HOW TO MEASURE STRESS IN HUMANS

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1. STRESS RESEARCH IN HUMANS?

a. What is stress?

Stress is a popular topic these days. There is rarely a week that passes without hearing or reading about stress and its deleterious effects on health. Given the negative impact of stress on human health, many types of stress management therapies have been put forward in order to decrease stress and to promote wellbeing. However, there is a great paradox in the field of stress research, and it relates to the fact that the popular definition of stress is very different from the scientific definition of stress. This inconsistency has left a multitude of people and experts talking about, and working on very different aspects of the stress system.

In popular terms, stress is mainly defined as *time pressure*. We feel stressed when we do not have the time to perform the tasks that we want to perform within a given period. The perception of time pressure usually triggers a set of physiological reactions that indicate that we are stressed. Although this popular definition of stress may trigger a stress response, it is important to acknowledge that in scientific terms stress is not equivalent to time pressure. Indeed, if this were true, every individual would feel stressed when pressured by time. However, many of us know that while there are some people who are extremely stressed by time pressure, there are others who thrive under time pressure. This shows that stress is a highly individualistic experience that does not depend on a particular event such as time pressure, but rather depends on specific psychological determinants that trigger a stress response.

b. Short historical background on stress

Prior to becoming part of our day-to-day conversations, the term "stress" was used by engineers to explain forces that can put strain on a structure. For example, one could place strain on a piece of metal in such a way that it would break like glass when it reached its stress threshold. In 1936, Hans Selye (reproduced in Selye, 1998) borrowed the term *stress* from the field of engineering and talked about stress as being a nonspecific phenomenon representing the intersection of symptoms produced by a wide variety of noxious agents. For many years, Selye tested various conditions (e.g., fasting, extreme cold, operative injuries, and drug administration) that would produce physical changes in the body that were representative of a stress response, such as enlargement of the adrenal gland, atrophy of the thymus, and gastric ulceration. Selye's view of the concept of stress was that the determinants of the stress response are non-specific. Thus, many unspecific conditions can put strain on the organism and lead to disease outcome, the same way that many unspecific conditions can put strain on a piece of metal and break it like glass.

Not all researchers agreed with Selye's model, particularly with the notion that the determinants of the stress response are non-specific. The reason for this was simple; While Selye spent his entire career working on physical stressors (e.g., heat, cold, and pain), we all know that some of the worst stressors we encounter in life are psychological in nature, and are induced by our interpretation of events. For this reason, a psychologist

named John Mason (see Mason, 1968) spent many years measuring stress hormone levels in people subjected to various conditions that he thought would be stressful. These experiments enabled Dr. Mason to describe the psychological characteristics that would make any condition stressful, to any individual exposed to it. This work was made possible in the early 1960's due to the development of new technology that allowed scientists to measure levels of hormones that are released during reactivity to a stressor. The release of stress hormones is made possible through activation of a neuroendocrine axis named the hypothalamic-pituitary-adrenal (HPA) axis.

c. The stress response

When a situation is interpreted as being stressful, it triggers the activation of the hypothalamic-pituitary-adrenal (HPA) axis whereby neurons in the hypothalamus, a brain structure often termed the "master gland", releases a hormone called corticotropin-releasing hormone (CRH). The release of CRH triggers the subsequent secretion and release of another hormone called adrenocorticotropin (ACTH) from the pituitary gland, also located in the brain. When ACTH is secreted by the pituitary gland, it travels in the blood and reaches the adrenal glands, which are located above the kidneys, and triggers secretion of the so-called stress hormones.

There are two main stress hormones, the glucocorticoids (called corticosterone in animals, and cortisol in humans), and the catecholamines (epinephrine and norepinephrine).

Under normal (non-stressed) conditions, cortisol secretion shows pronounced circadian rhythmycity, where concentrations are at their highest in the morning (the circadian peak), progressively decline from late afternoon to early nocturnal periods (the circadian trough), and show abrupt elevations after the first few hours of sleep.

