Hormonal measurement in psychobiological research

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Three peripherally circulating hormones that can be measured in saliva have received growing attention in psychobiology research. Cortisol and dehydoepiandrosterone (DHEA) are steroid hormones indicative of activity in the hypothalamic-pitutary-adrenal (HPA) axis. The third, the methoxyindole melatonin, is the hormonal product of the pineal neuroendocrine system. The development of reliable methods for salivary hormone assessment was a key turning point for psychobiology research as it enabled new approaches to the study of a wide range of individual difference factors. These biological indices provide meaningful objective measures that can be analysed in parallel to self-reported variables (e.g. stress/well-being) as well as sociodemographic, developmental, psychological and health variables. Saliva is an easy to access biological fluid, collection of which is convenient and does not require trained personnel. Indeed participants can be shown how to undertake self-collection of samples which enables repeated sampling in ambulatory studies (with resultant ecological validity) as well as experimental manipulations within the laboratory. The purpose of this chapter is to guide the psychobiology researcher on appropriate approaches and methodologies for using salivary hormone measures for meaningful investigation of a virtually limitless range of potential research questions.

Physiology

Together the three hormones reviewed in this chapter perform wide ranging and complex physiological roles; their study provides a remarkable insight in individual

differences in human functioning. Cortisol enables adjustment to a wide range of bodily and environmental demands (Fries et al., 2009). For example acute psychological demand (e.g. psychosocial stress) increases cortisol levels in a dose-response manner (Dickerson and Kemeny, 2004). Cortisol exerts powerful effects on the immune and cardiovascular (CV) systems, metabolism, mood and cognition (to name just a few). DHEA is associated with physical, sexual and emotional development as well as metabolism, CV function and memory. Melatonin is the main hormone of the night as it promotes sleep, it is also a power antioxidant and helps regulate the immune system and mood. So individual differences in the secretion of these hormones can provide insight into subtle changes in psychobiological function. These changes may not, at time of measurement, have translated into observable symptoms but may indicate vulnerability to clinical symptoms, should the changes be sustained. As such these hormone measures provide a useful pre-clinical indicator of the links between mind and body.

The hypothalamic-pituitary-adrenal (HPA) axis is a major neuroendocrine system; one of the pathways by which the brain can exert control over aspects of internal physiological activity. It is hierarchical in its organisation. The neural control centre is the hypothalamus, a region of the brain located below the thalamus within the limbic system, our emotional brain. The hypothalamus receives neuronal input from various modalities including the cognitive and emotional brain as well as visual information denoting dark-light transitions. In terms of its regulation over the HPA axis the paraventricular nuclei (PVN) of the hypothalamus performs a central role. In response to activating input signals the parvocellular cells of the PVN secrete a neuropeptide, corticotropin releasing factor (CRF) which in turn stimulates the release of adrenocorticotrophic hormone (ACTH) from corticotrophs in the anterior pituitary, an endocrine gland that sits just below the hypothalamus. ACTH is released into the general circulation as a hormone, to stimulate steroidogenic activity and peripheral hormone release from the adrenal cortex. Hence the hierarchical nature of the HPA axis.

In common with other patterns of anterior pituitary activity the HPA axis is pulsatile. Secretory episodes occurring at roughly hourly intervals. This is apparent for ACTH

and consequent cortisol secretion and likely reflects the activity of pulse generation in the hypothalamus. Pulsatile patterns of secretion are only evident in individual profiles based upon frequent (or continuous) blood sampling. They are lost for the majority of sampling protocols (including salivary sampling) and when data from multiple individuals is summed to reveal overall trends. The importance of pulsatility in cortisol function is attracting increasing attention, most notably by neuroendocrinologists studying animal models (e.g. Lightman and Conway-Campbell, 2010) however, although it is important to be aware of this interesting emerging area, analysis of hormone pulsitity is not possible in saliva samples and is hence beyond the scope of the majority of psychobiologists.

