Oskis et al., 2009; Pruessner et al., 1997; Wright & Steptoe, 2005). Other studies have also failed to find associations with the CAR and well-being measures, which is consistent with the current study.

The null findings in this young sample is consistent with another study which also failed to find associations between positive affect and lower cortisol in younger females (Jacobs et al., 2007). Together these studies suggest that the relationship between well-being and cortisol is not evident in healthy young females. Studies that have observed relationships between cortisol and well-being are in middle-aged and older individuals (e.g. Ryff et al., 2004; Steptoe et al., 2008; Steptoe & Wardle, 2005; Steptoe et al., 2005). This suggests that these associations are dependent on age of the sample, which supports the notion that the relationship between well-being and cortisol may change over the life span and may be particularly relevant to older individuals.

The main advantage of this study is the electronic-monitoring of adherence to the saliva sampling protocol. As seen in chapter 4 of this thesis, even moderate delays in sampling in the CAR period result in misleading CAR estimates. Inclusion of such erroneous CARs question the reliability of previous findings which have not accounted for non-adherence. This is the first study to account for the impact of moderate delays in associations of the CAR and psychosocial variables. Thus the lack of associations cannot be explained by non-adherence to the saliva sampling protocol since this was considered and accounted for in the analyses. The null findings may be attributed to the age of the participants such that well-being/ill-being is not associated with cortisol in young healthy females. It may be particular relevant for middle-aged or older individuals. Future research should directly compare associations between well-being and middle-aged or older individuals to understand how this relationship operates across the lifespan.

The study findings should be interpreted with caution. The intensive nature of the study meant that the sample size was quite modest. Participants were female students perceiving themselves as middle class and were educated. Further, individuals included exhibited either high or low levels of well-being on the trait measures. All these factors make it difficult to generalise findings. It would be interesting to examine the same associations in a male sample and in individuals exhibiting a range of well-being or ill-being scores.

In conclusion, this study failed to replicate previous associations between higher levels of well-being and patterns of cortisol secretion despite strict monitoring of adherence to protocol. This was not due to non-adherence since the study controlled for electronically monitored non-adherence. These findings may be attributed to the age of the sample, such that associations only emerge in middle-aged and older individuals, as previously shown. It must be considered that the findings reported come from a restricted and homogeneous sample of only modest size making it difficult to generalize findings.

Chapter 7 Study III

Exploring relationships between hair cortisol and well-being in young and old females

7.1 Introduction

Measurement of cortisol in saliva samples facilitates accurate investigation of the diurnal pattern of cortisol under ambulatory conditions and associations with psychosocial variables and health have been explored. Dysregulation of the HPA axis can include both high and low levels of cortisol as well as disrupted circadian rhythms. For example, chronic stress is associated with hyper-secretory cortisol patterns (high/flat cycles) and abnormally high overall cortisol levels are associated with major depression (Holsboer, 2000). Whilst hypo-secretory patterns (low/flat cycles) have been reported in patients with post-traumatic-stress-disorder (Rohleder, Joksimovic, Wolf, & Kirschbaum, 2004) and chronic fatigue syndrome (Crofford et al., 2004; Demitrack et al., 1991).

It is becoming increasingly evident that there are gender differences in cortisol secretory patterns. In earlier studies of the CAR, chronic stress was associated with a larger CAR in females compared with males (Schulz et al., 1998). Females were found to have higher cortisol in the morning compared to males (Wust et al., 2000). Additionally, on workdays, which are associated with more stress and less control and happiness CARs were higher compared to weekend days. The effect was more pronounced for females compared to males, on workdays (but not weekend days) suggesting that females may be particularly more sensitive to stress and anticipation of the day ahead than males (Kunz-Ebrecht et al., 2004). More recently, opposite patterns of cortisol secretory patterns for stress exposure in females are becoming increasingly evident. For example, chronically stressed females showed a smaller CAR and a lower

diurnal mean (O'Connor et al., 2009). Kumari et al. (2010) reported different cortisol patterns associated with stress in middle-aged individuals in terms of mean cortisol levels. Males exhibited higher mean cortisol levels and a reduced cortisol slope ('high and flat' cycle), whilst females had lower mean cortisol levels and a reduced cortisol slope ('low and flat cycle'). Likewise, depression was associated with hypo-cortisolemia in older females and hyper-cortisolemia in older males (Bremmer et al., 2007). Consistent with these findings, self-reported negative life events were associated with significantly lower cortisol levels in females (Witteveen et al., 2010). Further in a meta-analysis of cortisol and post-traumatic-stress-disorders, only females showed lower cortisol secretion and not in pooled samples of males and females (Meewisse, Reitsma, De Vries, Gersons, & Olff, 2007).

Typically the role of cortisol in ill-being and poor health are investigated. More recently (as discussed in chapter 6), the role of the HPA axis activity as a pathway mediating the relationship between well-being and health is being increasingly investigated. However, findings regarding the CAR are mixed, some studies showing the association between higher well-being and a smaller CAR (Steptoe et al., 2007), while others only find associations with mean levels of the CAR (Evans et al., 2007; Steptoe et al., 2007) or lower mean cortisol levels over the day (Ryff et al., 2004; Steptoe et al., 2008; Steptoe & Wardle, 2005). To the knowledge of the author, Study I (Experiment II, see chapter 6) of this thesis was the first to examine the role of the diurnal pattern of cortisol secretion in young healthy females (under the age of 30). In this study, no relationships between the diurnal pattern of cortisol and well-being or ill-being were observed. This lack of associations may be attributed to these females being young and healthy, supporting the neurotoxicity hypothesis (see Lupien et al., 2009), in which the effects of well-being or ill-being take a life time to impact on the HPA axis and therefore only becomes apparent in later life. It is proposed that in younger healthy individuals the HPA axis is more resilient to the effects of well-being/ill-being. Support for this is evident from changes in cortisol secretory patterns with age. Evidence suggests that cortisol changes with age, older individuals exhibit higher diurnal levels of cortisol than younger individuals (e.g. Raskind, Peskind, & Wilkinson, 1994). In a review of animal

and human studies chronic or repeated exposure to stress has enduring effects on the brain, through activation of the HPA axis and the release of glucocorticoids, with the highest impact on individuals going through age-related changes (i.e. adults and aging adults). In adulthood and old age the brain regions that undergo the most rapid decline as a result of aging are highly vulnerable to the effects of stress hormones, such as cortisol. However, the interacting vulnerability hypothesis (see Lupien et al., 2009) considers the way stress effects the brain in young individuals.

Gender differences are also evident for cortisol patterns and well-being. For instance, higher optimism was associated with less cortisol in the morning period for males. While females with high scores of optimism tended to show higher cortisol secretion in the post-awakening period (Lai et al., 2005). Also, females exhibiting low aggregated affect showed a low flat cycle whilst males shows a high flat cycle (Polk et al., 2005). Further, green space and average cortisol concentrations (but not the cortisol decline) differed in socially disadvantaged males and females. Higher neighborhood green space was related with higher cortisol levels in females but attenuated cortisol levels in males (Roe et al., 2013).

Although salivary cortisol enables measurement of the diurnal cortisol pattern, measure of cortsiol is acute with secretion of cortisol over minutes. The new method of assessment of cortisol in hair samples provides a retrospective trait measure of cortisol secretion over several months. Cortisol is incorporated into the hair shaft and hair grows approximately 1cm per month, thus 1cm of hair equates to one month of cortisol exposure (see chapter 2 for an overview of cortisol measurement in hair samples, including its validity as a measure of trait cortisol secretion). Unlike salivary cortisol there are no issues with non-adherence to the protocol since the researcher collects the hair sample. In this study measuring cortisol in hair enabled studying associations between cortisol and well-being in a larger sample allowing for comparison between young and old female samples. This is more difficult with salivary cortisol studies, largely due to participant non-adherence to the saliva sampling protocol, cost and timely nature of repeated saliva sampling. HCC has been investigated in

relations to ill-being, however, weak and inconsistent associations between HCC and stress-related psychosocial measures are observed even when the scores of these measures are consistently higher than controls (e.g. Stalder, Steudte, Alexander, et al., 2012; Van Uum et al., 2008; Yamada et al., 2007). Importantly, despite not showing associations between HCC and self-report measures, elevated HCC was evident in conditions assumed to exhibit increased levels of stress (Van Uum et al., 2008; Yamada et al., 2007). No studies have investigated associations between hair cortisol and well-being.

7.1.1 Aims

The aim of this study was to explore associations between HCC and well-being or ill-being in healthy females and to compare those associations between young and old females. The specific aims and objectives of the current study are displayed below (see Table 7.1).

Table 7.1 Specific aims and objectives of Study III

Aims	Objectives
Explore the structure of ill-being and subjective/psychological well-being	Conduct factor analysis to explore the structure of the trait ill-being and subjective/psychological well-being measures
Explore relationships between HCC and trait well-being independent of ill-being in young and old individuals	Explore associations between HCC and trait wellbeing and ill-being for the young and old sample. For any significant effects of well-being on HCC control for ill-being.
Investigate age differences in the relationship between HCC and well-being	Compare associations between HCC and well-being independent of ill-being between the young and old sample

7.1.2 Hypotheses

It was hypothesised that although there would be no relationship between HCC and well-being or ill-being in the young sample, there would be a significant relationship between HCC and well-being/ill-being in the old sample. The

direction of this relationship could not be predicted since both hyper- and hypo-secretory cortisol patterns have been observed in relation to well-being/ill-being in females.

7.2 Method

7.2.1 Participants

The young female participants (N = 91) were recruited from amongst the Psychology department at the University of Westminster, participant age ranged between 18-26 (19±2.1) years. The majority (86%) were single; ethnicity was mixed with the majority from a White (35%) or Asian background (38%). Only 16% were currently taking oral contraceptives. Requests from volunteers from among undergraduate psychology students were made via the Research Participation Scheme, in which participants received course credits for participating in the study. Wider recruitment was undertaken via announcements or flyers to postgraduate psychology students (including MSc and PhD level). Volunteers received no incentive to participate in the study. The older participants (N = 27) were recruited from among the University of the Third Age and Women's Institute. Participants recruited for the study were over the age of 65 (78±6.8) years and were retired or in part-time employment. The majority were widowed (52%) and all White British/Irish. The older participants were offered a small monetary award of a £10 high street voucher (7 participants received the voucher). All participants were selected on the basis that they were not pregnant, they had not taken any corticosteroids medication or had suffered from adrenocortical dysfunction in the last year and they had not taken any illicit drugs in the last 6 months. The majority of the sample (91%) was educated to at least A-level. The University of Westminster ethics committee approved the protocol and all participants provided informed written consent.

7.2.2 Psychosocial Measures

Information about demographic variables (age, smoking status, body mass index, ethnicity, education level, and employment status and medication or illness history) and hair-specific characteristics (washes per week, type of hair, hair treatment and natural hair colour) were obtained via self-report. Participants rated their general health as excellent, very good, good, fair or poor. In addition, they rated their perceived social status using the 1—10 'ladder' measure (Goodman et al., 2001). In relation to education, occupation and wealth participants indicated on a 1-10 step ladder their standing in society, with the top of the ladder represented a higher social standing.

Well-being and ill-being were assessed using validated scales (details provided in chapter 3). Subjective well-being was assessed using the 4-item subjective happiness scale (SHS; Lyubomirsky & Lepper, 1999) and the 5-item satisfaction with life scale (SWLS; Diener et al., 1985). Higher scores indicated higher subjective well-being. Eudemonic well-being was assessed by Ryff's well-being scales (Ryff, 1989), of which four dimensions were used including environmental mastery (EM), personal growth (PG), purpose in life (PIL), and self-acceptance (SA). Higher scores indicated higher psychological well-being. Ill-being was assessed using the 4-item perceived stress scale (PSS; Cohen et al., 1983) (PSS), and the 20-item depression, anxiety and stress scale (DASS; Lovibond & Lovibond, 1995). Higher scores indicated higher ill-being. All of the young participants completed these measures via an online questionnaire one week before or after providing the hair sample. The older participants had the choice of completing the questionnaire in the week before providing the hair sample via the online questionnaire or by pen-paper on the day of providing the hair sample.

7.2.3 Sample and collection preparation

Hair samples were taken near the scalp from a posterior vertex position. Cortisol concentrations were determined from the 3-cm hair segment most proximal to the scalp. Each segment was assumed to represent hair grown over a 3-month period, based on an average hair growth rate of 1-cm per month.

Hair samples were stored in labeled foil packages in a dry place. Samples were analysed by the Technical University in Dresden Germany. The wash and steroid extraction procedures followed the laboratory protocol described in detail in Stalder, Steudte, Miller, et al. (2012). Cortisol levels were determined using a commercially available immunoassay with chemiluminescence detection (CLIA, IBL-Hamburg, Germany), see Chapter 3 for more details.

7.2.4 Statistical Analysis

Comparisons of demographic, hair-related, health and psychological variables between the young and old groups were conducted. Factor analysis was conducted to explore the structure of the trait well-being and ill-being indicators. The well-being and ill-being indicators were analysed by means of principle component analysis, with varimax rotation. The various indicators of factorability indicated good factorability and the residuals indicated a good solution. For example, the KMO and Bartletts test was significant (statistic = .854, $p < .001$) indicating that there was a good amount of variance within the data explained by the factors. The anti-image covariance values were small which is indicative of good factor structure and the correlations showed that the KMO statistic for each variable included in factor analysis were all above .7 indicating that it was not necessary to drop any of the measures.

Kolmogorov–Smirnov tests revealed that cortisol data were not normally distributed. Log transformations were applied which effectively reduced the skewness statistic, and for three outliers log transformed values were winsorised. For illustration purposes Figure 7.4 presents the means and standard errors in original units (pg/mg). Pearson correlations or tests of differences were conducted between the demographic, well-being and ill-being components and HCC.

To assess the relationship between HCC and well-being and ill-being Pearson's correlations were conducted separately for the young and old groups. Mixed modelling regression analyses were conducted to investigate the relationship between HCC and well-being or ill-being components and participant age.

Using the mixed modelling command interaction effects between well-being or ill-being and age on HCC were also investigated.

7.3 Results

7.3.1 Participant Characteristics

Table 7.2 illustrates differences in participant characteristics such as demographic, and health variables among the young and old groups. No group differences emerged in perceived social status, although there was a trend for the older individuals to perceive their social status higher on the ladder measure. The older group reported their health status to be poorer in comparison to the younger group. There were few occasional or ex-smokers therefore smoking was recorded as a dichotomous variable to indicate current smoker or non-smoker. There were significantly more individuals categorized as a current smoker in the younger group. Ethnicity was mixed in the young sample with the majority from a white (49%) or Asian (32%) background. All females in the older group were white.

Table 7.2 Demographic, and health characteristics of young and old samples

	Young	Old	Group differences
SES M (SD)	5.9 (1.4)	6.5 (1.4)	$F = 3.849$, $p = .052$
White ethnicity N (%)	31 (35)	27 (100)	$X^2 = 32.875$, p < .001
Self-reported health status M (SD)	2.58 (0.9)	3.12 (0.9)	$F = 7.177$, p = .008
Taking regular Medication N (%)	13 (14)	19 (76)	$X^2 = 36.914$, p = <.001
Diagnosed illness N (%)	9 (10)	12 (50)	$X^2 = 19.580$, p <.001
Non-smoker N (%)	70 (73)	26 (100)	$X^2 = 6.564$, p = .010

There were no effects of medication use or suffering from an illness, on HCC ($p > .05$). Additionally hair characteristics, such as, hair treatments (e.g. colour, tints or perms), hair type (e.g. curly, wavy or straight), hair colour or number of hair washes per week did not influence HCC.

7.3.2 Structure of well-being and ill-being indicators

The measures of well-being and ill-being exhibited good reliability statistics (see Table 7.3). The well-being and ill-being indicators correlated in the expected direction (see Table 7.4). The well-being indicators were positively associated with each other and the ill-being measures were positively associated with each other. The well-being measures were negatively associated with the ill-being measures; except for the DASS stress measure, which was not associated with either personal growth or purpose in life. These associations suggest shared variance among the well-being and ill-being measures.