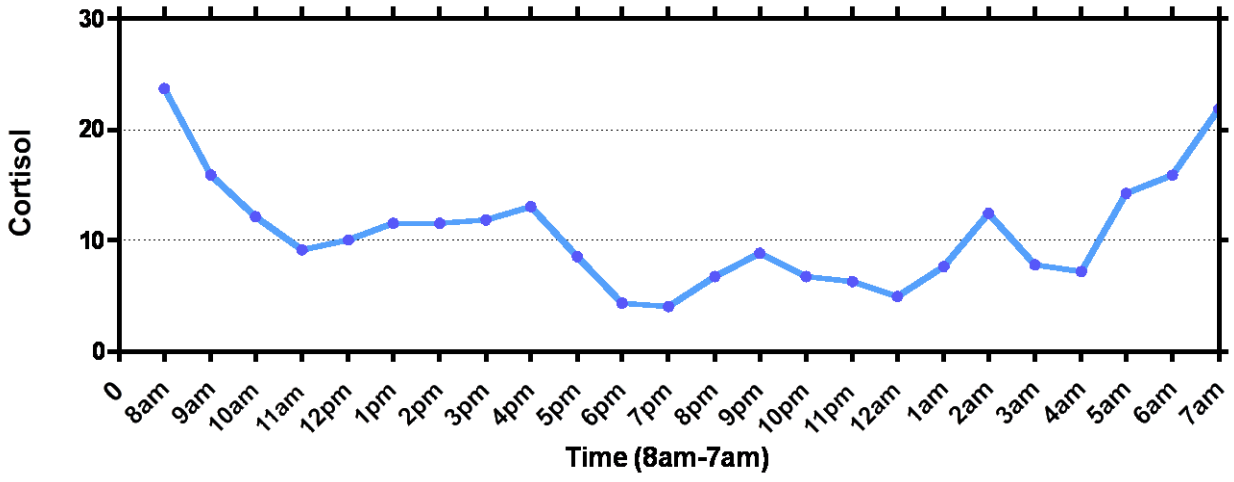


Figure. Example of the circadian rhythm of serum cortisol levels.

The acute secretion of glucocorticoids and catecholamines constitutes the primary mediators in the chain of hormonal events triggered in response to stress. When these two hormones are secreted in response to stress, they act on the body to give rise to the fight-or-flight response whereby one would, for instance, experience an increase in heart rate and blood pressure.

d. Psychological determinants of a stress response in humans

By summarizing the results of studies that measured the circulating levels of stress hormones before and after individuals were exposed to various situations that were deemed to be stressful (e.g., air-traffic controllers or parachute jumping), Mason (1968) was able to describe four main psychological determinants that would induce a stress response in any individual exposed to them. Using this methodology, he showed that in order for a situation to induce a stress response, it has to be interpreted as being novel, and/or unpredictable, it must be threatening to one's ego and/or the individual must have the feeling that he/she does not have control over the situation. Although this work led to a general debate between Selye and Mason (Selye, 1975), further studies confirmed that the determinants of the stress response are highly specific, and therefore, potentially predictable and measurable.

2. HOW CAN WE MEASURE STRESS?

a. Psychological Questionnaires

The field of psychology concentrates on the measurement of abstract concepts, such as language, cognition, personality and emotions, to name a few. Given that the determinants of a stress response as defined by Mason were psychological in nature, the concept of stress became part of the group of psychological concepts that can be measured. Once developed in the field of psychology, stress became measurable by the use of questionnaires. To this end, psychologists developed questionnaires that cover a wide range of psychological symptoms that can be induced by exposure to chronic stress.

There are a wide variety of questionnaires that have been developed to assess the psychological factors that are associated with stress in humans. The Centre for Studies on Human Stress presents a large database containing the majority of stress questionnaires available in the scientific literature.

b. Physiological Measures of Stress

The interpretation of a situation as being stressful leads to the activation of the hypothalamic-pituitary-adrenal (HPA) axis, and to the ultimate secretion of cortisol and catecholamines in humans. The end products of HPA activation (cortisol and catecholamines) are easily measurable in blood, urine and saliva.

This is not the case for other markers of HPA activation such as ACTH and CRF levels. ACTH can only be measured in blood and CRF can only be measured in cerebrospinal fluid. Given the ease with which one can assess the end products of HPA activation, several studies are now using cortisol and proxy measures of sympathetic activation (catecholamine, alpha-amylase) as validated physiological measures of stress in humans.

Cortisol and catecholamines can be measured in blood. Catecholamines cannot be easily measured in saliva, so researchers have developed proxy measures of sympathetic activation that can be assessed using non-invasive methods.

Studies now show that cortisol can also be sampled in saliva, a technique that has been preferred by researchers for its non-invasive advantage. In addition, as opposed to blood sampling, saliva collection does not require the collaboration of skilled personnel for the installation of a catheter, allowing for an uncomplicated and trouble-free sample collection.