Accurate and meaningful assessment of cortisol in saliva samples was the first of these hormones to be validated (see Kirschbaum and Hellhamer 1989). Consequently salivary cortisol is the most intensively studied, however interest in salivary DHEA is substantial and is gradually gathering ground for salivary melatonin. Cortisol is a lipid-soluble hormone and passes freely through cellular plasma membranes. In the blood circulation it is mostly bound to plasma proteins, a specific cortisol binding globulin termed 'transcortin' and to some extent albumin. Some 90% of blood cortisol is protein bound, mostly to transcortin. This serves to transport the molecule in the circulation and also acts as a reservoir. At target tissue a dynamic equilibrium will operate between circulatory protein-bound steroid, free cortisol and cortisol receptors expressed within the cells. Only free cortisol is biologically active; available for receptor binding. Blood cortisol assay measures total cortisol in a sample with no distinction between bound and free. Over the past several decades and particularly in psychobiology research the body fluid of choice for cortisol assessment has been saliva. As previously mentioned this medium provides the obvious advantage of sampling convenience, which can be done simply by the participant without clinical attention or trauma and at repeated intervals of close proximity over the time course of a study interest. Additionally saliva contains only the free component of circulating cortisol, since free cortisol equilibrates across the membrane barriers of the salivary gland. Salivary cortisol is a convenient measure of circulating free cortisol. In this it provides a more sensitive index of endocrine signalling within the HPA axis, not encumbered by the large amounts of

protein bound steroid circulating inactively. In an analogous way much of the DHEA in the blood circulation is conjugated as the sulphate (DHEA-S). This forms a large reservoir, comparable to the protein bound cortisol component in blood. Unconjugated DHEA is physiologically active and this is the component usually reported as the salivary measure

The adrenal cortex is composed of three distinct zones; the mid zone, zona fasciculate is the largest and synthesises and secretes the glucocorticoid cortisol. Inner to this is the zona reticularis which secretes the adrenal androgen DHEA whereas an outer zone, the zona glomerulosa produces mineralocorticoids primarily aldosterone. All are derived from the steroid precursor pregnenolone, itself derived from cholesterol. The pathway of steroidogenesis depends upon the enzyme activity in each zone. ACTH exerts its stimulatory influence at the early undifferentiated stage, cholesterol conversion to pregnenolone. ACTH is thought the only secretagogue for cortisol and DHEA. Hence the HPA axis is the stimulatory axis for the two major peripheral steroid hormones cortisol and DHEA.

A major difference between cortisol and DHEA activity is developmental. Secretory patterns of cortisol are established in infancy and remain relatively stable throughout adult life, although disturbances are associated with older age, adverse life circumstances, physical and mental illnesses (see later). By contrast DHEA secretion is lacking in infancy and childhood up until near puberty. Preceding the onset of puberty (menarche in girls) by a year or so the adrenal undergoes a maturation, referred to as adrenarche, when the zona reticularis begins to secrete DHEA. DHEA is responsible for the growth of pubic and axillary hair in both boys and girls, stimulates the prepubertal growth spurt and contributes to a shift in the focus of psychological attachment from parent to peer (Oskis et al., 2015). DHEA secretion, continues to increase through adolescence and early adulthood until late twenties; declining from middle into older age.

Melatonin is distinct from both cortisol and DHEA being synthesized from tryptophan, once taken up from the circulation and transformed to serotonin. Serotonin is converted into melatonin by a two-step process involving the sequential activities of

two enzymes, the rate-limiting serotonin-N-acetyl transferase, and hydroxyindole-O-methyl transferase. Noradrenergic neurones stimulate pinealocytes in the pineal to secrete the hormone, which has high lipid and water solubility which facilitates passage across cell membranes enabling access to body fluids including saliva. The peripheral hormone profile of melatonin faithfully reflects active secretion as no pineal storage of melatonin is available. The secretion of melatonin occurs almost exclusively at night in the dark, with maximum levels around 3:00-4:00 a.m.; diurnal levels are low and often undetectable (Claustrata et al, 2005). Maternal melatonin crosses the placenta and is one of the maternal rhythmic signals capable of synchronizing the fetal biological clock. After maturation, melatonin production reaches the highest levels at the age of 3-6 years. In contrast to DHEA melatonin secretion decreases with the onset of puberty (Crowley et al, 2012). With aging, the melatonin rhythm progressively dampens, and can be completely abolished in advanced age (Claustrata et al, 2005).