Table 7.3 Reliability and descriptives statistics for psychosocial measures

Scale	α	Mean (SD)	Min-max
SHS	.828	5.0 (1.1)	3-7
SWLS	.851	23.8 (6.0)	8-35
EM	.833	37.7 (7.6)	19-53
PG	.776	40.6 (6.7)	27-53
PIL	.751	38.5 (7.0)	21-54
SA	.849	37.3 (8.3)	16-54
PSS	.749	6.5 (2.9)	0-15
DASS stress	.836	13.0 (8.3)	0-38
DASS anxiety	.778	8.1 (7.5)	0-36
DASS depression	.859	8.7 (8.0)	0-40

SHS = subjective happiness scale; SWLS = satisfaction with life scale; EM = environmental mastery; PG = personal growth; PIL = purpose in life; SA = self-acceptance, PSS = perceived stress scale; DASS-D = Depression; DASS-A = anxiety; DASS-S = stress.

Table 7.4 Pearson's correlations for the well-being and ill-being indicators

	SWLS	EM	PG	PIL	SA	PSS	DASS-S	DASS-A	DASS-D
SHS	.492*	.525*	.388*	.528*	.566*	-.423*	-.432*	-.342*	-.589*
SWLS	.626*	.259*	.259*	.466*	.670*	-.530*	-.309*	-.269**	-.450
EM			.464*	.603*	.727*	-.626*	-.502*	-.507*	-.536*
PG				.754*	.581*	-.254*	-.126	-.186*	-.326*
PIL					.655*	-.330*	-.134	-.186***	-.414*
SA						-.539*	-.434*	-.370*	-.607*
PSS							.507*	.451*	.550*
DASS-S								.632*	.696*
DASS-A									.602*

* < .0001, ** p, .01, *** p < .05. SHS = subjective happiness scale; SWLS = satisfaction with life scale; EM = environmental mastery; PG = personal growth; PIL = purpose in life; SA = self-acceptance, PSS = perceived stress scale; DASS-D = Depression; DASS-A = anxiety; DASS-S = stress.

In order to explicitly test the structure of the well-being and ill-being indicators exploratory factor analysis was conducted by means of principle component analysis. Two components with an eigenvalue greater than 1.0 were found. The screen plot (see Figure 7.1) also indicated two components.

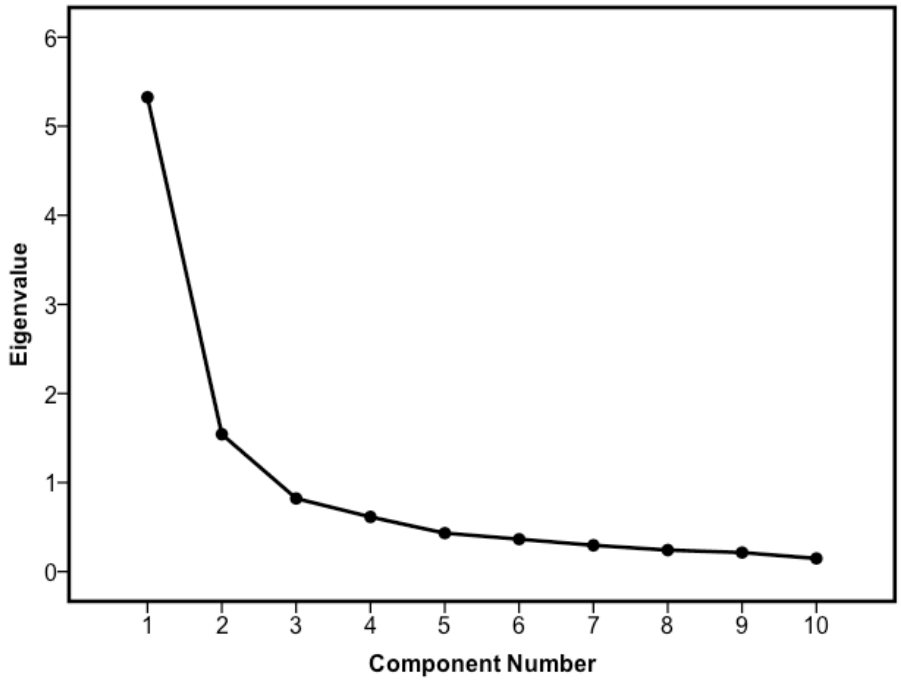

Figure 7.1 Scree plot showing the eigenvalues (> 1)

Table 7.5 displays the component matrix, which shows the loadings of each measure on the components 1 and 2 prior to rotation. The majority of measures were correlated with component 1. The pattern matrix shows the factor loadings after rotation, it shows the unique relationship between each component and each measure. The PSS, depression, anxiety and stress (DASS) scales are strongly associated with component 1. The SHS, SWLS, EM, PG, PIL and SA are strongly associated with component 2.

Table 7.5 Factor analysis of measures of SWB, PWB and IB.

	Component Matrix		Rotated component matrix	
Measure	Component		Component	
	1	2	1	2
SHS	.746	-.032	-.567	.486
SWLS	.716	.118	-.442	.475
EM	.848	.070	-.572	.630
PG	.576	.608	-.006	.838
PIL	.688	.608	-.087	.914
SA	.858	.236	-.465	.758
PSS	-.729	.210	.676	-.344
DASS-D	-.804	.299	.791	-.331
DASS-A	-.620	.504	.797	-.055
DASS-S	-.660	.583	.880	-.025

Note. Presented is the component matrix showing the correlations between components 1 and 2 before rotation and the rotated component matrix correlation between component 1 and 2 after rotation. SWB = subjective well-being; PWB = psychological well-being; IB = ill-being; SHS = subjective happiness scale; SWLS = satisfaction with life scale; EM = environmental mastery; PG = personal growth; PIL = purpose in life; SA = self-acceptance, PSS = perceived stress scale; DASS-D = Depression; DASS-A = anxiety; DASS-S = stress.

The two components accounted for 69% of the total variance explained by the solution to the factor analysis. Component 1 accounted for 53% and component 2 accounted for 15%. Figure 7.2 shows the component plot in rotated space. Component 1 represents ill-being and component 2 represents well-being. On the components a positive values indicates a high score and negative value indicates a low score. These components were explored in relation to demographic variables and HCC in the young and old groups.

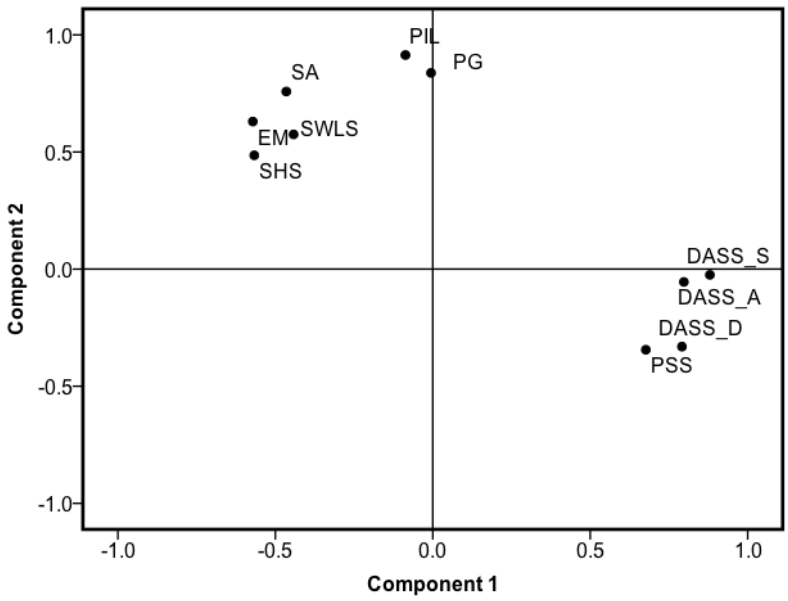

Figure 7.2 Component plot in rotated space

Differences in scores on the well-being and ill-being components among the young and old groups were investigated. Scores on the well-being component did not differ between the two groups (F = 1.513, df = 1, 115, p = .218). Scores on the ill-being component were significantly higher for the younger group (F = 13.895, df = 1, 115p < .001). Figure 7.3 displays the differences in means (±SEM) of the well-being and ill-being components.

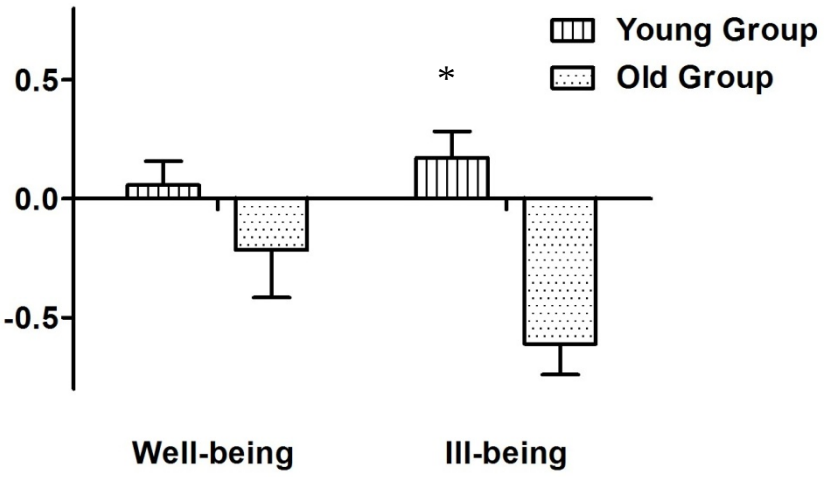

Figure 7.3 Means (± SEM) differences in well-being and ill-being components for young and old groups *p < .001

The well-being component was not associated with perceived social status (*r* = .140, *p* = 152) or self-reported health status (*r* = -.150, *p* = .108). Also there were no differences in well-being scores between smokers and non-smokers (*F* = .025, *df* = 1, 114, p = .874) or white and other ethnic groups (F = .186, *df* = 1, 112, p = .667). Ill-being was associated with lower perceived social status (*r* = -.353, *p* < .001) and poorer self-reported health (*r* = .195, *p* = .036). Ill-being was higher in smokers compared to non-smokers (*F* = 24.868, *df* = 1, 114, *p* = <.001) and lower in white females compared to other ethnic groups (*F* = 6.166, *df* = 1, 112, *p* = .015).

7.3.3 Hair cortisol concentrations: relationships with participant characteristics

Differences in HCC between the young and old groups were investigated. HCC differed between the young and old groups (*F* = 5.087, *df* = 1, 114, *p* = .026). The older group secreted significantly more cortisol over the past three months compared to the younger group (see Figure 7.4). HCC was not significantly associated self-reported health status (*r* = .065, *p* = .494) or with perceived social status (*r* = -.001, *p* = .992). HCC did not significantly differ among smokers or non-smokers (*F* = 1.486, *df* = 1, 111, *p* = .225).

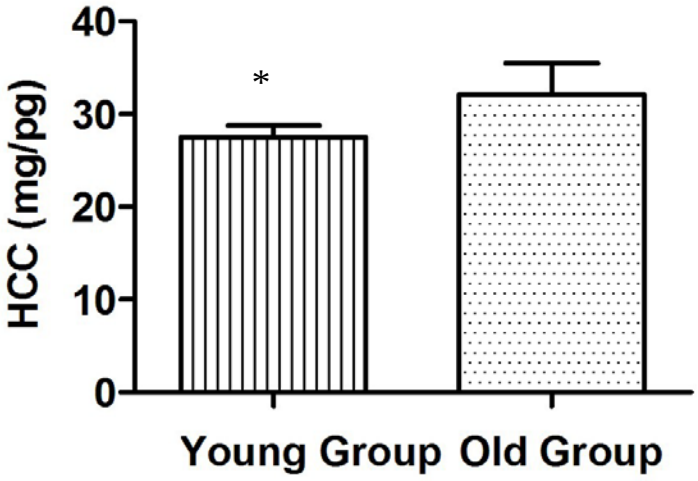

Figure 7.4 Mean (±SEM) differences in HCC for young and old groups *p < .001

7.3.4 Hair cortisol concentrations: relationships with well-being and ill-being components

Relationships between HCC and the well-being and ill-being components were conducted separately for the young and old groups. In the young group, HCC was not significantly related with the well-being (*r* = .140, *p* = .193) or ill-being (*r* = .159, *p* = .139) components. In the older group, HCC was significantly positively related with the well-being component (r = .501, p =.009) but not the ill-being component (r = -.229, p = .260). In older individuals higher HCC was associated with higher levels of well-being (see Figure 7.5).

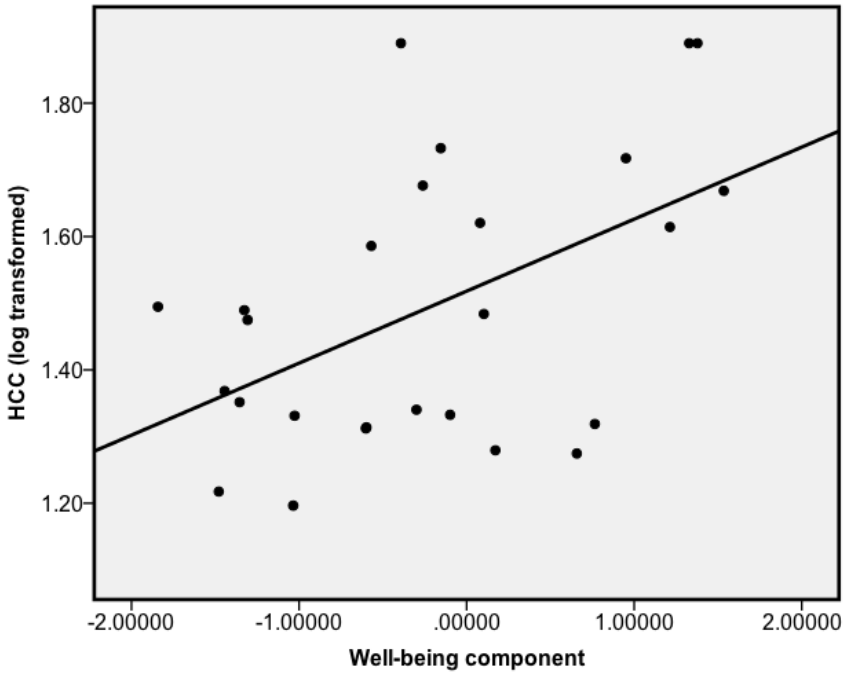

Figure 7.5 Scatterplot showing the relationship between HCC and well-being in older individuals - higher HCC is associated with experiencing higher well-being

7.3.5 Age differences: associations between HCC and well-being/ill-being

The relationship between HCC and well-being in both age groups was investigated by means of mixed regression analysis. This analysis was deemed most appropriate since an interaction between two continuous variables was being explored. The questions asked of the data were whether there were main effects of both well-being and age on HCC and if there was an interaction effect of well-being and age on HCC.

There was a main effect of participant age on HCC (F = 9.649, df = 1, 109, p = .002). The younger group secreted significantly less cortisol (M = 0.11, SE = .002) than the older group. There was a main effect of well-being on HCC (F = 12.144, df = 1, 109, p = .001). An increase in scores on the well-being component was associated with increases in HCC (M = 0.108, SE = .032). A significant interaction between age and well-being score on HCC was found (F = 5.829, df = 1,109, p = .017). The interaction effect shows that higher well-being was associated with higher HCC in the old group but not the young group (see Figure 7.6.). The same analysis was conducted exploring the effect of ill-being and age on HCC. There was no main effect of ill-being (F = 0.913, df = 1, 109, p = .350) or age (F = .882, df = 1, 109, p = .341) on HCC. There was a trend for an interaction effect of ill-being and age on HCC (F = 3.369, df = 1, 109, p = .069). When ill-being was included in the analysis of age and well-being effects on HCC in order to ensure the effects observed were independent of ill-being, the results were unchanged.