Moreover, new biospecimens for assessment of stress hormones are now being developed in humans. One biospecimen that is very interesting is hair samples. Hairs grow 1 cm per month, and new methodology are now being developed in order to measure cortisol levels in hair, using enzyme immunoassays. The interesting aspect of this new biospecimen is that cortisol levels are accumulating in hair samples, providing a measure of the accumulation of stress hormones over time in humans. By analyzing samples of 3 cm of hair, one is potentially able to assess exposure to stress in humans within the last 3 months. In this way, hair becomes a retrospective calendar of stress hormone production.

Below, we briefly describe the various methods used to assess biomarkers of stress in humans.

i. Autonomic measures as biomarkers of stress

Blood pressure : Blood pressure is a measure of the force that blood exerts on the walls of blood vessels. When blood pressure (a sympathetic parameter) is measured, two numbers appear, e.g. 120/80 mmHg. The first number, 120, represents the systolic pressure, which occurs when the heart pushes blood out of the arteries. The second number, 80, represents the diastolic pressure, which is the pressure of the heart at rest. Blood pressure can be easily measured using a simple vital signs monitor.

Vagal tone : Vagal tone represents the parasympathetic impulse that would apply a brake to decrease heart rate during both resting and reactive conditions. This measure requires the use of more advanced electronic devices and the installation of leads.

Salivary alpha-amylase: Recently, alpha-amylase has been described as a potential indicator of noradrenergic activity (Chatterton et al. 1996; Nater et al., 2005; Rohleder et al. 2004). Alpha-amylase is an enzyme that is produced under sympathetic innervations and can be collected in saliva. There is growing body of evidence suggesting that the level of salivary alpha-amylase (sAA) increases with physiological stress, such as exercise (Li & Gleeson, 2004; Walsh et al., 1999). Recent studies also indicate that the Trier Social Stress Test (TSST), a validated psychosocial stressor (Kirschbaum et al., 1993) can also elicit an increase in sAA following stress exposure (Nater et al., 2006; Nater et al., 2005; Rohleder et al., 2004). Given earlier observations that sAA concentration increases with sympathetic stimulation and salivary flow rate increases with parasympathetic stimulation (Garrett, 1999), as well as the fact that significant correlations have been shown between sAA and catecholamine levels (Chatterton et al., 1996), it has been proposed that sAA may be a potential biomarker for gauging the level of activity of the sympathetic nervous system (Nater et al., 2006; Nater et al., 2005; Rohleder et al., 2004). Further support for this hypothesis came from van Stegeren and collaborators (van Stegeren et al., 2006) who demonstrated that participants who were given beta-blockers prior to an fMRI psychosocial stress paradigm exhibited a significantly lower level of sAA, heart rate and blood pressure throughout the entire paradigm, when compared to placebo subjects.

ii. Measures of hypothalamic-pituitary-adrenal activity

1. Diurnal cortisol secretion

Under natural unstimulated conditions, the secretion of cortisol follows a circadian rhythm characterized by a peak in the early morning hours, followed by declining cortisol concentrations throughout the day, reaching the lowest levels during the late evening. This rhythm is influenced by altered sleep patterns and exposure to daily life stressors (Smyth et al., 1998; van Eck, Berkhof, Nicolson, & Sulon, 1996). While this pattern of diurnal cortisol secretion has been widely published, it has been shown that individuals may deviate from this "typical pattern". Indeed, both inter-individual differences and intra-individual differences in the diurnal pattern of cortisol secretion have been reported.

2. Calculation of diurnal cortisol subgroups

Smyth and colleagues (1997) have developed a method to assess individual differences in the diurnal pattern of cortisol secretion. These calculations allow one to assess stability and characteristic of the diurnal cortisol slope over a 2-day period. Specifically, based on a 2-day sampling period, individuals may be categorized into one of three possible subgroups: the Typical diurnal subgroup, in which the individual displays the typical cortisol peak and decline throughout the day (as described above); the Flat diurnal subgroup, in which evening cortisol levels fail to decline to the common nadir phase and remain relatively elevated; finally, the Inconsistent subgroup, in which individuals display both the Typical and Flat pattern on alternate days (e.g. flat on day one and

typical on day two). Using this method, Smyth et al (1997) reported that 51% of the population present a "Typical" and consistent decline in cortisol levels on both days, while 17% of the population displayed a "Flat" cortisol cycle on both days, and 31% exhibited an "Inconsistent" cycle.