Circadian regulation

Healthy regulation of cortisol, DHEA, melatonin is overseen by the brain's biological clock: the suprachiasmatic nucleus (SCN). The SCN generates circadian (24 hour) secretory patterns of all three hormones. Using careful methodology as described below, daytime (diurnal) patterns are accessible to psychobiologists using saliva sampling. There is increasing evidence that aberrant circadian cycles are a common factor in a wide range of clinical mental and physical health conditions (Dickmeis et al, 2009; Wulff et al, 2010; Jagganarth et al, 2013). By careful assessment of these hormones from saliva samples it is possible to gain an insight into circadian function of populations. However what makes this approach so worthwhile is that aberrant diurnal hormone can be detected in sub-clinical syndromes i.e. at risk populations can show aberrant diurnal hormone profiles prior to onset of symptoms (e.g. Oskis et al, 2011). This is interesting as it suggests that dysfunction of circadian function may precede and predict pathology, rather than be a consequence thereof. The diurnal profile of these hormones has been shown to be responsive to modification by interventions and can thus be used to evaluate changes in circadian physiology prior to observable change in symptomology. In other words the neuroendocrine system is

an excellent model for studying the transmission of circadian information in the body (Kalsbeek et al, 2012).

The SCN is located in the hypothalamus and is the body's 24 hour endogenous oscillator (Kriegsfeld et al, 2003). Activity of genes within every cell of the SCN switch on and off over an approximately 24 hour period by using transcription-translation feedback loops. These genes generate proteins that send messages to surrounding cells controlling function – in this way the SCN has a powerful effect on brain and body function. The individual cells of the SCN are synchronised with each other and the external environment by a range of zeitgebers. The most powerful of these cues is light; others include body temperature and social activities such as eating. Light is a particularly powerful zeitgeber as the SCN is strategically located over the optic chiasmic (the crossing of the optic nerves) such that light messages from non-vision light receptors in the back of the retina (retinal ganglion cells) terminate there and inform the cells of the SCN of day and night. Most bodily functions oscillate around the 24 hour day night cycle. Aging and a wide range of psychopathology is associated with dysfunction in circadian function, which can be studied by careful examination of secretory patters of hormones from saliva samples.

Cortisol has a marked circadian pattern of secretion, with lowest levels (i.e. the nadir) a few hours after the onset of night-time sleep. Levels gradually rise during the later phases of night-time sleep; the moment of awakening initiates a large burst in cortisol secretion, which is at its maximum when waking with ambient light (i.e. at dawn). The cortisol awakening response (CAR) generates the highest levels (i.e. the acrophase) in daily cortisol secretion, peaking between 30-45 minutes following morning awakening. Marked day differences in the size of the CAR have been reported; this particular aspect of the circadian pattern of cortisol secretion is particularly sensitive to daily variation in state variables such as ambient light and psychological state (Stalder et al, 2009; Clow et al, 2010). Following this peak there is a marked decline in cortisol and between 3 hours after awakening and bedtime there is a gradual decline in cortisol secretion, in readiness to bedtime and night-time sleep. The cortisol diurnal decline is not sensitive to light and daily variation in this aspect is less marked than for the CAR in healthy participants (Edwards et al, 2001).

The circadian secretory pattern of melatonin is the opposite to that of cortisol as it reaches its maximum during night-time sleep and is low or even absent during the day: melatonin secretion is inhibited by light (Vakkuri, 1985). The relatively recent introduction of validated assays for the assessment of melatonin levels from saliva samples (Voultsios et al, 1997) means there is a more limited literature on the dynamics of melatonin secretion across the day using repeated saliva sampling. Most studies have utilised blood sampling, often at a single time point. Recently however it has been shown that the post-awakening pattern of melatonin secretion is consistent across days for healthy individuals. However there is no melatonin equivalent of the CAR (Ramachandran et al, 2016): levels of melatonin gradually fall after awakening (29% in the first 45 minutes compared to a 112% rise in cortisol over the same post awakening period) and remain low until night-time sleep when there is a marked surge. Changes in, and levels of, post awakening cortisol and melatonin are reported to be unrelated. This is not surprising as the hormones originate from separate synthetic pathways, as described above. However there are reports that the amplitude of day-to-night changes in cortisol and melatonin are related (Corbalan-Tutau et al, 2014).