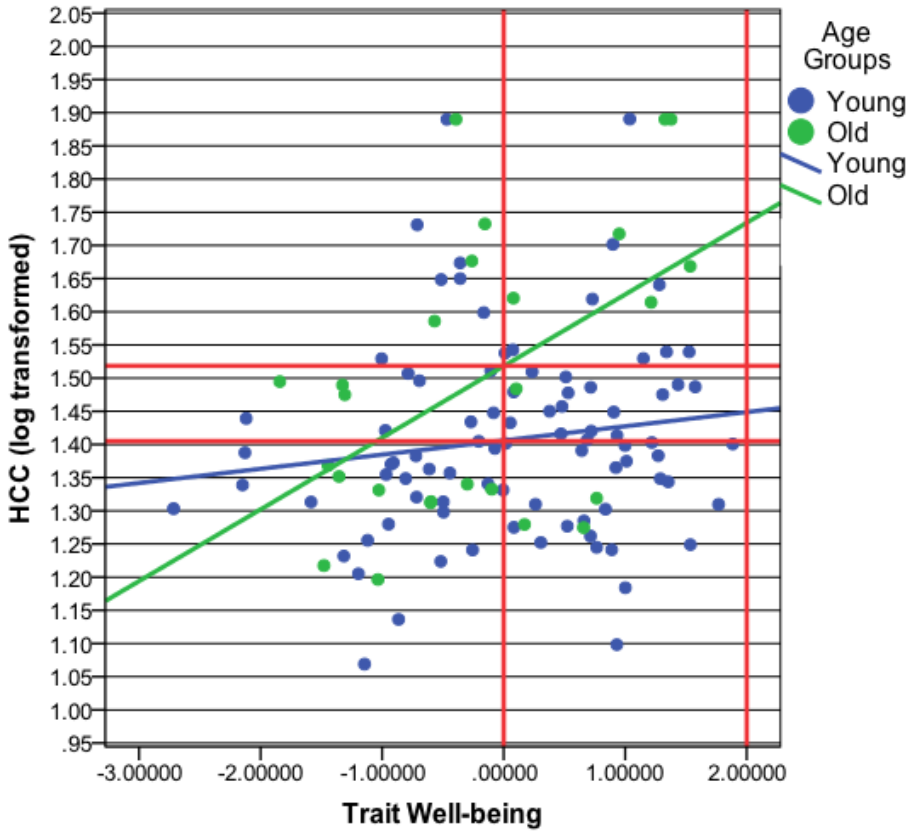

Figure 7.6 Interaction effect of age and well-being on HCC
It shows higher well-being is associated with higher HCC in the older individuals but not for younger individuals. The red vertical lines represent X=0 (and also X= 2) and the red horizontal lines represent the correct intercept points

7.4 Discussion

The main aim of this study was to compare the association between HCC and well-being or ill-being in two distinct age groups; younger (aged between 18-26 years) and older (ager +65 years) females. As hypothesized no relationship with HCC and well-being or ill-being were evident in the group of young females. Whilst for the older group, higher levels of well-being were associated with higher HCC, but no association for ill-being and HCC was found. The relationship found between HCC and well-being was independent of ill-being suggesting that well-being has a unique relationship with cortisol in the older age group.

Previously studies have shown that higher levels of psychological well-being was related with lower cortisol in older females (Ryff et al., 2004). Also aggregated ratings of positive affect were associated with lower mean daily

cortisol in middle-aged males and females (Steptoe et al., 2008; Steptoe et al., 2005). In Study I (Experiment II) of this thesis (chapter 6) cortisol and well-being were not related in healthy young females. The current study was the first to directly investigate age-differences in the relationship between well-being and cortisol. The finding that well-being or ill-being are not associated with HCC in young healthy females is consistent with findings from Study I (Experiment II). These findings provide evidence for the neurotoxicity hypothesis. In a healthy population without any psychopathology well-being or ill-being may not exert their effects on the HPA axis early in adulthood. In this study, the younger females exhibited significantly lower levels of ill-being than the older group but this was not enough to exert effects on the HPA axis. The finding that well-being is associated with HCC only in older individuals suggests that the effects of well-being are not exerted until later in life in healthy individuals. The finding that ill-being is not related with HCC in these older females suggests that the effects of well-being are a stronger predictor of cortisol activity than ill-being.

There was a trend for an interaction between age and ill-being on cortisol. The direction of the relationship between ill-being and cortisol in older females is in line with previous literature suggesting that lower levels of cortisol is associated with ill-being in females (e.g. Bremmer et al., 2007; Kumari et al., 2010; Witteveen et al., 2010). In a larger sample or with poorer levels of ill-being this interaction may have been significant thus a relationship between ill-being and HCC cannot be ruled out. However, this is consistent with previous studies, in which weak or no relationships are found for psychosocial stress measures and HCC (see Stalder, Steudte, Alexander, et al., 2012). It may also be possible that in normal healthy populations it is difficult to relate aberrant cortisol levels with trait variables. Further to this, the lack of association may be a methodological issue with the measurement of trait variables and hair cortisol. The ill-being measures used in this study are limited by a short recall period whereas the measure of hair cortisol is over several months. As suggested previously, alterations in long-term cortisol secretion as measured by HCC may assess a distinct aspect of chronic stress exposure than captured by individuals' subjective experience (Stalder, Steudte, Alexander, et al., 2012). No study to date has investigated associations between well-being and HCC. This is the

first study to show that well-being is related with HCC. The well-being measures used in this study do not specify a recall period; participant's general feelings of well-being were assessed and so may relate more strongly to HCC. A more intensive study of measuring ill-being using the EMA method may help understand how ill-being such as stress and depression are related with HCC.

Smoking has been associated with higher cortisol levels (Badrick et al., 2007), however, this is unlikely to explain the finding of higher cortisol in older females since all older females reported being non-smokers. Others have found that higher cortisol is associated with sleep problems, such as shorter sleep duration (Spiegel, Leproult, & Van Cauter, 1999). This is an important consideration since impairments in sleep quality (e.g. poorer sleep efficiency, greater sleep disturbances) are associated with aging (for a review, see Bloom et al., 2009). From this study it cannot be determined if sleep quality had a role in higher HCC as sleep quality was not investigated so this association cannot be ruled out completely.

In this sample of healthy females it is possible that higher cortisol secretion is indicative of being busier and energised. It may be that cortisol is acting as an energiser in older females with higher levels of well-being. Evidence for cortisol as an energiser is suggestive from studies showing that hypo-cortisolism is associated with pathologies characterized by exhaustion and fatigue. For example low levels of cortisol are implicated in chronic fatigue syndrome (Crofford et al., 2004; Demitrack et al., 1991). Further to this low levels of cortisol are associated with fatigue in normal healthy populations and not just clinical populations. For instance, low cortisol in the morning predicted fatigue later in the day in (Adam et al., 2006). In a longitudinal analysis of the Whitehall II cohort of community dwelling adults, lower waking cortisol levels and flatter daily cortisol slope was associated with fatigue (Kumari et al., 2009).

Evidence for cortisol acting as a energiser in normal healthy populations comes from the finding that a more responsive HPA axis in terms of higher cortisol levels was associated with better physical functioning in older males (Gardner et al., 2011). Thus the findings indicate that higher cortisol levels in a normal healthy older sample may be indicative of a healthier HPA axis. The CAR is

also thought of as a ‘boost’ enabling individuals to deal with the upcoming demands of the day. Adam et al. (2006) suggests that the CAR is an adaptive response that helps individuals to meet anticipated demands of the day ahead. Evidence to support this comes from larger CARs evident in healthy individuals (Kudielka & Kirschbaum, 2003) and the CAR can be larger on work compared to week days (Kunz-Ebrecht et al., 2004; Schlotz et al., 2004). Further the CAR was associated with study-day anticipations of the level of obligations; days anticipated to be busier were associated with an elevated CAR (Stalder, Evans, et al., 2010; Stalder et al., 2011). Of course HCC is a blunt measure, as it does not illuminate any dynamic of cortisol secretion: we cannot deduce associations with the CAR or any other aspect of the circadian cycle. However it is clear that HCC can be determined by a variety of different circadian patterns and low post awakening cortisol may be an important contributor in this study. Such a possibility deserves further investigation.

In this study higher well-being was restorative of cortisol levels in older females. This is consistent with previous studies showing that in females higher cortisol was associated with positive affect (Polk et al., 2005) and green space (Roe et al., 2013). Thus in this sample of older healthy females well-being was associated with healthy cortisol levels in terms of having higher levels of cortisol which may be characteristic of being highly functioning, outgoing and busy and the finding that ill-being was not associated with HCC suggests that the females in this study were well balanced and not at risk of cortisol pathologies.

Although this study was cross-sectional in design and therefore cannot demonstrate causality, it provides evidence to help untangle the complex interaction between age, well-being and health. This study was limited by the unequal group sizes of the young and old females. However, the effect of well-being was strong enough to be detected in this small group of older females. The sample may not be representative of the population as a whole; the older females in this study were of middle social-economic status as perceived by themselves and were healthy and highly functioning. Thus, the relationship observed between well-being and cortisol may not extend to other female populations. The young group was ethnically diverse, but the females in the old group were all white. To ensure that results were not influenced by ethnicity in

the younger sample, comparison of cortisol, well-being and ill-being were made between the different ethnic groups. Only ill-being differed between ethnicity group, White individuals exhibited lower scores on ill-being compared to other ethnic groups. Analyses were conducted on just white young females and the result remained unchanged therefore, it is unlikely that the mixed ethnic groups in amongst the younger females influenced the findings.

Future work could examine the cognitive function of this population of individuals exhibiting better well-being and higher cortisol. Previous research has shown that poorer cognitive performance is associated with an attenuated CAR and lower decline in cortisol over the day (Evans et al., 2011). It is likely that in this sample of healthy females the high cortisol levels would not be at a high enough level to impact on cognitive function and they are within the healthy range and are not dangerous levels instead they are restorative and healthy. Table 7.6 compares HCC of the current sample with study of alcoholics. Determination of HCC was in the same laboratory so values can be compared. HCC is higher than healthy controls, however, the controls were a younger sample and HCC is known to be higher in older adults (Dettenborn, Tietze, et al., 2012). The HCC was not high enough as found in the alcoholic patients. This provides support for the assumption that the high HCC found in the current study are within healthy range.

Table 7.6 HCC in healthy adults and clinical samples

	HCC mg/pg M, SD	Age of sample
Current study	30.0 ± 11.8	78 ± 6.8
Healthy older females		
[a]Alcoholics	52 ± 43	44 ± 11
Matched controls	16 ± 13	44 ± 11

[a] values from (Stalder, Kirschbaum, et al., 2010)

It is unknown from this study at what age the effects of well-being start to exert its effects on the HPA axis since comparisons were only made between young and older females. Future research would benefit from including a group of middle-aged individuals or even better a longitudinal analysis to tease out the point in which well-being or ill-being exert its effect on the HPA axis.

In conclusion, this study showed that in these healthy young females, there were no relationships between hair cortisol and ill-being or well-being. This indicated that healthy young females tend to be resilient, and thus no impact on their physiology, in terms of cortisol. In these healthy older females, ill-being was not related with hair cortisol either. However in this group there were associations between higher hair cortisol and well-being. The results provide support for the neurotoxicity hypothesis. They indicate that in healthy aging well-being, rather than ill-being, is more closely associated with the health-related hormone cortisol. These results indicate that high levels of well-being in older people may have a role as an energizer.

Chapter 8 General Discussion

The importance of cortisol cannot be doubted; it is the only hormone that we cannot live without. It is a product of neuroendocrine system activity, released in response to internal and external triggers that activate the HPA axis. However, what makes cortisol particularly interesting and important for healthy functioning is its primary and essential role as a master hormone with responsibility for regulating physiological function around the 24-hour light/dark cycle. In healthy individuals, cortisol exhibits a marked circadian rhythm characterised by peak levels following morning awakening (known as the cortisol awakening response: CAR) and declining levels thereafter, reaching lowest levels in early sleep. Dysregulation of cortisol secretion is attributed to the cumulative effect of chronic stress and is thought to be a prime mediator of associations between stress and adverse health outcomes (Clow & Hamer, 2010). More recently, cortisol is thought to mediate the effects of well-being on favourable health outcomes (Dockray & Steptoe, 2010). The current work expands on previous published literature on the role of cortisol in the beneficial effects of well-being in healthy females. Along with this it highlights and identifies methodological issues involved in measurement of the CAR. In doing so it challenges previous findings of what constitutes a tolerable delay in saliva sampling for measurement of the CAR and informs best practice methodology for the ever-increasing interest in CAR research. The studies presented in this thesis should be viewed as components of an overall research programme. The rationale for study II and III were borne out of findings from study I. Accordingly, in this chapter an attempt is made to discuss and integrate the results of the three studies as a whole.

8.1 Participant non-adherence to saliva sampling protocol

Participants were encouraged to collect saliva samples according to the desired times (i.e. 0, 15, 30 and 45 min, and 3 and 12 hr post-awakening). They were encouraged to be honest and accurate in reporting their awakening and saliva

sampling times and were aware that electronic devices would be used to verify these timings. Participants were adherent to the protocol in terms of reporting accurate saliva sampling times, and collecting samples (after awakening) according to the desired protocol intervals. This is mostly likely due to participants 'aware status', previous research shows that informing participants of electronically monitoring saliva sampling times increases their adherence to protocol in terms of collecting samples in accord with the desired sampling times and reporting accurate saliva sampling times (Broderick et al., 2004; Kudielka et al., 2003). Together these findings provide support for informing participants of electronic monitoring as a method of increasing their adherence to the protocol.

Participant self-reported awakening times were not accurate; self-reports were significantly later than actigraph. This is consistent with previous research showing discrepancies in awakening times between self-reported and actigraph (DeSantis et al., 2010; Dockray et al., 2008). This resulted in a delay in collecting the first 'awakening' sample, and delay for the CAR period was attributed to this period, since S2-4 (15, 30 and 45 min post-awakening) were collected very closely in accordance with the desired 15 minute intervals. The fact that participants were accurate and honest in reporting their saliva sampling times suggests that they were not deliberately reporting inaccurate awakening times. Participants' self-reported awakening times may have been accurate to the best of their knowledge, but they were not aware of the exact timing of awakening and this may be related with a co-incident sleep inertia, but this needs to be tested in future research.

Moderate delay (average 8 min) between awakening and collection of the 'awakening' sample resulted in misleading CAR measurements; no difference in cortisol concentrations at S1, but magnitude of the cortisol increase was overestimated in the first 45 minutes post-awakening and the peak in cortisol was earlier at 30 minutes compared to the more typical female peak at 45 minute post-awakening (Oskis et al., 2009). These findings differ from previous research showing opposite effects of delay on the CAR (i.e. higher S1 and attenuated CAR). The novelty of these findings is that previously thought 'tolerable' moderate delays lead to erroneous CAR measurements and raises

serious concerns over the validity and reliability of previous research on the CAR magnitude and the more recent interest in timing of the CAR peak. These findings were indicative of a hypothesised time lag in cortisol secretion between awakening and the start of increased cortisol secretion characteristic of the CAR. This hypothesis was tested with frequent saliva sampling in the CAR period. Sampling every five minutes for 30 minutes following awakening, showed that cortisol does not start to significantly increase until 15 minutes post-awakening. The impact of inaccuracy of participant-estimated awakening time and the consequent delay in collection of S1 on CAR measurement emphasises the need for electronic measures of awakening in CAR research; self-reported estimates at this time are not sufficiently reliable. The effect of delay on the CAR was not due to situational or participant variables (e.g. study day, awakening time, psychosocial variables). A person could be adherent on one day and non-adherent on the other day.

The observed 10 minute time lag between awakening and rise in cortisol levels explains the finding that awakening cortisol levels were not different between the adherent and moderately non-adherent day data in Study I (Experiment I). A moderate delay (5-15mins) in collection of samples would lead to the assumed 'awakening' sample being collected during the time lag period when cortisol levels are relatively stable (i.e. no dynamic increase during this time). The over-estimated CAR magnitude found in Study I (Experiment I) would be a consequence of the real-time CAR-assessment period being shifted just sufficiently along the time axis to maximise the average level of cortisol measured in subsequent samples relative to the first sample but not being shifted sufficiently for that average to be influenced unduly by significantly lower post-peak values (unlike for longer delays > 15 min). The peak of cortisol secretion would occur up to 15 minutes earlier than if the first sample was actually collected at the moment of awakening, which explains why the timing of the peak is earlier. This time lag could be explained in terms of the ultradian pulsatile pattern of HPA activity and the conversion of cholesterol to cortisol following stimulation of ACTH at the adrenal cortex.

Normative cortisol concentrations for the CAR have been reported in healthy adults; however the values of salivary free cortisol concentration vary between

studies (see Clow et al., 2004). The cortisol values in Study I fell within the range of previous studies (4.7-18.5 nmol/l for awakening sample, and 8.6-21.9 nmol/l for samples collected at 30 min post-awakening), however values in Study I are generally in the lower range for normative data (see Table 8.1). This variation may be due to the different assay protocols, an issue that has been explored in an inter-laboratory comparison employing different methods for determination of salivary cortisol (Garde, Hansen, & Nikolajsen, 2003).

Despite these variations in absolute values the reported *change* in cortisol concentration from awakening till 30 minutes post-awakening in healthy adults is relatively consistent between studies with an approximate average increase of 9.3 nmol/l (±3.1 nmol/ l), ranging between 4.0-15.0 nmol/l, representing percentage increases of between 50-160% (see Clow et al., 2004). The mean increase is lower in study I is significantly lower than the normative data (see table I). This could be due to the mean increase in Study I being based on adherent data (< 5 min delay in collection of awakening sample), whereas the normative data may include moderate delays in collecting the awakening sample resulting in overestimation in mean increase during the first 45 minutes post awakening (as shown above).

Table 8.1 Cortisol concentrations and mean increase from 0 to 30 min post-awakening for adherent data from Study I and normative values derived from 12 published studies (see Clow et al., 2004)

	Saliva samples (collected post-awakening)				Cortisol MnInc (nmol/l)
	0 min	15 min	30 min	45 min	
Study I[a]	8.3	9.9	11.2	12.4	3.0
Mean (±SD) of normative data	11.6(4.6)	16.3(5.6)	20.0 (5.9)	18.9 (6.5)	6.8

[a] Adherent data in Study I (< 5 min delay between awakening and collection of S1)

Delay in collecting saliva samples across the day (at least at 3 and 12 hrs post-awakening) did not impact on the diurnal decline or mean diurnal cortisol. This was expected given the steady decline in cortisol levels over the day. This is consistent with another study, which showed that delays in random spot samples collected over the day did not result in misleading diurnal cortisol

measures (Jacobs et al., 2005). However, other studies have shown that the diurnal decline was flatter when sampling was delayed but the diurnal decline was anchored from the CAR, which was flat due to delays (>10-15 min) in the post-awakening period (Broderick et al., 2004; Kudielka et al., 2003). It is a convenient finding that non-adherence is not an issue for diurnal cortisol measures and may help explain why more consistent relationships with the diurnal cortisol profile and psychosocial variables have been found compared with the CAR. In this study the diurnal cortisol profile was calculated as the simple measure of decline from 3 to 12 hr post-awakening. Additional samples points (i.e. 6, and 9 hrs post-awakening) provide the best measure of the diurnal cortisol profile. It cannot be determined from this study that delay in collection of saliva samples for these times would result in misleading measurements of diurnal cortisol profile measured with all these samples.

8.1.1 Measurement of cortisol in hair samples

Cortisol in hair samples is a relatively new area of research. It is important to examine how HCC relates to previously published studies. In study III, two distinct age groups were recruited, a young and old group, HCC differed among these groups. HCC was examined for the young women in comparison to a published study. In study III the sample were young healthy female students, and HCC was examined in relation to healthy young adults. The comparison sample were mostly university students (i.e. similar age range), utilised the same inclusion criteria and laboratory for determining HCC (see Stalder, Steudte, Alexander, et al., 2012), making it an appropriate comparison. Table 8.2 presents the HCC for the three samples. HCC obtained in study III of this thesis were generally higher than HCC obtained in the study of Stalder, Steudte, Alexander, et al. (2012). This may be due to differences in time of year of sampling between the two studies. The students in the study III of this thesis provide their hair samples during the first three months of university, reflecting the summer and autumn period. Although in this study the authors did not specify the time of year the study took place (Stalder, Steudte, Alexander, et al., 2012) it may be that samples were collected following a more intensive period of the academic year.

Table 8.2 HCC values for two samples of young healthy adults

Sample of students	Hair cortisol in pg/mg M (SD)
Study III of this thesis	27.5 (12.3)
Stalder, Steudte, Alexander et al. (2012)	20.0 (11.6)

In study III, two distinct age groups of females were recruited, and differences in HCC were found in line with previous research. For example, Dettenborn, Tietze et al. (2012) found higher HCC in older individuals. This suggests that studies with samples with a wide age range need to consider differences in HCC with age. In line with previous findings (Dettenborn, Tietze, et al., 2012; Stalder, Steudte, Alexander, et al., 2012) smoking status, medication intake, hair colour, hair treatments had no influence on HCC.

8.1.2 Measurement of well-being and ill-being

In Study I (Experiment II) and III, measures of self-reported trait well-being or ill-being were obtained. Factor analysis showed that ill-being and well-being were inversely correlated but were indeed separate components. This provides support for the 'independence' hypothesis and further supports the argument of measuring ill-being alongside well-being in order to test if well-being is uniquely related to cortisol measures. The factor analysis did not reveal separate aspects of subjective or psychological well-being. The subjective and psychological measures included in both studies loaded on the same component in the factor analysis of Study I (Experiment II) and Study III. These findings suggest that subjective and psychological well-being were not distinct aspects of well-being. Accordingly the well-being or ill-being measures were explored in relation to the cortisol measures obtained in this thesis.

To assess day-to-day variations in positive and negative mood in Study I: (Experiment II), the EMA method was used. This method was chosen since it is regarded as the gold standard for measurement of mood in natural settings, since questionnaire measures may be influenced by retrospective biases, availability heuristics and other factors (Kahneman & Krueger, 2006). Further,

more robust associations between well-being and biology have previously been found when affect is assessed repeatedly in everyday life with the EMA method than with questionnaire measures (Steptoe et al., 2007). Typically EMA ratings are completed using pen-pencil diaries. In Study I (Experiment II) electronic mood ratings were obtained to ensure participants were compliant and mood ratings were completed in real time. Using the actiwatch-score device, participants were signalled at random times between 10:00 and 21:00, to complete eight positive and negative mood ratings. Previously, electronic EMA ratings using the actiwatch-score device have been shown to be successful in assessing fatigue. For instance, using the actiwatch-score device participants completed 87% of responses. In Study I (Experiment II) the response rate was slightly lower, 73% of participants completed the eight mood ratings at least four times on each study day. This lower response rate may be explained by the demand placed on participants; participants were required to complete eight mood ratings five times a day. In the study assessing fatigue, participants were only required to complete one item three times over the day. Considering the increased demand placed participants in Study I (Experiment II) the response rate for the EMA mood ratings were relatively high.

8.2 Cortisol measures: relationships with well-being/ill-being

In Study I (Experiment II) and Study III associations between the cortisol measures and well-being or ill-being were investigated. In Study I (Experiment II) salivary cortisol enabled measurement of the CAR and diurnal cortisol profile. It has been discussed throughout this thesis, the importance of adherence to the saliva sampling protocol. Given the misleading CAR estimates obtained from including non-adherent data, analyses involving the CAR were conducted on data in which long delays between awakening and collection of S1 were excluded (i.e. > 15 min), and moderate delays (5-15 min) were controlled for statistically. Although non-adherence did not have an impact on the diurnal cortisol profile or mean diurnal cortisol, to ensure accuracy, analyses were also conducted controlling for delayed sampling for S5 and S6.

In healthy young females, no relationships with the CAR and well-being or ill-being were evident. This is consistent with previous studies, which also failed

to observe relationships between well-being and the CAR in older individuals (Evans et al., 2007; Steptoe et al., 2008). However, it contrasts with others who found a smaller CAR associated with higher levels of happiness in middle-aged males (Steptoe et al., 2007). The difference in findings may be attributed to the age and gender of the sample. Neither were there any associations between the diurnal profile of cortisol or mean diurnal cortisol and well-being or ill-being observed. Again this contrast with previous research showing associations between higher levels of trait well-being and a stepper decline in cortisol across the day (Evans et al., 2007; Ryff et al., 2004) and associations between higher levels of happiness and lower cortisol across the day (Steptoe & Wardle, 2005; Steptoe et al., 2005). Differences in findings may be attributed to the different methodologies applied between studies. Findings are consistent with Jacobs et al. (2007) who also did not observe an associations between well-being and lower cortisol in younger healthy females. It is likely that the relationship between cortisol and well-being is not evident in young healthy individuals.

Females in this study were recruited based on their levels of trait well-being or ill-being. This was necessary to increase the statistical power of the study given the large amount of data collected per participant. Although the high well-being and low well-being participants recruited onto the study exhibited significantly different levels of well-being and ill-being, there was little range and variation of state well-being and ill-being. The recruitment of participants exhibiting low levels of well-being was difficult since they were likely to be taking medication or have received a diagnosed illness and due the influence of medications on salivary cortisol they were not recruited onto the study. Participants who were taking medication may have been exhibiting significantly lower levels of well-being or higher levels of ill-being, and state well-being may have been more varied in these individuals. It cannot be ruled out that diurnal cortisol patterns may have been associated with well-being or ill-being in participants exhibiting more variation in well-being or ill-being over the four study days.

To directly test the hypothesis that associations between well-being and cortisol are not evident in healthy young females, associations were compared in young and old participants (Study III). There are clear advantages of measurement of cortisol in saliva, one of which is the repeated saliva sampling in the domestic

setting enabling measurement of the diurnal pattern of cortisol in participant's natural settings conferring high ecological validity. However, the issue of participant non-adherence to protocol presents a major problem in this line of research. Given the cost and complexity of monitoring adherence to the saliva protocol, the new measure of cortisol in hair samples presented an appropriate alternative to salivary cortisol. It provided a trait measure of cortisol secretion with a one-off hair sample. Although it provides a trait measure of cortisol and does not provide information of the diurnal cortisol patterns it is a good starting point for comparing associations between well-being and cortisol in young and old females. In line with Study I (Experiment II) HCC was not associated with well-being or ill-being in the young healthy female sample. But in the older group well-being was associated with higher HCC, however ill-being was not associated with HCC in this group. Recruitment of females in study III was less strict than the criteria applied to Study I, only individuals that suffered from any condition of adrenocortical dysfunction and/or took any glucocorticoid-containing medication were excluded. Further, given the ease of measuring cortisol in hair sample, a larger sample was recruited which meant selection of high and low well-being was not necessary. However, the difficulty in terms of recruitment in this study was the recruitment of older participants resulting in the smaller number of participants in this age group.

These findings provide evidence that within a healthy female population associations between well-being and cortisol are evident only in older females and not young females. Together these findings provide support for the neurotoxicity hypothesis from a positive aspect. In a healthy population without any psychopathology the effects of well-being may not exert their effects on the HPA axis until later in adulthood. Ill-being and aberrant cortisol profiles have been found in younger adults this could be explained by the interacting vulnerability hypothesis (see Lupien et al., 2009) which suggests that in vulnerable individuals (e.g. insecure attachment styles and/or exposure to early life stress) the HPA-axis is programmed during childhood to make it more vulnerable to dysregulation. In vulnerable individuals dysfunction of the HPA-axis may precede and predict later psychopathology (e.g. ill-being, low well-being aberrant cortisol profiles). Measures of vulnerability (such as attachment style and life events) may tease out vulnerable individuals and different

associations between ill-being or well-being and cortisol measures may be observed to those reported here.

These findings are based on all female adults and results may not generalise to male adults. Gender differences are evident in diurnal cortisol patterns, and thus an all female sample was deemed necessary since within a modest sized sample comparisons between males and females would not have been possible. The finding that higher levels of well-being were associated with higher HCC adds to the growing body of literature showing gender differences between cortisol patterns and associations with ill-being or well-being measures (Bremmer et al., 2007; Kumari et al., 2010; Witteveen et al., 2010). Previous reports of hyper-cortisolism and well-being are evident for females, whereas males show the opposite (Roe et al., 2013). Such gender differences in the cortisol secretory patterns may account for some of the variation in findings in the literature, as such future studies should account for gender differences either by having a large enough sample to make comparisons between gender or restrict sample to one gender so that a greater understanding of the cortisol secretory activity in relations to psychosocial variables can be gained. It is an interesting finding that higher cortisol secretion is associated with well-being. It is possible that in healthy females higher cortisol secretion is indicative of being busier and energised. Table 8.3 provides a summary of the strengths and novelty of the programme of research

Table 8.3 A summary of the strengths and novelty of the programme of research

Encouraging adherence to saliva sampling protocol	- One of the first studies to use electronic devices to measure both awakening and saliva sampling times and not simply rely on self-reports. - The real sampling times identified non-adherent data, which was accounted for in analyses involving diurnal cortisol patterns and psychosocial variables. - Self-reports of awakening were inaccurate and resulted in delayed collection of the first 'awakening' sample - Informing participants that their saliva sampling times will be verified by an electronic device increased participants accuracy in their self-reports - Prompts (via text messages) were provided to participants to remind them to collect the day saliva samples, prepare for their upcoming study day and wear actiwatch-score to bed the night prior to study day
Measurement of the diurnal cortisol patterns (CAR and diurnal decline)	- Saliva sampling was synchronised to awakening and not clock time - The CAR was measured in saliva samples at 0, 15, 30 and 45 min post-awakening providing optimal assessment of the CAR on each study day - Diurnal decline was measured as the decline from 3 hr to 12 hour post-awakening and did not include the CAR period - Measurement of diurnal cortisol patterns was on four study days which allowed for statistical analyses of state as well as trait variation
Electronically determined non-adherence and the CAR	- Challenges the assumption that delay between awakening and collection of S1 less than 10-15 min are acceptable to obtain accurate measures of the CAR magnitude - Confirmed the prediction that delays between awakening and collection of S1 of just 8 min (5-15 min) do not impact on 'awakening' cortisol levels, but they do result in overestimated CAR magnitude and earlier timing of the peak in cortisol - First study to investigate impact of sampling delay on the timing of the CAR peak, and growing interest in timing of CAR peak is evident
CAR time lag	- First study to measure salivary cortisol intensively in the immediate post-awakening period (i.e. every 5 min for the first 30 min following awakening) - First study to show that cortisol does not rise linearly immediately after awakening. Increase in cortisol is observed 15 minutes following awakening

Non-adherence and diurnal cortisol measures	- First study to measure diurnal decline or mean diurnal cortisol measured at 3 and 12 hours post-awakening and show it was not influenced by delay in collecting samples
Expands well-being research	- Traditionally the focus of psychology research has been on ill-being. This work attempts to understand how well-being exerts its effects on good health - Ill-being and well-being were independent components, highlights the need for investigating ill-being along well-being - Pays particular attention to the definition of well-being – assesses both subjective and psychological aspects of well-being, which have been shown to be related differently to physiological functions (but not in this work)
Measurement of well-being	- Measures trait and state well-being or ill-being. Most studies focus on trait measures of well-being failing to assess the day-to-day variations in mood, which may be more strongly related with diurnal cortisol patterns - EMA method used to assess state well-being, uses electronic devices to ensure that participants complete mood ratings at the desired times and not retrospectively
Trait measure of cortisol	- Utilised a new measure of cortisol, HCC provided a retrospective assessment of cortisol secretion - Previous studies have failed to link HCC with psychosocial measures, such as, stress, anxiety and depression. First study to observe relationships between HCC and measures of well-being but not ill-being
Cortisol patterns and well-being across the life span	- Relationships between diurnal patterns of cortisol and well-being in healthy participants are evident at later points in life (i.e. older age). But does not extend to younger healthy females. - Support for neurotoxicity hypothesis from a positive aspect (i.e. the effects of well-being are not evident until later in life)
Statistical analysis	- Instead of using traditional approaches (i.e. general linear model), the new approach of mixed regression analysis was used, which is the method deemed most appropriate for multiple repeated measures over time.