In contrast to melatonin the circadian pattern of DHEA secretion is more likely to be associated with cortisol as both hormones originate from the same gland, albeit discrete layers within that gland. Indeed as ACTH is the secretagogue of both cortisol and DHEA it is unsurprising that both hormones show similar circadian profiles: highest in the morning with decreasing levels during the day and night. Levels and patterns of DHEA secretion are remarkably consistent across days in healthy participants (Hucklebridge et al, 2005). However there is no observable post awakening surge in DHEA secretion; levels are highest upon awakening and remain stable during the first 45 minutes post awakening. DHEA levels do fall following awakening, reaching a diurnal low prior to sleep. Another distinguishing feature between diurnal patterns of cortisol and DHEA is that post awakening DHEA strongly predicts daytime levels of that hormone whereas post awakening cortisol does not (Oskis et al, 2012). It is argued that DHEA more closely reflects levels of the secretagogue ACTH (i.e. HPA axis activation) than cortisol, secretion of which is

dependent upon ACTH availability but is fine-tuned by direct sympathetic nervous system innervation to the zona fasciculate of the adrenal cortex (Buijs et al, 2003; Clow et al, 2010).

Recommendations for hormone measurement

A number of technical issues relating to salivary hormone measurement deserve consideration. Common illnesses (i.e. colds and flu) can affect the immune system and sensitivity of the HPA axis. Consequently participants should be advised to only continue with the requested saliva sampling if they are feeling symptom-free. Avoid testing on participants who are taking prescribed medications as these influence basal cortisol and DHEA (e.g. Granger et al., 2009; Kroboth et al., 1999). If testing on participants who are taking medication record these and control for this in statistical analysis. Exclude all participants with a diagnosed neuroendocrine disorder (e.g. Cushing's and Addison's disease; associated with over- and underproduction of cortisol, respectively). Exclude all participants taking steroid-based medication (e.g. to alleviate asthma, Crohn's disease, rheumatoid arthritis) as the medication will mask any endogenous patterns of cortisol secretion. Other common behaviours such as vigorous physical activity, meals, smoking, caffeine and alcohol consumption can all affect activity of the HPA axis and should be avoided (see Table 7.1 for additional information and references).

In early studies the most commonly used assay was commercial radioimmunoassay (RIA) originally developed for blood measurement. Assay volumes were adjusted to increase sensitivity; cortisol in saliva is only 5-10% of total circulating cortisol. More recently commercial assay kits developed specifically for saliva and covering the appropriate assay range have become available for all three hormones here discussed. Enzyme linked immunosorbent assay (ELISA) has tended to supplant RIA. Assays should be undertaken in duplicate to ensure accurate measurement; the percentage coefficient of variation (%CV) between duplicates should be reported and variation greater than 10% should be repeated to confirm accurate results. Each assay plate should also always run a low and high 'control' sample (i.e. samples of known concentration provided by the assay suppliers; again assayed in duplicate).

This enables analysis of inter-plate reliability, reported when publishing results and should be no more than 10%CV.

Along with advances in measurement technique a number of saliva collection methodologies have been developed. The simplest and in many ways the most practical for salivary cortisol assessment is the Salivette device. This relies on a cotton dental role that is placed in the mouth for about a minute to absorb saliva. It is transferred to a small collection vial then centrifuged to obtain a clear sample. Passive drool (typically using a small section of plastic straw, into an Eppendorf vial) is required when DHEA or melatonin is to be measured. Care should be taken when labelling saliva collection devices. Tubes can become wet and be stored for long periods, so robust clear labelling is essential. Samples should be frozen (-20^{0}C) as soon as possible after collection and so stored until assay. In ambulatory studies participants are recommended to store in their home freezer as soon as is practical. Research suggests that salivary cortisol is relatively stable at room temperature for up to 5 days, after which concentrations begin to decline. If convenient samples can be mailed back to the researcher during this time period (Clements et al, 1998). Participants are instructed to take nil by mouth, other than water, and not to smoke for at least 30 min prior to sampling. Sample protocols should be designed to avoid proximity to meal times.

Assessment of hormonal circadian patterns

Repeated saliva sampling can enable accurate assessment of the dynamics of the diurnal secretory pattern of all hormones. Ecological validity can be enhanced by self-collection of saliva samples within the domestic setting. Participants can be instructed (most efficiently in a face-to-face interview with the researcher) on the methods for saliva sampling and the requirement to adhere to strict timing requirements. In this way participants can practice using the sampling devices and ask questions. There are several important points to emphasise about the methodology here; overlooking these points can invalidate the quality of data collected and rightly jeopardise opportunities to publish. As pointed out in the section above only cortisol can be accurately measured following collection of saliva using salivette sampling devices. These devices use a cotton swab to absorb saliva.