8.3 Future directions

The focus of this work has been upon reliable measurement of cortisol and relationships with trait psychosocial variables and daily variations in positive and negative mood in a healthy female sample. Future research on diurnal patterns of cortisol needs to consider the methodological issues raised in this thesis and adopt similar methodological rigor. The importance of participant adherence to protocol has been emphasised. Thorough briefing of the protocol, emphasising the importance of participant adherence is not enough for accurate measurement of the CAR. Accurate measurement of the CAR requires accurate awakening cortisol levels at S1. Self-reported awakening times are inaccurate which may not be through fault of the participant but due to them being in a state of sleep inertia. More objective measures of awakening can easily be obtained using electronic devices such as actigraph, which can be worn on the wrist with relatively no discomfort. Self-reports alone will not identify moderate but significant delays between awakening and collection of S1.

Informing participants that their adherence to the protocol is being electronically monitored increases their adherence to the saliva sampling protocol. In Study I, (Experiment I) although participants delayed collecting the first 'awakening' sample it was proposed not to be intentional since participants collected the remaining samples within the desired interval time (and sampling time was synchronised to their inaccurate sampling time). Thus in terms of collecting their samples from the time that participants were awake on the whole participants did this correctly. The finding that saliva sampling times were accurate should be interpreted cautiously. The sample was comprised of undergraduate students who were receiving credits from their participation and so may have been motivated to be honest regarding their self-reported adherence to protocol. However, this is unlikely to be the case since financial incentives have not be shown to increase participant adherence to the saliva sampling protocol (Halpern et al., 2012). Also, this was a sample of young females; it is necessary to investigate if accuracy of self-reports is evident in a male sample or older adults. It is the recommendation here that, wherever possible saliva sampling times should be electronically monitored with awakening times since using these devices together will provide real sampling

times. If cost is an issue, at the very least if electronic devices such as track caps are not available dummy devices should be used and participants should be informed that their sampling times are being monitored electronically.

It may be conceived at first that CAR research is more difficult with the finding that moderate delays between awakening and collection of S1, however, it is the recommendation of this work that real sampling times (obtained with electronically determined awakening and saliva sampling times) can be used to adjust data involving the CAR and not waste the data. Analyses involving timing of the CAR peak can map the actual timing to participants real sampling times and calculation of the CAR magnitude can be conducted using real sampling times rather than the protocol desired times.

Although participant non-adherence is not a problem for the analysis of hair cortisol, this area of research is relatively new. Studies in this thesis have demonstrated how sensitive the CAR is to non-adherence, which has only recently been considered despite the extensive research on the CAR. This highlights how important it is to understand the measure being used. Methodological considerations should continue to be explored with regards to HCC as it is possible that there may be variables that distort results from HCC. One such variable could be the assumed time line of cortisol based on the average growth of hair. Currently 1-cm of hair is assumed to equate to one month cortisol secretion. However, it could be possible that variations in hair growth could influence HCC. Thus, future research should investigate how different factors influence HCC before providing a body research of associations between HCC and psychosocial variables that may be meaningless and limited by inconsistent and conflicting findings as is the case with the CAR literature.

The causal pathways between well-being and health cannot be determined from day-to-day or cross-sectional studies. It is crucial to understand the pathways mediating these associations, so as to formulate better methods of reducing morbidity and enhancing the well-being and sustained productivity of the nation. It is possible that the effects of well-being on health work through stimulating healthy cortisol patterns and if these persist in the long-term it will result in favorable health outcomes. However, it is also possible that cortisol patterns

could influence mood. For example, medications that reduce cortisol may lead to improvements in mood, although the evidence is very inconsistent (Gallagher et al., 2008). The causal pathways can be best untangled through manipulating mood through intervention studies. Although our happiness is determined in part by genetic and developmental factors, and in part by life circumstances, there is growing evidence that intentional activities and cognitions also contribute (Lyubomirsky et al., 2005). A growing body of evidence demonstrates the effectiveness of positive psychological interventions in enhancing individual's mood (Seligman et al., 2005). Intervention studies in which well-being is enhanced by deliberate actions could determine the effects on cortisol. Future research will need to determine if the techniques for enhancing well-being has beneficial effects on cortisol. Comparisons between young and older groups are only the first step to investigating the associations between well-being and cortisol over the lifespan, longitudinal studies are needed to determine this over the life span. Longitudinal evidence for the effects of well-being on health are evident but longitudinal investigation of the physiological pathways mediating this relationship across the life span is needed. This is an exciting field of research that offers promise both in the understanding of connections between the mind and body, and for the relief of suffering and the postponement of ill health.

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Appendices

Appendix 1
Information sheet and consent form for Study I (Phase I): online questionnaire

The 'happi' Study
Health and Positive Psychological Interventions
Participant information sheet

What is the study about?

This study explores the relationship between health, attachment styles and well-being, looking at how our attachment styles relate to our well-being and the way well-being relates to our health. We believe that secure attachment styles are related to positive well-being and positive well-being is associated with good health, while more negative emotional states may contribute to ill-health.

What will happen during the study?

This study involves completing an online questionnaire about yourself, your well-being and your health, which should take approximately 30 minutes to complete.

Who can take part?

This study is being carried out with full- and part-time students; if you have access to a computer (public or private) with internet connection you can take part.

What if I change my mind during the study?

You have the right to withdraw from the study at any time, including withdrawing your data prior to publication, without explanation.

What happens to the information?

Any information you give will be treated in the strictest confidence and used only for this study. All information you provide will be stored in such a manner that no specific details will be linked to individuals. Although we will not be able to give feedback on individual data, we will provide all participants with a summary of the overall findings.

Please Copy and paste this information so that you can either print or save it so you can refer to it if you need to.

Part II: Consent:

Please select when you have read the above information. By selecting this box you are confirming that you understand the study and that you agree to participate in it.

Do you agree to take part in the study? Yes ○

Please do not hesitate to contact Nina Smyth if you have any questions or problems

020 7911 5000 ext. 2184 or nina.smyth@my.westminster.ac.uk

Appendix 2:
Information sheet and consent form for Study I (Phase II): salivary cortisol & mood phase

University of Westminster

The 'happi' Study:
Health and Positive Psychological Interventions
Participant information sheet

Part I: Study Overview:

What is the study about?

This study explores the association between low and high wellbeing and cortisol. Cortisol is known as a stress hormone, as it is produced in response to psychological stress. It is also essential for normal physiological functioning. It has a marked 24hour rhythm, with peak levels about 45 minutes following awakening and low levels in the early part of sleep. Abnormal cortisol rhythms have been implicated in psychological (e.g. depression) and physical ill health. Cortisol is also thought to play a role in the association between well-being and good health. The study is part of a PhD being undertaken by Nina Smyth at the University of Westminster, Psychology Department. The main supervisor is Professor Angela Clow.

What will happen during the study?

The hormone cortisol can be measured in saliva. The study requires you to take saliva samples every 15 minutes for the first 45mins after awakening and samples at approximately 3 and 12 hours after awakening on each study day. You will be required to rate your mood throughout each study day. Additionally, you will also complete an evening and morning diary about your anticipations, sleep and saliva sampling times. You will also be asked to wear a watch to bed which will record your awakening time. To monitor saliva sampling times, the cotton swabs used on saliva sampling will be stored in an electronic cap, each opening of the cap will indicate the time you collected the sample.

You will be provided with detailed instructions (verbal and written) on the research procedure, saliva sampling packs for each study day and the study equipment. You will be able to ask as many questions as you like and contact the researcher with problems if and when they arise throughout the process of the study.

Who can take part?

Full- or part-time students that participated in the online 'happi' questionnaire. Students must be healthy, not suffering from any serious medical or psychiatric illness or taking prescribed medication.

What if I change my mind during the study?

You have the right to withdraw from the study at any time, including withdrawing your data without explanation.

What happens to the information?

Any information you give will be treated in the strictest confidence and used only for this study. All information you provide will be stored in such a manner so that no specific details will be linked to individuals. Your saliva will only be tested for the hormone cortisol. Although we will not be able to give feedback on individual data, we will provide all participants with a summary of the overall findings.

Please do not hesitate to contact Nina Smyth if you have any questions or problems

020 7911 5000 ext. 2184 or nina.smyth@my.westminster.ac.uk

Part II: Consent:

	Please circle
Have you read the information sheet about this study?	**YES / NO**
Have you had the opportunity to ask questions, and received satisfactory answers to your questions?	**YES / NO**
Have you received enough information about the study?	**YES / NO**
Do you agree to your saliva being tested for the hormone cortisol?	**YES / NO**
Do you understand that you are free to withdraw from the study at any time, without giving a reason for withdrawal?	**YES / NO**
Do you agree with the publication of the results of this study in appropriate outlets?	**YES / NO**
Do you agree to take part in the study?	**YES / NO**
Do you agree to be contacted about future research?	**YES / NO**

	Print name	Signature	Date
Participant			
Researcher			

Please provide your contact details; this will be stored separately from any data you provide so that your responses remain anonymous

ID (student / staff)	
Name	
Tel	
Email	

Appendix 3:
Information sheet and consent form for Study II

Part I: Study Overview:

What is the study about?

This study is investigating cortisol secretion within the first 30 min of awakening. In healthy individuals cortisol exhibits a marked circadian rhythm characterised by peak levels following morning awakening (known as the cortisol awakening response: CAR) and declining levels thereafter, reaching lowest levels in early sleep. We assume that cortisol arises immediately on awakening, however, this has not been directly tested, thus the aim of the study aimed to explore this. The study is part of a PhD being undertaken by Nina Smyth at the University of Westminster, Psychology Department. The main supervisor is Professor Angela Clow.

What will happen during the study?

The hormone cortisol can be measured in saliva. The study requires you to collect saliva samples every 5 minutes for the first 30 mins after awakening. Additionally, you will also complete a diary recoding your awakening and saliva sampling times. You will also be asked to wear a watch to bed which will record your awakening time. To monitor saliva sampling times, the cotton swabs used on saliva sampling will be stored in an electronic cap, each opening of the cap will indicate the time you collected the sample. You will be provided with detailed instructions on the research procedure, saliva sampling packs for each study day and the study equipment. You will be able to ask as many questions as you like and contact the researcher with problems if and when they arise throughout the process of the study.

Who can take part?

Male or female adults who are healthy, not suffering from any serious medical or psychiatric illness or taking prescribed medication.

What if I change my mind during the study?

You have the right to withdraw from the study at any time, including withdrawing your data without explanation.

What happens to the information?

Any information you give will be treated in the strictest confidence and used only for this study. All information you provide will be stored in such a manner so that no specific details will be linked to individuals. Your saliva will only be tested for the hormone cortisol. Although we will not be able to give feedback on individual data, we will provide all participants with a summary of the overall findings.

Please do not hesitate to contact Nina Smyth if you have any questions or problems: 020 7911 5000 ext. 2184 or nina.smyth@my.westminster.ac.uk

Part II: Consent:

	Please circle
Have you read the information sheet about this study?	**YES / NO**
Have you had the opportunity to ask questions, and received satisfactory answers to your questions?	**YES / NO**
Have you received enough information about the study?	**YES / NO**
Do you agree to your saliva being tested for the hormone cortisol	**YES / NO**
Do you understand that you are free to withdraw from the study at any time, without giving a reason for withdrawal?	**YES / NO**
Do you agree with the publication of the results of this study in appropriate outlets?	**YES / NO**
Do you agree to take part in the study?	**YES / NO**
Do you agree to be contacted about future research?	**YES / NO**

	Print name	Signature	Date
Participant			
Researcher			

Please provide your contact details; this will be stored separately from any data you provide so that your responses remain anonymous

ID (student / staff)	
Name	
Tel	
Email	

Appendix 4:
Information sheet and consent form for Study III

University of Westminster Roots of Stress

ROOTS OF STRESS:

Hair cortisol concentrations in relation to age, stress and genotype.

PARTICIPANT INFORMATION SHEET

Part I: Study Overview:

What is the study about?

Cortisol is a stress hormone - it is produced in response to stressful situations, however, it is also essential for normal functioning. Aberrant levels of cortisol have been implicated in poorer physical and mental health (e.g. cardiovascular disease, depression) particularly in older people. In this study we will explore the relationship between current as well as early life stress and cortisol levels. We are interested in this because research indicates that early life stress, as well as recent life stress, may be an important determinant of well-being. Another important role is genetics; research shows that genes and environment interact to predict vulnerability to the effects of stress. Brain-derived neurotropic factor (BDNF) is an important brain chemical. Its role is to promote brain health and it is known as a 'nerve growth factor'. Genes influence the levels of BDNF in the brain and depending upon your BDNF genetic makeup life stress may have different effects throughout life. The study will explore the relationships between the BDNF genotype and environmental stress across the lifespan. It is part of a PhD being undertaken by Nina Smyth at the University of Westminster, Psychology Department. The main supervisor is Professor Angela Clow.

What will happen during the study?

The hormone cortisol and the BDNF-gene can be measured in hair samples. We will collect a small piece of hair from as close as possible to your scalp. The required amount of hair is quite small (see picture below), which is cut from the posterior region of your scalp, without leaving a trace. We will also ask you to provide information on yourself (i.e. birth weight, age etc) hair characteristics, your current well-being/ill-being and your experience of early life stress this should take approximately 30 minutes to complete.

You will be provided with detailed instructions (verbal and written) on the research procedure. You will be able to ask any questions you have about this research and you can contact the researcher with problems if and when they arise.

Example of hair sampling process

Who can take part?

We are looking for females between 18 and 26 years of age and older women over 65 years of age. Participants must be healthy, not suffering from any serious medical or psychiatric illness or taking prescribed medication containing corticosteroid during the last year. Also participants should not be pregnant or abuse drugs.

What if I change my mind during the study?

You have the right to withdraw from the study at any time, including withdrawing your data without explanation.

What happens to the information?

All of the data will be treated in the strictest confidence and will be stored in such a manner so that no specific details will be linked to you. Although we cannot give feedback on individual data, if you wish we can provide you with a summary of the overall findings.

Please do not hesitate to contact Nina Smyth if you have any questions or problems

020 7911 5000 ext. 2184 or rootsofstress@gmail.com

Part II: Consent:

	Please circle
Have you read the information sheet about this study?	**YES / NO**
Have you had the opportunity to ask questions, and received satisfactory answers to your questions?	**YES / NO**
Have you received enough information about the study?	**YES / NO**
Do you agree to your hair being tested for the hormone cortisol?	**YES / NO**
Do you agree to your hair being tested for the BDNF gene?	**YES / NO**
Do you understand that you are free to withdraw from the study at any time, without giving a reason for withdrawal?	**YES / NO**
Do you agree with the publication of the results of this study in appropriate outlets?	**YES / NO**
Do you agree to take part in the study?	**YES / NO**
Do you agree to be contacted about future research?	**YES / NO**

Consent to store and use hair samples in future research.

	Please circle
I give permission for my hair sample to be stored after this research.	YES / NO
AND (if the sample is to be stored) I give my permission for my hair sample to be stored and used in future research of any type which has been properly approved	YES / NO

	Print name	Signature	Date
Participant			
Researcher			

Please provide your contact details; this will be stored separately from any data you provide so that your responses remain anonymous

Name	
Tel	
Email	

Appendix 5
Study advert for Study I (Phase I): online questionnaire

Appendix 6
Study advert for Study I (Phase II): salivary cortisol and mood phase

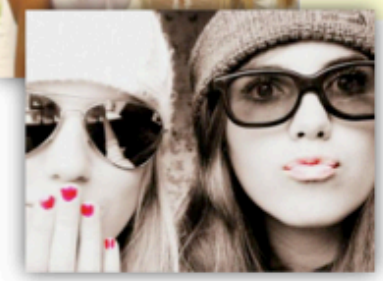
Appendix 7:
Study advert for Study III

Roots of Stress

Don't Pull your Hair Out!!

Feel so stressed you want to pull your hair out? Do Not! from hair it is possible to measure how much stress you're under! And this is what we are interested in!

Roots of Stress is a new research project, under the superivision of professor Angela Clow and conducted by Psychophysiology and Stress Research Group at University of Westminster, Department of Psychology. The study aims to understand how stressful past life events can affect our present **health and well-being**. To do this, we will measure the hormone cortisol and stress related genes in relation to self-reported health, well-being, stress, mood and life events.



WE ARE LOOKING FOR VOLUNTEERS

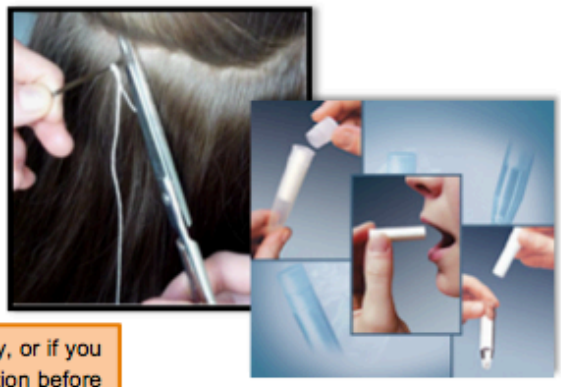
Would like to participate to our study??! We are looking for females between 18 and 26 years of age

We would offer you a £10 high-street voucher for your kind contribution

www.rootsofstress.webs.com

WHAT IS REQUIRED:

In this study we will ask you to complete a few questionnaires on mood, stress, well-being and life events. We will also cut a small sample of hair to measure your stress hormone and take one saliva sample for the analyses of some stress related genes.



If you are interested in participating in this study, or if you have any questions or would like more information before deciding, please contact our research team at:

rootsofstress@gmail.com

Nina Smyth or Matilde Bianchin 02079115000 Ext. 2184

UNIVERSITY OF WESTMINSTER

Roots of Stress

Appendix 8:
Article for Study III

Roots of Stress

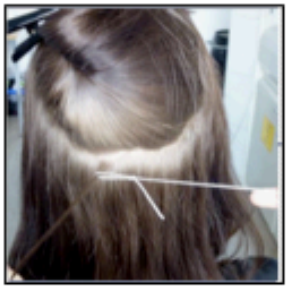
Don't Pull Your Hair Out!

Stress is something that everybody experiences in their life time.
What happens to us when we experience stress?
How do we know if it is still having an effect on us?
Our **body** can tell us.

In situations of major stress, our body releases the hormone cortisol; this is a normal response to stress. But when stress and cortisol production is prolonged over time it has many consequences on our mental and physical health. Extended stress over years can lead to a body that is less resilient to cope with future stress. This can starts in the early stage of life, resulting in poor health and well-being in the future.

Roots of Stress is a new research project, conducted by the internationally known Psychophysiology and Stress Research Group in the Department of Psychology, under the supervision of Professor Angela Clow. The study aims to understand how stressful past life events can affect our present health and well-being. To do this, we will measure the hormone cortisol in relation to self-reported health, well-being, stress, mood and life events.

We will measure cortisol in hair samples; this is a fascinating and new technique which has only recently been developed by researchers. Previous methods that measured cortisol in body fluids (e.g. saliva and blood) allowed researchers to study the acute production of cortisol up to 24 hours upon sampling. In hair samples we can measure the production of the hormone cortisol up to the previous 6 months. Cortisol is slowly incorporated into the hair shaft from the blood stream hence the hair shows a month by month calendar of cortisol production, providing a fuller understanding of periods of stress.



We are now looking for **volunteers** to participate to our study! You will be asked to complete a few questionnaires on mood, stress, well-being and life events. We will also cut a small sample of hair to measure your stress hormone.
We are looking for **FEMALES** between **18 and 26 years of age** and older women over **65 years of age**. You can ask to your *grandmothers, aunties or friends* if they would like to participate with you to our study!

If you are interested in participating in this study, or if you have any questions, curiosities or would like more information before deciding, please contact our research team at:

rootsofstress@gmail.com

Nina Smyth or Matilde Bianchin
02079115000 Ext. 2184

Roots of Stress

Appendix 9:
Letter to University of the third age

Participant address

Professor Angela Clow
Department of Psychology
University of Westminster
309 Regent Street
London W1B 2UW

Dear …..

Thank you again for participating in the West Focus study at the University of Westminster. Your participation in this study was very valuable, directly leading to 4 scientific publications as well as several conference presentations. The work has generated much interest in the scientific community and advanced understanding of the links between well-being and health in active aging. Of course no single study can answer all the questions! As a result we are now seeking to explore the lifespan in more depth, investigating a wider range of age groups. We are hoping that this new study may be of interest to you. This time however we are seeking female volunteers only (sorry chaps!). This is to simplify the study as males and females have been shown to have differing relationships between well-being and health. So, in the first instance we are seeking to understand females and then return to study males at a later date. The study has been approved by ethics committee at the University of Westminster.

The study involves measuring the hormone cortisol again but this time it is much easier to participate. In the last study you kindly collected several saliva samples over the course of 2 days. This time we want to measure cortisol in a single small sample of your hair. The procedure requires cutting a small hair sample near the scalp at the back of the head and leaves no visible sign. This new technique provides a measure of cortisol secretion over several months (1-cm equates to 1 months hair growth and cumulative cortisol secretion). The study would also involve you completing a questionnaire about your early life and your current well-being. Completion of the questionnaire would take approximately 20-30 minutes. If you wish you can ask to your female friends and relatives if they would like to participate with you to the study.

Enclosed is some extra information on the research study for your interest. If you are interested in this study we would need you to come to the University of Westminster, located in central London, Regents Street (about 100 yards from Oxford Circus tube station). If you like you could come with a friend, who may also like to participate.

We would offer you a £10 high-street voucher for your kind contribution. If you are interested in participating, or if you have any questions, curiosities or would like more information before deciding, please contact our research team:

Nina Smyth or Matilde Bianchin by email **rootsofstress@gmail.com or by** telephone on **02079115000 Ext. 2184** or myself on **clowa@wmin.ac.uk.**

Thank you for your time, and we look forward to hearing from you.

Best Regards

Professor Angela Clow
Roots of stress research team.
University of Westminster,
Psychophysiology and Stress Research Team.

Appendix 10
Study guidelines for Study I (Phase II)

STUDY SAMPLING GUIDELINES

Project ID _____

Date ____/____/____

If you have questions at any time,
Please contact Nina Smyth on
020 7911 5000 Ext. 2184 (business hours)
07796177392 (out of hours)
nina.smyth@my.westminster.ac.uk.

Study Overview:

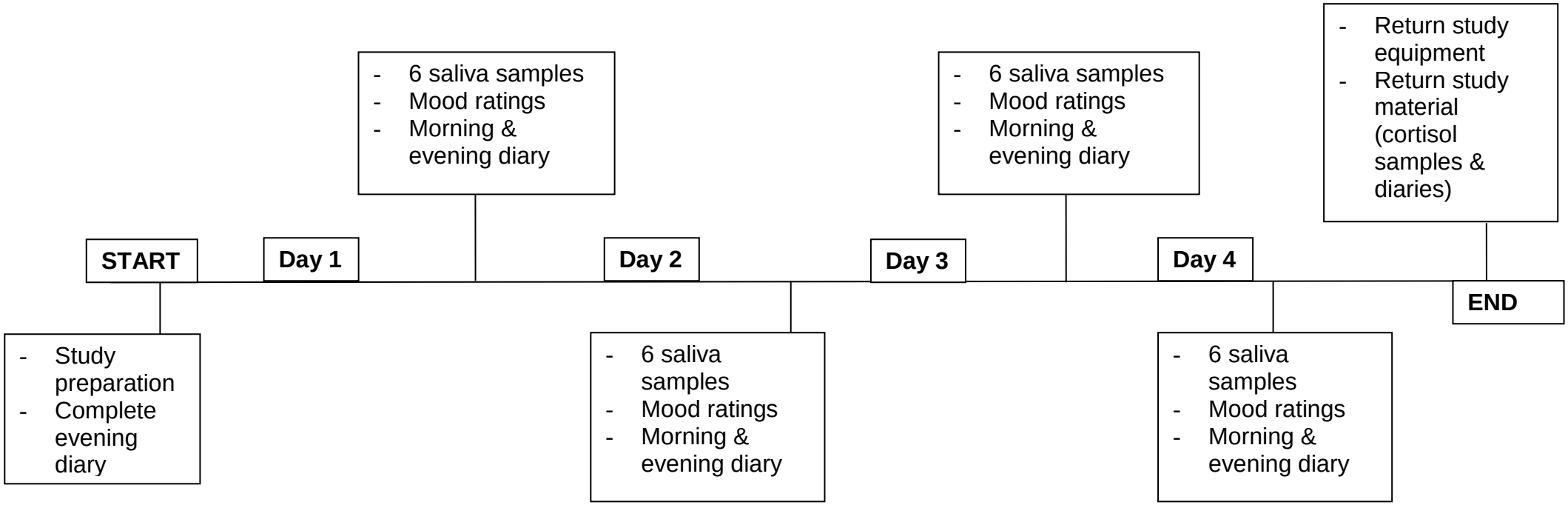
What is involved in the study:

Over the course of the study week, you will collect saliva samples on four days: these include two weekdays (Tuesday and Wednesday) and the weekend (Saturday and Sunday). During these days you will collect six saliva samples throughout the day, these times will be based on your waking time.

Throughout these study days you will be asked to rate your mood using the Actiwatch-Score.
Additionally you will complete an evening diary the evenings prior to your study days plus the study days and a morning diary after your fourth cortisol sample. You will be sent an SMS message to your mobile phone reminding you to prepare for the study day and complete your evening diary. You will also be sent an SMS message during the morning to remind you to complete your morning diary.

You will be given an Actiwatch-score which you'll be required to wear the nights prior to your study days and during your study days. You will be given four cortisol packs; each pack will include six salivettes (labelled for each sampling day and time). You will also be provided with a MEMSCap bottle which will contain 24 cotton swabs. To prepare for your study days you should ensure that you are wearing the Actiwatch-score when you go to bed and place the salivette tubes and MEMSCap bottle next to your bed to ensure that you collect your morning sample as soon as you wake up.

Study protocol:



Saliva sampling instructions:

Do not eat or drink, smoke, brush your teeth or exercise for 30 minutes before you collect each of the saliva samples. You can drink water only.

Open the MEMSCap bottle and take one cotton swab, place the cotton swab in your mouth. Close the MEMSCap bottle.

Gently chew on the swab until it is soaked, this will usually take about 2 minutes.

Once the swab is soaked, place it back in the salivette tube (**NOT** the MEMSCap bottle), trying not to use your hands. Put the cap on securely, and place the tube in the plastic bag provided for that sampling day.

Store the tube in a cold place or in a refrigerator.

Placing the cotton swab in the salivette tube:

DO: Place in the smaller inner tube

DO NOT: don't remove the smaller, inner tube and place the swab in the larger inner tube.

Cortisol Packs:

You have 4 cortisol packs, one for each study day, it is very important that you put your cortisol tubes in the appropriate bags. The packs are colour coded:

Day 1 (Tuesday) = Baby blue
Day 2 (Wednesday) = Yellow
Day 3 (Saturday) = Grey
Day 4 (Sunday) = Green

It is very important that you place the cotton tubes in the appropriate salivette tubes. Each tube is clearly labelled and is colour coded:

Tube 1 (0 mins) = Purple
Tube 2 (15 mins) = Green
Tube 3 (30 mins) = Blue
Tube 4 (45 mins) = Orange
Tube 5 (3 hrs) = Pink
Tube 6 (12 hrs) = Red

MEMSCaps Bottles

A total of 24 cotton swabs (total for the study week) are stored in the MEMSCaps bottle. Only open this bottle when you are collecting your saliva samples.

Place the cotton swab in the salivette tube for that sampling time and day.

DO NOT put it back in this bottle.

These bottles record the times the bottle was opened, this will be used to check that you collected the saliva sample at the appropriate time.

Saliva cortisol sampling times:

On each of the study days you will collect six saliva samples, the times depend on your waking time. The first samples will be collected as soon as you wake, please do this **STRAIGHT** away, even if you are a little sleepy. Please do not dose back to sleep because you will need to complete your samples 15, 30, and 45 minutes after you wake up. Then 3, & 12 hours after you wake up.

This table gives you an overview of each of the saliva sampling times (left side) and gives you examples of calculating your sampling times based on your waking times (right side). Your sampling times will vary on weekdays and the weekend because you may get up later on the weekend, so please calculate the times based on your awakening.

Sampling times			Examples of calculating your sampling times based on your waking times	
Sample No.	**Sample Time**	**Instructions**	**e.g. weekday sampling times**	**e.g. weekend sampling times**
Sample 1	Awakening	This first sample should be collected **as soon as you wake up** and **before** you **get out of bed.** Even if you are still half asleep you should still take it.	6.00	9.00
Practice				
Sample 2	Waking plus 15 minutes	This second sample should be collected 15 minutes after you have woken up.	6.15	9.15
Practice				
Sample 3	Waking plus 30 minutes	Take this sample 30 minutes after your awakening sample.	6.30	9.30
Practice				
Sample 4	Waking plus 45 minutes	Take this sample 45 minutes after your awakening sample.	6.45	9.45
Practice				
Sample 5	Waking plus 3 hours	Take this sample 3 hours after your awakening sample.	9.00	12.00
Practice				
Sample 6	Waking plus 12 hours	Take this sample 12 hours after your awakening sample.	18.00	21.00

Mood ratings using the Actiwatch-Score:

Do's & don'ts:

DO:
Wear this watch whilst in bed (the watch records your activity which will indicate your sleep and waking times).
Wear the watch throughout the day (the watch needs to be worn throughout the study days because you will be presented with five random prompts asking you to rate your mood.

DO NOT:
Wear the watch in the shower because it is not waterproof.

Using the watch to rate your mood:

Between the hours of 10 am and 9 pm you will be presented with five random prompts. These prompts will present you with a series of questions that are about your mood in the last hour.

For each question a letter will appear on the screen this letter will symbolise a mood adjective, for each of these you should rate the extent to which you have experienced them in the last hour on a scale 1 (not at all) to 5 (very much).
The mood adjectives will be presented in the following order and will be presented by the following letters.

Pleased → P
Stressed → S (this looks like a 5 on the screen)
Down → d
In control → C
Happy → H
Busy → b
Frustrated → F
Goal directed → g

Refer to these codes if you do not remember what each letter stands for

Rate the extent have you felt the following in the last hour? 1 = not at all - 5 very much
P = Pleased
S = Stressed
d = Down
C = In control
H = Happy
b = Busy
F = Frustrated
g = Goal directed

How to rate your mood using the watch:

The watch will beep to prompt you to complete your mood ratings.

Press the Ⓢ on the watch, this will start the scale, starting with the number 1

Press the Ⓢ to move through the scale

When the number that represents your mood appears on the screen wait for the Actiwatch-score to record this number, this will take a few seconds.

If you pass the number that you want, carry on pressing the Ⓢ to take you through the numbers

When the prompt is presented the watch will beep, it will only beep for about a minute or so, so please try and respond straight away otherwise that data will be missing.

This screen will present you with each of the mood adjectives.

Use this button to move between the mood ratings. Once you have the number that you want leave it for 5 seconds and the watch will record it and will present you with

Diaries:

Morning Diary:

The morning diary asks you to report your calculated and actual saliva sampling times for the morning and about your sleep and anticipations of the day ahead.

You need to complete this on the mornings of the four study days (after the forth saliva sample). You will be reminded by an SMS message to complete this.

Evening Diary:

The evening diary asks you to report your calculated and actual saliva sampling times (fifth & sixth saliva samples) and about your anticipations of the following day.

You need to complete the evening diary at the following times:

		Type of questions to answer
Preparation for study day 1	Monday	Anticipations of the following day
Study day 1	Tuesday	Times of cortisol samples Anticipations of the following day
Study day 2	Wednesday	Times of cortisol samples
Preparation for study day 3	Friday	Anticipations of the following day
Study day 3	Saturday	Times of cortisol samples Anticipations of the following day
Study day 4	Sunday	Times of cortisol samples

You will receive a text message reminding you to complete the evening diary.