However both DHEA and melatonin stick to these swabs so for studies of these hormones it is necessary to collect saliva via fee drooling into small containers (e.g. Eppendorf tubes) cortisol can also be accurately measured from saliva collected using these devices. The other major consideration when measuring diurnal patterns of these hormones is to collect an adequate number of samples to enable analyses of the changing dynamics of secretion. As these secretory dynamics are closely associated with sleep/wake patterns it is necessary to provide clear instructions on sample timing relative to individual awakening time. This is especially true for cortisol as the CAR is a period of very rapid change in cortisol secretion, initiated at the moment of awakening. Delay between awakening and collection of the first sample by as little of just over 5 minutes can generate inaccurate assessment (Smyth et al, 2013). As participants are frequently not good at sampling within such a narrow time frame it has recently been agreed by a group of experts that electronic monitoring of awakening and sampling is a necessary requirement in CAR research (see Stalder et al, 2016). Whilst this may seem demanding it is necessary when measuring the CAR. In contrast the post-awakening patterns of melatonin and DHEA secretion are less volatile, and if measured without cortisol it is not necessary to employ these strict measures. Likewise measurement of the day decline in all hormones, after the immediate post-awakening period, is less prone to the negative effects of moderate sampling delay. In this case self-reported diary records of awakening and sampling times is acceptable. However if morning samples are to be captured as part of the profile it is usual practice to instruct participants to collect samples in sets of hours post-awakening (e.g. 3,6,9,and 12 hours post awakening) rather than at set clock times. This is necessary as there is the potential for large individual variation in awakening times between participants (e.g. 5.30am-10.00am). This means that a sample collected, say at 12.00 noon, could be much as 6.5hours, or as little as 2 hours, post awaking. Interpretation of these values would be limited as the day decline would be ‘polluted’ with the CAR in the last instance, meaning the results for these 2 participants are not comparable. It is of course also necessary to abide by the rules of nil by mouth, other than water, for at least 30 minutes prior to each sampling.

Although the diurnal patterns of these hormones are reported to be relatively reproducible from day to day it is strongly advisable to sample across *at least* 2 typical weekdays in order to reliably capture the 'typical' or 'trait-like' profile of the individuals in the target populations. This is necessary to offset the possibility of random events interfering with underlying profiles. If using these hormone profiles as an outcome measure in an intervention evaluation it would be necessary to demonstrate that the difference between pre and post the intervention were greater than between 2 consecutive days. For cortisol profiles this is especially a requirement when assessing the CAR, which has the most marked state variation of all measures considered here (see Stalder et al, 2016).

There is a wide and diverse literature on the relationship between diurnal patterns of these hormones (especially for cortisol) and physical and mental health outcomes. Whilst outside the scope of this brief chapter a common finding to emerge is that flattening of the diurnal profile of these hormones is associated with worse outcome, whatever that might be. Indeed the consistent finding of flat cycles (i.e. less change in concentration from morning to evening (for cortisol and DHEA) and between day and night (for melatonin) is indicative of aging, chronic stress and morbidity in general (e.g. Stephton et al, 2000; Corbalan-Tutau et al, 2014, respectively).
In summary careful assessment of the diurnal pattern of neuroendocrine function from saliva samples provides a useful insight into circadian function, dysregulation of which is a prominent factor in aging, chronic stress, physical and mental health disorders.

Assessment of HPA axis reactivity

The HPA axis response to an acute stress challenge has been associated with a range of stress-related disorders. For example in children and adolescents increased cortisol reactivity has been shown to be associated with behaviours and emotional problems associated with depression (Dockray et al, 2009). In contrast older patients with depression (mean age=62.3 years) have been reported to have an attenuated cortisol stress response (Taylor et al, 22006). It has been proposed that chronic stress, with associated changes in HPA axis reactivity, is associated with changes in the structure and function of the hippocampus (via changes in neurogenesis) and

that this in turn leads to reduced negative feedback on the HPA axis, generating a vicious circle of psychopathology influential in the genesis of clinical depression (and a range of physical health conditions such as CV disease) (Mahar et al, 2014). Such theories makes it valuable to investigate individual differences in sensitivity of the HPA axis to stress-stimulation, as a marker of vulnerability to stress-related disorder.