Study days & Sampling times:

The study will run for one week, your four sampling days are on a **Tuesday** (_____), **Wednesday** (_____), **Saturday** (_____), and **Sunday** (_____). On these days you will be required to collect salivary cortisol, rate your mood and complete the diaries. Apart from completing the evening diary the day before your study days (Monday and Friday) you do not need to collect saliva samples, rate your mood or complete the morning diary on the remaining days of the week. You will need to complete the evening diary the evenings prior to the first and third sampling day (you will be reminded to do so with an SMS message). On these evenings you will also need to make sure that you are wearing the Actiwatch-score and that your cortisol pack and MEMSCap bottle is placed by your bed.

Study Days:

This is an overview of what you will need to do throughout the study week:

Type of Day	Date	What you need to do
Preparation for study day 1	Monday (_____)	Complete evening diary Wear Actiwatch-Score to bed Place bottles & salivettes next to bed
Study day 1	Tuesday (_____)	6 Cortisol samples Momentary mood ratings Morning diary Evening diary
Study day 2	Wednesday (_____)	6 Cortisol samples Momentary mood ratings Morning diary Evening diary – only questions about saliva samples
Preparation for study day 3	Friday (_____)	Complete evening diary Wear Actiwatch-Score to bed Place bottles & salivettes next to bed
Study day 3	Saturday (_____)	6 Cortisol samples Momentary mood ratings Morning diary Evening diary
Study day 4	Sunday (_____)	6 Cortisol samples Momentary mood ratings Morning diary Evening diary – only questions about saliva samples

Returning your Saliva Samples, diaries & equipment

At the end of the study week you will need to return your saliva samples, the study equipment and your diaries.

These need to be returned to:
Nina Smyth
Regents St Campus
Room 556 (upper 5th floor)
020 7911 5000 ext. 2184
nina.smyth@my.westminster.ac.uk

A convenient time can be arranged to drop these back, it is very important that these are returned straight after the study so the equipment can be used for other participants

Thank you for taking the time to read the sampling guidelines. It is very important that you understand the instructions and are able to clearly follow the sampling instructions.

The content of this guidebook has be covered thoroughly in the initial introductory meeting however, if you have any questions regarding the procedure of this research project, or need to clarify something, this guidebook will serve as a useful reminder of all necessary information to complete this study.

However, should you have any additional questions not covered by the content of this guidebook, please do not hesitate to contact Nina Smyth on any of the following numbers:

020 7911 5000 ext. 2184

07796177392 (out of hours)

Or by email:

nina.smyth@my.westminster.ac.uk

Alternatively pop into room 556

Thank you very much for participating in this study!

Appendix 11
Self report diary used by participants in Study I (phase II)

Pre Study day 1: .....

EVENING DIARY:
Please complete this the evening before your first study day

How busy do you expect tomorrow to be?

1 = not at all busy – 5 = very busy.....

How do you feel about tomorrow?

1 = very negative - 5 = very positive .....

Study Day 1: .....

MORNING DIARY:
Please complete after your **45 minutes** post-awakening sample

What time did you wake up? .....

Please calculate your collection times and state the actual times that you collected your saliva samples. Please be honest as you can.

Tube	Collection time (post-awakening)	Calculated collection time (24hr)	Actual collection time (24hr)	Problems with sampling
1	Awake	0 min		
2	15 mins			
3	30 mins			
4	45 mins			

Your honesty is very important to us in analysing the data. Therefore, please state your actual collection times for each sample, even if it is different to the designated time, and answer the questions as accurately as possible.

What was the approximate time you went to sleep latest night?

.....(24 hr)

How easy was it for you to determine the exact moment you woke up?

1 = very difficult, 5 = very easy .....

How did you wake this morning?

(please circle)
Alarm clock / radio
Somebody I asked to wake me
Noises
Just woke

Compared to usual, how was your quality of sleep last night?

Much better than usual
A bit better than usual
Same as usual
A bit worse than usual
Much worse than usual

How busy do you expect the day ahead to be?

1 = not at all busy – 5 = very busy .....

How do you feel about the day ahead?

1 = very negative - 5 = very positive .....

EVENING DIARY:

Please complete after your **12 hours** post-awakening sample

Please calculate your collection times and state the actual times that you collected your saliva samples, please be honest as you can

Tube	Collection time (post-awakening)	Calculated collection time (24hr)	Actual collection time (24hr)	Problems with sampling
5	3 hr			
6	12 hr			

How busy do you expect tomorrow to be?

1 = not at all busy – 5 = very busy.....

How do you feel about tomorrow?

1 = very negative - 5 = very positive .....

Study Day 2: .....

MORNING DIARY:

Please complete after your **45 minutes** post-awakening sample

What time did you wake up? .....

Please calculate your collection times and state the actual times that you collected your saliva samples. Please be honest as you can.

Tube	Collection time (post-awakening)	Calculated collection time (24hr)	Actual collection time (24hr)	Problems with sampling
1	Awake	0 min		
2	15 mins			
3	30 mins			
4	45 mins			

Your honesty is very important to us in analysing the data. Therefore, please state your actual collection times for each sample, even if it is different to the designated time, and answer the questions as accurately as possible.

What was the approximate time you went to sleep latest night?

.....(24 hr)

How easy was it for you to determine the exact moment you woke up?

1 = very difficult, 5 = very easy .....

How did you wake this morning?

(please circle)
Alarm clock / radio
Somebody I asked to wake me
Noises
Just woke

Compared to usual, how was your quality of sleep last night?

Much better than usual
A bit better than usual
Same as usual
A bit worse than usual
Much worse than usual

How busy do you expect the day ahead to be?

1 = not at all busy – 5 = very busy .....

How do you feel about the day ahead?

1 = very negative - 5 = very positive .....

EVENING DIARY:

Please complete after your **12 hours** post-awakening sample

Please calculate your collection times and state the actual times that you collected your saliva samples, please be honest as you can

Tube	Collection time (post-awakening)	Calculated collection time (24hr)	Actual collection time (24hr)	Problems with sampling
5	3 hr			
6	12 hr			

Pre Study day 3: .....

EVENING DIARY:

Please complete this the evening before your third study day

How busy do you expect tomorrow to be?

1 = not at all busy – 5 = very busy.....

How do you feel about tomorrow?

1 = very negative - 5 = very positive .....

Study Day 3: .....

MORNING DIARY:

Please complete after your **45 minutes** post-awakening sample

What time did you wake up? .....

Please calculate your collection times and state the actual times that you collected your saliva samples. Please be honest as you can.

Tube	Collection time (post-awakening)	Calculated collection time (24hr)	Actual collection time (24hr)	Problems with sampling
1	Awake	0 min		
2	15 mins			
3	30 mins			
4	45 mins			

Your honesty is very important to us in analysing the data. Therefore, please state your actual collection times for each sample, even if it is different to the designated time, and answer the questions as accurately as possible.

What was the approximate time you went to sleep latest night?

.....(24 hr)

How easy was it for you to determine the exact moment you woke up?

1 = very difficult, 5 = very easy .....

How did you wake this morning?

(please circle)
Alarm clock / radio
Somebody I asked to wake me
Noises
Just woke

Compared to usual, how was your quality of sleep last night?

Much better than usual
A bit better than usual
Same as usual
A bit worse than usual
Much worse than usual

How busy do you expect the day ahead to be?

1 = not at all busy – 5 = very busy .....

How do you feel about the day ahead?

1 = very negative - 5 = very positive .....

EVENING DIARY:

Please complete after your **12 hours** post-awakening sample

Please calculate your collection times and state the actual times that you collected your saliva samples, please be honest as you can

Tube	Collection time (post-awakening)	Calculated collection time (24hr)	Actual collection time (24hr)	Problems with sampling
5	3 hr			
6	12 hr			

How busy do you expect tomorrow to be?

1 = not at all busy – 5 = very busy.....

How do you feel about tomorrow?

1 = very negative - 5 = very positive .....

Day 4: .....

MORNING DIARY:
Please complete after your **45 minutes** post-awakening sample

What time did you wake up? .....

Please calculate your collection times and state the actual times that you collected your saliva samples. Please be honest as you can.

Tube	Collection time (post-awakening)	Calculated collection time (24hr)	Actual collection time (24hr)	Problems with sampling
1	Awake	0 min		
2	15 mins			
3	30 mins			
4	45 mins			

Your honesty is very important to us in analysing the data. Therefore, please state your actual collection times for each sample, even if it is different to the designated time, and answer the questions as accurately as possible.

What was the approximate time you went to sleep latest night?

.....(24 hr)

How easy was it for you to determine the exact moment you woke up?

1 = very difficult, 5 = very easy .....

How did you wake this morning?

(please circle)
Alarm clock / radio
Somebody I asked to wake me
Noises
Just woke

Compared to usual, how was your quality of sleep last night?

Much better than usual
A bit better than usual
Same as usual
A bit worse than usual
Much worse than usual

How busy do you expect the day ahead to be?

1 = not at all busy – 5 = very busy .....

How do you feel about the day ahead?

1 = very negative - 5 = very positive .....

EVENING DIARY:

Please complete after your **12 hours** post-awakening sample

Please calculate your collection times and state the actual times that you collected your saliva samples, please be honest as you can

Tube	Collection time (post-awakening)	Calculated collection time (24hr)	Actual collection time (24hr)	Problems with sampling
5	3 hr			
6	12 hr			

Appendix 12
Self-reported diary used by participants in Study II

Participants ID …….
Date…….

Recording Saliva Sampling Collection:

What time did you wake up? .....
What time did you go to bed? .....

Tube	Sampling Time	Calculated Collection time	Actual collection time	Any problems with sampling
1	Awake	0 min		
2	5 mins			
3	10 mins			
4	15 mins			
5	20 mins			
6	25 mins			
7	30 mins			
8	35 mins			
9	40 mins			
10	45 mins			
11	60 mins			

Please describe how you are feeling this morning (i.e. how your sleep was last night, how awake/drowsy you are feeling, how easy it was to determine when you were awake)
.....
.....
Please describe what it feels like to participate in the study, and report any problems with the protocol.
.....
.....

Appendix 13
Demographic questions

This section has a series of questions that ask about you and your current situation.

Participant/student ID: ……

How old are you? ……

What is your sex? ○ Male
○ Female
○ Do not wish to answer

What is your marital status?

○	Currently married and living together, or living together in a marital-like situation
○	Single
○	Separated/divorced/formerly lived with someone in a marital-like situation
○	Widowed
○	Do not wish to answer

To which of these ethnic groups do you consider you belong?

○	White British	○	White and Black Caribbean	○	Indian
○	White Irish	○	White and Black African	○	Pakistani
○	Any other white background	○	White and Asian	○	Bangladeshi
○	Caribbean	○	Any other Mixed background	○	Any other Asian background
○	African	○	Chinese	○	Any other
○	Any other Black background	○	Do not wish to answer		

What is your student status?

- ○ Full-time
- ○ Part-time
- ○ Do not wish to answer

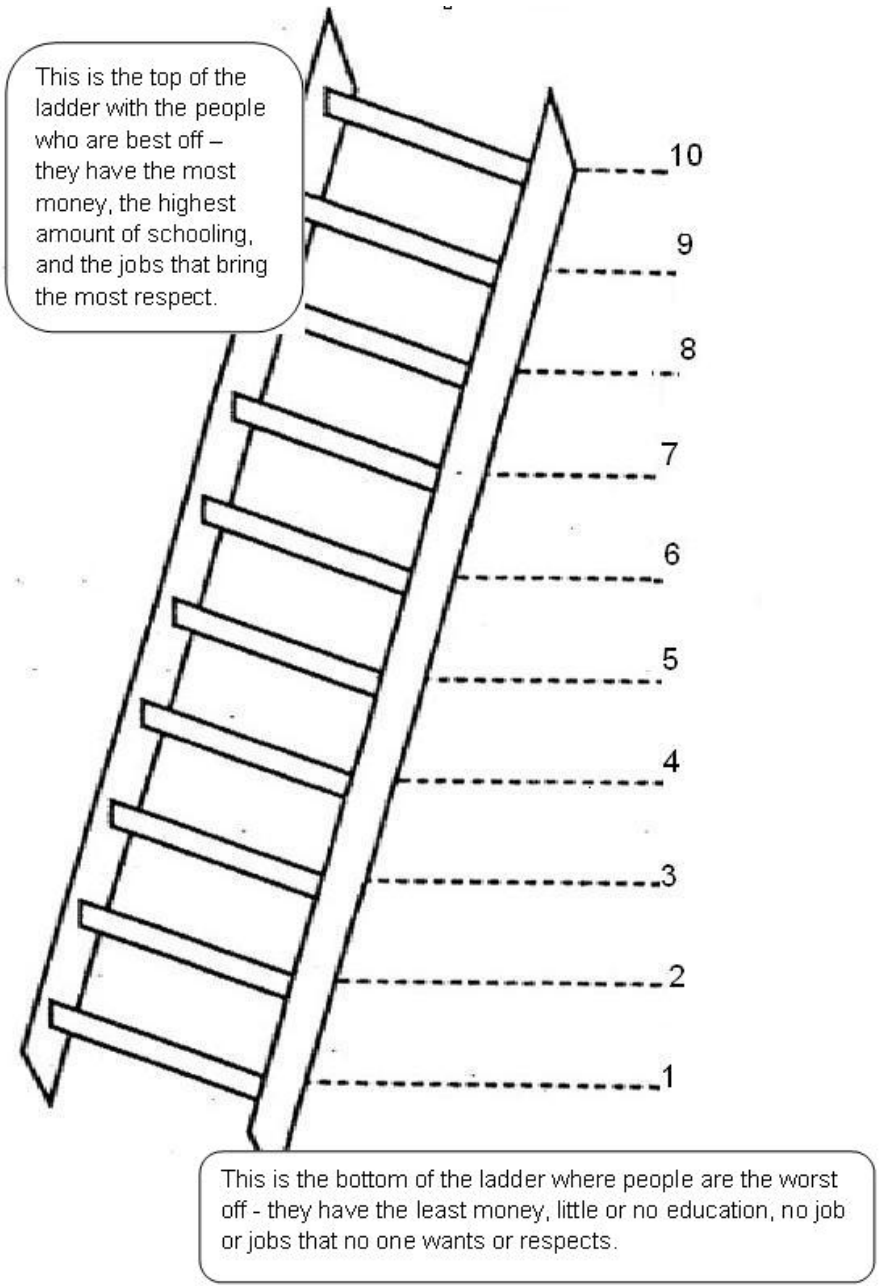
Are you in paid employment?

- ○ Full-time
- ○ Part-time
- ○ Do not wish to answer

What are your highest educational qualifications? Please select your highest qualification?

○	None	○	Modern apprenticeship
○	CSEs or equivalent	○	Diploma
○	GCSEs, O Levels, etc or equivalent	○	Degree
○	A levels	○	Postgraduate (e.g. MBA, PhD)
○	HNC/HND	○	Other (please specify)
○	GNVQ	○	Do not wish to answer

Imagine that this ladder pictures how society is set up. At the top of the ladder are the people who are best off – they have the most money, the highest amount of schooling, and the jobs that bring the most respect. At the bottom are people who are the worst off - they have the least money, little or no education, no job or jobs that no one wants or respects. Now think about your family. Please tell us where you think you would be on this ladder. What step of the ladder best represents where you would be on the ladder ……..



Please specify your **cigarette/tobacco smoking status**

- ○ Current smoker
- ○ Occasional Smoker
- ○ Ex-smoker
- ○ Never smoked
- ○ Do not wish to answer

Are you on any prescribed medication?

- ○ No
- ○ Yes (please specify)
- ○ Do not wish to answer

In general, how would you say that your health has been in the past month?