In laboratory settings acute activation of the HPA axis is typically studied by presenting individuals with a brief (~10 minute) psychosocial stressor. Protocols that contain the following elements (1) active engagement in the task (e.g. cognitive tasks, public-speaking or mental arithmetic); (2) lack of control; and (3) social-evaluative threat (e.g. aspects of the 'self' appears to be negatively judged by others) are the most effective at eliciting a stress response (Dickerson & Kemeny, 2004). The most common and widely standardized protocol used is the Trier Social Stress Test (TSST). It includes a brief anticipation period and a 10 minute test period in which participants engage in a public speaking and mental arithmetic task in front of an unsympathetic authoritarian audience (Kirschbaum, Pirke, et al., 1993; Smyth et al., 2013 for an overview). Several studies have consistently demonstrated that the TSST produces large and reliable HPA axis responses in both healthy and clinical populations (Dickerson & Kemeny, 2004; Kirschbaum, Pirke, et al., 1993; McRae et al., 2006). Typically, cortisol has been the main physiological measure of interest however, more recently, the importance of DHEA and DHEA-S have been highlighted. To our knowledge melatonin has not been studied in this context.

Activation of the HPA axis in response to stress is immediate, but the peak of cortisol secretion is reached approximately 30 minutes following onset of the stressor, returning to baseline in the following hour (Dickerson & Kemeny, 2004). Less focus has been on the secretion of DHEA/DHEA-S in response to psychosocial stressors, however, studies that have investigated this show that on average plasma and salivary DHEA/DHEA-S peaks 20 min following onset of the stressor (Izawa et al., 2008; Lennartsson et al., 2012) and returns to baseline levels 40-50 min following the stressor (Izawa et al., 2008). DHEA responses have been shown to be moderately associated with cortisol (Izawa et al., 2008). The finding that DHEA activation precedes cortisol by approximately 10 min (Izawa et al., 2008;

Lennartsson et al., 2012) is surprising since the secretion of these hormones are dependent upon the same secretagoge: ACTH. The time difference might reflect differential latency in steroidogenic pathways. ACTH stimulates the first step (cholesterol to pregnenolone). Subsequent conversion to DHEA involves fewer enzyme-mediated biosynthetic stages than the cortisol pathway. Due to changes in basal levels of both DHEA and cortisol in the morning (Hucklebridge et al, 2005) and the post-prandial period (e.g. Lovallo et al., 2005; Svec & Shawar, 1997) it is recommended that stress reactivity testing takes place in the afternoon (at least 1 hour after lunch) and time of testing should be consistent throughout the study (Dickerson & Kemeny, 2004; Kudielka et al., 2004). To capture the full profile of the stress response and the peak of cortisol or DHEA saliva samples should be collected at 10–15 min before and immediately prior to the stressor onset and again 10- to 15-min intervals for about 45–60 min after the end of the stressor (Kirschbaum, Pirke, et al., 1993). Following food, drink, physical exercise or smoking cortisol and DHEA levels increase (e.g. Lovallo et al., 2005; Svec & Shawar, 1997; Mendelson et al., 2005; Kirschbaum et al., 1992), thus participants should remain nil-by-mouth (except water) and free from exercise for 30 min prior to saliva sampling period.

More recently a group version of the TSST has been developed and found to reliably activate the HPA axis: TSST-G (von Dawans et al. 2011). The same elements of motivated task performance, lack of control and socioevaluative threat are in place however, instead of individual testing, 6 participants are tested together, in sequence. In turn, each participant gives a 2 minute talk, after which each participant completes the mental arithmetic task for 80 seconds. This version of the TSST may be of particular interest to experimental social psychologists as it is possible to manipulate the social context of the groups, e.g. to examine the impact of social identity (e.g. Häusser et al, 2012). It has also been used to examine individual differences in stress reactivity without social manipulation (Smyth et al, 2015).

When studying stress reactivity in relation to psychological individual difference variables it is important to take account of various demographic and developmental factors, which if ignored might contaminate data analysis. Table 1 provides a brief

overview of such factors when examining cortisol and DHEA responses to stress. The table includes initial basal levels, when relevant, as well as responsivity.

Table 7.1. Some variables to consider when measuring salivary cortisol and DHEA responses to stress

	(a) Cortisol	(b) DHEA
Age	• Studies show that older adults have higher basal cortisol levels and some show an enhanced response to psychosocial stressor Kudielka 1999; Nicolson, 1997) but findings are mixed (Kudielka, 2004; Rohleder, 2002).	• DHEA basal levels are age dependent: from 1 month old levels decline, rise to mark the onset of adrenarche, peaking between 20-40 years old and decline thereafter reaching lowest levels between 70-80 years old, at approximately 20-30% of peak levels. (Kroboth et al., 1999). • Magnitude of DHEA/DHEA-S stress response is reduced with age (Lennartsson et al., 2012).
Gender	• Males reliably show larger responses (e.g. 200-400% increases) compared with females (50-150% increases) following psychological stressors {Kudielka et al, 2009).	• Basal levels are lower in males compared with females (see Kroboth et al., 1999). • No significant differences between males and females in DHEA stress reactivity levels. For DHEA-S increases were significantly higher for males (ranging from 5% to 47%) compared with females (ranging from 2% to 40%) (Lennartsson et al., 2012).
Menstrual Cycle	• Larger responses observed during the luteal phase whilst smaller responses in	• Self-reported menstrual phase did not affect DHEA levels or response patterns

	the follicular phase or in women taking oral contraceptives (Kudielka et al, 2009)	(Lennartsson et al., 2012).
Puberty Stage/hormonal status	• The cortisol response to a psychological stressor is associated with the transition to adolescence: blunted in boys and increased in girls {Gunnar, 2009 #1349}.	• DHEA and DHEA-S secretion rises rapidly during puberty in both boys and girls (Smith 1975). • Higher DHEA levels observed in older and more puberty advanced adolescences and this effect is more pronounced in girls compared with boys (Shirtcliff et al., 2007). • Mixed results that DHEA responsively to stressors are associated with age and puberty e.g. higher DHEA responses in adolescents more puberty developed for a public speaking task but not in the conflict discussion paradigm (Shirtcliff et al., 2007).
Alcohol consumption	• Blunted stress responses associated with chronic alcohol consumption {Lovallo, 2000 #1352}. • Results for social drinkers and those with a family history more mixed (see Kudielka et al 2009).	• In students that binge drink, DHEA levels did not significantly increase following a stressor (impossible task) (Wemm et al., 2013).
Smoking	• Habitual smokers have chronically elevated cortisol levels but a blunted response to a standardised stressor (Kudielka et al,	• High doses of nicotine induces elevated DHEA levels (Mendelson et al., 2005). • DHEA levels are higher in

	2009).	smokers compared with non-smokers Nonsmokers (Pomerleau et al, 1992; Field et al, 1994; del Arbol et al, 2000; al'Absi et al, 2003).
Medication	• Medication use should be recorded – numerous and ever-changing range of medications impact on salivary cortisol, see Granger et al {, 2009 #1361} for the pathways involved. • Medications can impact cortisol responses to psychological stressors in both patients and healthy controls. {Fries, 2006 #1356;Makatsori, 2004 #1357;Pariante, 2004 #1358}.	• Medication use should be recorded – numerous and ever-changing range of medications impact on DHEA levels, see Kroboth et al. (1999) for an overview.
Physical exercise	• Review?? Or key study?	• Exercise is associated with higher basal DHEA (see Kroboth et al., 1999).

Conclusions

In conclusion, the development of salivary assays for cortisol, DHEA and melatonin determination has provided the opportunity to gain valuable insight into individual differences in circadian rhythms and stress reactivity. The ease and widespread acceptability of saliva sampling has enabled the study of small infants to the very old. Differences in circadian function and/or stress reactivity can be detectable prior clinical manifestation of symptoms. Such observations enable the development of theoretical frameworks centered on stress-related vulnerability that can be tested in longitudinal lifespan studies and inform a wide range of physical and mental health clinical syndromes. The use of saliva samples in this way is relatively simple and attention to the methodological considerations summarized in this chapter will ensure the validity of results generated and hence help advance this fascinating and important area of research.

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