- ○ Excellent
- ○ Very good
- ○ Good
- ○ Fair
- ○ Poor
- ○ Do not wish to answer

Appendix 14
Hair characteristics

Medication history	
List any prescribed medication	
Any corticosteroids medication in the last year?	○ Yes ○ No
Any history of illicit Drug history in the last 6 months e.g. cannabis etc	○ No ○ Yes (please specify) …… …… ……
Illness history	
Any diagnosed illnesses	○ No ○ Yes (please specify) …… …… ……
Have you suffered from adrenocortical dysfunction in the last year?	○ No ○ Yes (please specify) …… …… ……

Roots of Stress

Participant ID: ……..Date of research Session: ……

A FEW QUESTIONS ABOUT YOU

Age …….... DOB …….....

Pregnant …….....

A FEW QUESTIO

NS ABOUR YOUR HAIR

What is your natural hair colour?

…….....

Is your hair naturally *(please tick)*

- ○ curly
- ○ straight
- ○ wavy

On average how many times a week do you wash your hair?

…….....

Do you use colour or tint products to change the colour of your hair?

Yes/No

If yes, approximately when was last time you did this? ……

Is the colour or tint:

- ○ Permanent (e.g. peroxide)
- ○ Semi-permanent (e.g. (e.g. washes out typically in 4-5 shampoos)
- ○ Temporary (e.g. wash out typically after 1 shampoo)

Do you bleach your hair (e.g. paroxide)?

If yes, when was last time you bleached your hair? .....

If this is known, please specify the name and degree of lightning?

……

Do you use any of the following hair treatments *(please tick and specify how often)*

○ Hair drying

……

○ Hair straightening

……

○ Heated rollers

……

○ Hair growth stimulators

……

○ Hair products e.g. gel, spray etc

……

○ Other *(please specify)*

……

Please specify any other hair treatments you have used in the last 6 months.

……

Appendix 15
Subjective Happiness Scale

For each of the following statements and/or questions, please select the response that you feel is most appropriate in describing you.
For each of the following statements and/or questions, please select the response that you feel is most appropriate in describing you.

In general, I consider myself						
Not a very happy Person 1	2	3	4	5	6	**A very happy person** 7
○	○	○	○	○	○	○

Compared to most of my peers, I consider myself						
Less happy 1	2	3	4	5	6	**More happy** 7
○	○	○	○	○	○	○

Some people are generally very happy. They enjoy life regardless of what is going on, getting the most out of everything. To what extent does this characterisation describe you?						
Not at all 1	2	3	4	5	6	**A great deal** 7
○	○	○	○	○	○	○

Some people are generally not very happy. Although they are not depressed, they never seem as happy as they might be. To what extent does this characterisation describe you?						
Not at all 1	2	3	4	5	6	**A great deal** 7
○	○	○	○	○	○	○

Appendix 16
Positive and negative affect schedule

Below are a number of words that describe different feelings and emotions. Read each word and then indicate how much you felt that way during the past WEEK by selecting the appropriate box for that word.

	very slightly / not at all	a little	moderately	quite a bit	extremely	Do not wish to answer
Interested	○	○	○	○	○	○
Upset	○	○	○	○	○	○
Scared	○	○	○	○	○	○
Proud	○	○	○	○	○	○
Ashamed	○	○	○	○	○	○
Determined	○	○	○	○	○	○
Active	○	○	○	○	○	○
Distressed	○	○	○	○	○	○
Strong	○	○	○	○	○	○
Hostile	○	○	○	○	○	○
Irritable	○	○	○	○	○	○
Inspired	○	○	○	○	○	○
Attentive	○	○	○	○	○	○
Afraid	○	○	○	○	○	○
Excited	○	○	○	○	○	○
Guilty	○	○	○	○	○	○
Enthusiastic	○	○	○	○	○	○
Alert	○	○	○	○	○	○
Nervous	○	○	○	○	○	○
Jittery	○	○	○	○	○	○

Appendix 17
Satisfaction with Life Scale

Below are five statements with which you may agree or disagree. Using the 1-7 scale below, Please indicate how much you agree or disagree with the following statements your agreement with each item. Please be open and honest in your responding.

	Strongly disagree	**Disagree**	**Slightly disagree**	**Neither disagree or agree**	**Slightly agree**	**Agree**	**Strongly agree**
	1	2	3	4	5	6	7
In most ways my life is close to my ideal	○	○	○	○	○	○	○
The conditions of my life are excellent	○	○	○	○	○	○	○
I am satisfied with my life	○	○	○	○	○	○	○
So far I have gotten the important things I want in life	○	○	○	○	○	○	○
If I could live my life over again I would change almost nothing	○	○	○	○	○	○	○

Appendix 18
Meaning in Life Scale

Please take a moment to think about what makes your life and existence feel important and significant to you. Please respond to the following statements as truthfully and accurately as you can, and also please remember that these are very subjective questions and that there are no right or wrong answers. Please select the response that describes you the most:

	Absolutely untrue 1	Mostly untrue 2	Somewhat untrue 3	Can't say true or false 4	Somewhat true 5	Mostly true 6	Absolutely true 7
I understand my life's meaning	○	○	○	○	○	○	○
I am looking for something that makes my life feel meaningful	○	○	○	○	○	○	○
I am always looking to find my life's purpose	○	○	○	○	○	○	○
My life has a clear sense of purpose	○	○	○	○	○	○	○
I have a good sense of what makes my life meaningful	○	○	○	○	○	○	○
I have discovered a satisfying life purpose	○	○	○	○	○	○	○
I am always searching for something that makes my life feel significant	○	○	○	○	○	○	○
I am seeking a purpose or mission in my life	○	○	○	○	○	○	○
My life has no clear purpose	○	○	○	○	○	○	○
I am searching for meaning in my life	○	○	○	○	○	○	○

Appendix 19
Ryffs' scales of psychological well-being

For each of the following items, indicate how often you have felt like this by selecting an item, using the response choices listed just below.

	Strongly disagree	Moderately disagree	Slightly disagree	Slightly agree	Moderately agree	Strongly agree
In general I feel I am in charge of the situation in which I live.	○	○	○	○	○	○
I am not interested in activities that will expand my horizons.	○	○	○	○	○	○
I live life one day at a time and don't really think about the future.	○	○	○	○	○	○
When I look at the story of my life, I am pleased with how things have turned out.	○	○	○	○	○	○
The demands of everyday life often get me down.	○	○	○	○	○	○
I don't want to try new ways of doing things – my life is fine the way it is.	○	○	○	○	○	○
I tend to focus on the present, because the future nearly always brings me problems.	○	○	○	○	○	○
In general, I feel confident and positive about myself.	○	○	○	○	○	○

	Strongly disagree	Moderately disagree	Slightly disagree	Slightly agree	Moderately agree	Strongly agree
I do not fit very well with the people and community around me.						
I think it is important to have new experiences that challenge how you think about yourself and the world.	○	○	○	○	○	○
My daily activities often seem trivial and unimportant to me.	○	○	○	○	○	○
I feel like many of the people I know have gotten more out of life than I have.	○	○	○	○	○	○
I am quite good at managing the many responsibilities of my daily life.	○	○	○	○	○	○
When I think about it, I haven't really improved much as a person over the years.	○	○	○	○	○	○
I don't have a good sense of what it is I'm trying to accomplish in life.	○	○	○	○	○	○
I like most aspects of my personality.	○	○	○	○	○	○
I often feel overwhelmed by my responsibilities.	○	○	○	○	○	○
I have the sense that I have developed a lot as a person over time.	○	○	○	○	○	○

	Strongly disagree	Moderately disagree	Slightly disagree	Slightly agree	Moderately agree	Strongly agree
I used to set goals for myself, but now that seems like a waste of time	○	○	○	○	○	○
I made some mistakes in the past, but I feel that all in all everything has worked out for the best.	○	○	○	○	○	○
I generally do a good job of taking care of my personal finances and affairs.	○	○	○	○	○	○
I do not enjoy being in new situations that require me to change my old familiar ways of doing things.	○	○	○	○	○	○
I enjoy making plans for the future and working to make them a reality.	○	○	○	○	○	○
In many ways, I feel disappointed about my achievements in life.	○	○	○	○	○	○
I am good at juggling my time so that I can fit everything in that needs to get done.	○	○	○	○	○	○
For me, life has been a continuous process of learning, changing and growth.	○	○	○	○	○	○
I am an active person in carrying out the plans I set for myself.	○	○	○	○	○	○

	Strongly disagree	Moderately disagree	Slightly disagree	Slightly agree	Moderately agree	Strongly agree
My attitude about myself is probably not as positive as most people feel about themselves.	○	○	○	○	○	○
I have difficulty arranging my life in a way that is satisfying to me.	○	○	○	○	○	○
I gave up trying to make big improvements or changes in my life a long time ago.	○	○	○	○	○	○
Some people wander aimlessly through life, but I am not one of them.	○	○	○	○	○	○
The past had its ups and downs, but in general, I wouldn't want to change it.	○	○	○	○	○	○
I have been able to build a home and lifestyle that is much to my liking.	○	○	○	○	○	○
There is truth to the saying that you can't teach an old dog new tricks.	○	○	○	○	○	○
I sometimes feel as if I've done all there is to do in life.	○	○	○	○	○	○
When I compare myself to friends and acquaintances, it makes me feel good about how I am.	○	○	○	○	○	○

Appendix 20
Perceived Stress Scale

These questions ask you about your feelings and thoughts during the **last month**. In each case, you will be asked to indicate how often you felt or thought a certain way. Although some of these questions are similar, there are differences between them and you should treat each one as a separate question. The best approach is to answer each question fairly quickly. That is, don't try to count up the number of times you felt a particular way, but rather indicate the alternative that seems like a reasonable estimate. Please select the response that is most appropriate for you:

In the last month, how often have you	Never 0	Almost never 1	Sometimes 2	Fairly often 3	Very often 4
been upset because of something that happened un-expectantly?	○	○	○	○	○
felt that you were unable to control the important things in your life?	○	○	○	○	○
felt nervous and 'stressed'?	○	○	○	○	○
felt confident about your ability to handle your personal problems?	○	○	○	○	○
felt that things were going your way?	○	○	○	○	○
found that you could not cope with all things that you had to do?	○	○	○	○	○
been able to control irritations in your life?	○	○	○	○	○
felt that you were on top of things?	○	○	○	○	○
been angered because of things that happened that were outside of your control?	○	○	○	○	○
felt difficulties were piling up so high that you could not overcome them?	○	○	○	○	○

Items 2, 4, 5, 10 = 4 item PSS

Appendix 21
Centre for Epidemologic Studies Depression Scale

Below is a list of the ways you might have felt or behaved in the past week. For each of the following items, indicate how often you have felt like this in the past week by circling one response, using the response choices listed just below.

	Rarely or none of the time (less than 1 day)	Some or a little of the time (1-2 days)	Occasionally / a moderate amount of time (3-4 days)	Most or all of the time (5-7 days)
I was bothered by things that don't usually bother me.	○	○	○	○
I did not feel like eating; my appetite was poor.	○	○	○	○
I felt that I could not shake off the blues even with help from my family or friends.	○	○	○	○
I felt that I was just as good as other people.	○	○	○	○
I had trouble keeping my mind on what I was doing.	○	○	○	○
I felt depressed.	○	○	○	○
I felt that everything I did was an effort.	○	○	○	○
I felt hopeful about the future.	○	○	○	○
I thought my life had been a failure.	○	○	○	○
I felt fearful.	○	○	○	○
My sleep was restless.	○	○	○	○
I was happy.	○	○	○	○
I talked less than usual.	○	○	○	○
I felt lonely.	○	○	○	○
People were unfriendly.	○	○	○	○
I enjoyed life.	○	○	○	○
I had crying spells.	○	○	○	○
I felt sad.	○	○	○	○
I felt that people dislike me.	○	○	○	○
I could not get going.	○	○	○	○

Appendix 22
Depression Anxiety and Stress Scale

Please read each statement and circle a number 0, 1, 2 or 3 which indicates how much the statement applied to you *over the past week*. There are no right or wrong answers. Do not spend too much time on any statement.

	Did not apply to me 0	**Applied to me to some degree, or some of the time** 1	**Applied to me to a considerable degree, or a good part of time** 2	**Applied to me very much, or most of the time** 3
I found it hard to wind down	O	O	O	O
I was aware of dryness of my mouth	O	O	O	O
I couldn't seem to experience any positive feeling at all	O	O	O	O
I experienced breathing difficulty (eg, excessively rapid breathing, breathlessness in the absence of physical exertion)	O	O	O	O
I found it difficult to work up the initiative to do things	O	O	O	O
I tended to over-react to situations	O	O	O	O
I experienced trembling (eg, in the hands)	O	O	O	O
I felt that I was using a lot of nervous energy	O	O	O	O

	Did not apply to me 0	Applied to me to some degree, or some of the time 1	Applied to me to a considerable degree, or a good part of time 2	Applied to me very much, or most of the time 3
I was worried about situations in which I might panic and make a fool of myself	O	O	O	O
I felt that I had nothing to look forward to	O	O	O	O
I found myself getting agitated	O	O	O	O
I found it difficult to relax	O	O	O	O
I felt down-hearted and blue	O	O	O	O
I was intolerant of anything that kept me from getting on with what I was doing	O	O	O	O
I felt I was close to panic	O	O	O	O
I was unable to become enthusiastic about anything	O	O	O	O
I felt I wasn't worth much as a person	O	O	O	O
I felt that I was rather touchy	O	O	O	O
I was aware of the action of my heart in the absence of physicaL exertion (eg, sense of heart rate increase, heart missing a beat)	O	O	O	O

	Did not apply to me 0	Applied to me to some degree, or some of the time 1	Applied to me to a considerable degree, or a good part of time 2	Applied to me very much, or most of the time 3
I felt scared without any good reason	O	O	O	O
I felt that life was meaningless	O	O	O	O

Appendix 23
Extra tables for Study I (Experiment II)

Table 1 Situational or demographic effects on the CAR and awakening level

	MnInc		S1 (SQRT)		Diurnal decline		Average cortisol	
Fixed effect	Coefficient (SE)	p	Coefficient (±SEM)	p	Coefficient (±SEM)	p	Coefficient (±SEM)	p
Intercept	6.529 (2.538)	<.003	1.961 (0.378)	<.001	2.743 (1.773)	<.001	4.691 (1.028)	<.001
Day of study	-0.459 (0.913)	.617	0.218 (0.128)	.095	0.169 (0.660)	.798	-0.159 (0.389)	.684
Study start tues	-0.540 (1.223)	.661	-0.0450 (0.235)	.849	1.128 (0.772)	.149	0.569 (0.722)	.435
Weekend	-0.742 (2.061)	.720	0.394 (0.300)	.193	1.410 (0.151)	.354	1.028 (0.954)	.283

Table 2 Main effect and interaction effect of well-being/ill-being on cortisol measures

Fixed effect	MnInc Coefficient (SE)	p	S1 (SQRT) Coefficient (±SEM)	p	Diurnal decline Coefficient (±SEM)	p	Average cortisol Coefficient (±SEM)	p
Intercept	5.188 (0.854)	<.001	2.491 (0.154)	<.001	4.484 (0.413)	<.001	5.211 (0.392)	<.001
State WB	1.028 (1.570)	.514	0.202 (0.241)	.405	-0.742 (0.996)	.458	-0.320 (0.674)	.636
State IB	-0.665 (1.243)	.549	-0.070 (0.194)	.720	0.487 (0.896)	.544	0.816 (0.601)	.177
State WB X State IB	0.1997 (2.251)	.929	0.493 (0.139)	.889	2.512 (1.458)	.087	0.341 (1.026)	.740
Intercept	5.671 (0.862)	<.001	2.453 (0.158)	,.001	4.090 (0.410)	<.001	5.097 (0.386)	<.001
Trait WB	0.111 (.495)	.824	-0.509 (0.098)	.607	-0.257 (0.326)	.434	0.149 (0.307)	.048
Trait IB	0.367 (0.495)	.574	0.064 (0.128)	.621	-0.079 (0.414)	.849	-0.151 (0.391)	.702
Trait WB X Trait IB	1.231 (0.612)	.049	-0.050 (0.118)	.673	-0.393 (0.369)	.294	-0.392 (0.348)	.266