In order to obtain these three subgroups within a given population, 3 calculation steps are followed:

STEP 1: Determine the cortisol slope of Day 1 and Day 2: First, one must log-transform all cortisol values for both sampling days. Once values are transformed, the slope for Day 1 and Day 2 must be calculated.
STEP 2: Determine which individuals display a consistent profile (flat or typical) and which individuals display an inconsistent profile: In order to do this, one must take the difference score between the slopes of Day 1 and Day 2 (i.e. slope Day 2 – slope Day 1). From this difference score, calculate the standard deviation (SD). If the absolute difference score is greater than 1 SD, the diurnal cycle for that individual is characterized as Inconsistent. Why? Because there is a significant difference (greater than 1 SD) between their Day 1 and Day 2 slope.
STEP 3: For the remaining individuals (i.e. those who have not been categorized as Inconsistent), determine which individuals display a Typical cycle and which individuals display a Flat cycle: In order to do this, one must obtain the average of the two slopes. From the average score, those who display a slope that is more positive than the (-)SD are labelled as Flat (i.e. less of a decline in cortisol over time) and those who display a slope that is more negative than the (-)SD are labelled Typical (i.e. more of a decline in cortisol over time).

Below is an example of calculations:

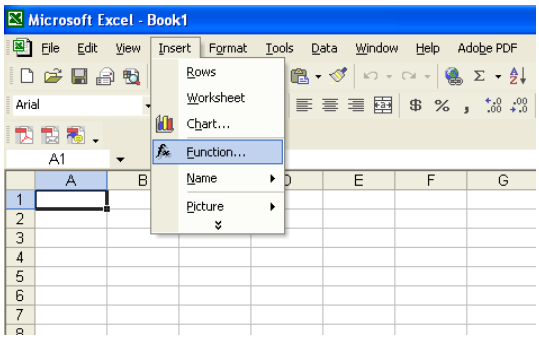
We have collected 4 saliva samples in 10 participants on Day 1 and Day 2. On both days, samples were collected at 9:00, 12:00, 15:00, 18:00. Table 1, shows the log-transformed concentrations on each day for 10 participants.

Table 1: Diurnal cortisol secretion on two days in 10 participants. Samples were collected at 9:00, 12:00, 15:00 and 18:00 on both days. Cortisol concentrations were log-transformed prior to calculations of slopes.

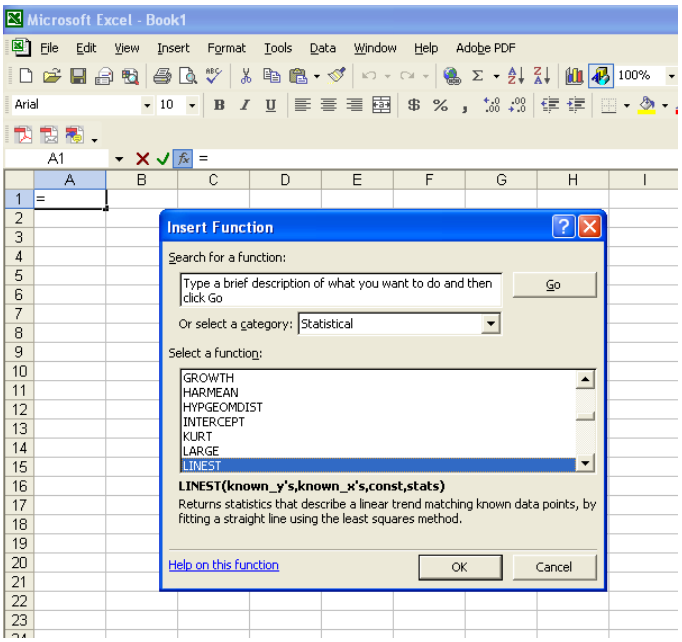
	Day One				Day Two			
code	9	12	15	18	9	12	15	18
F01	0.75	0.5	0.3	0.2	0.72	0.51	0.47	0.23
F02	0.65	0.45	0.27	0.21	0.47	0.49	0.3	0.35
F03	0.62	0.51	0.46	0.42	0.79	0.59	0.41	0.22
F04	0.74	0.8	0.57	0.4	0.65	0.45	0.27	0.21
F05	0.72	0.5	0.4	0.23	0.74	0.54	0.39	0.16
F06	0.81	0.64	0.43	0.24	0.76	0.47	0.26	0.13
F07	0.79	0.56	0.46	0.28	0.72	0.5	0.42	0.3
F08	0.69	0.76	0.6	0.59	0.64	0.75	0.59	0.56
F09	0.59	0.58	0.42	0.37	0.76	0.6	0.42	0.27
F10	0.4	0.63	0.54	0.31	0.52	0.46	0.48	0.33

Using preset formulas in Excel, it is possible to assess the slope for both days for all participants.

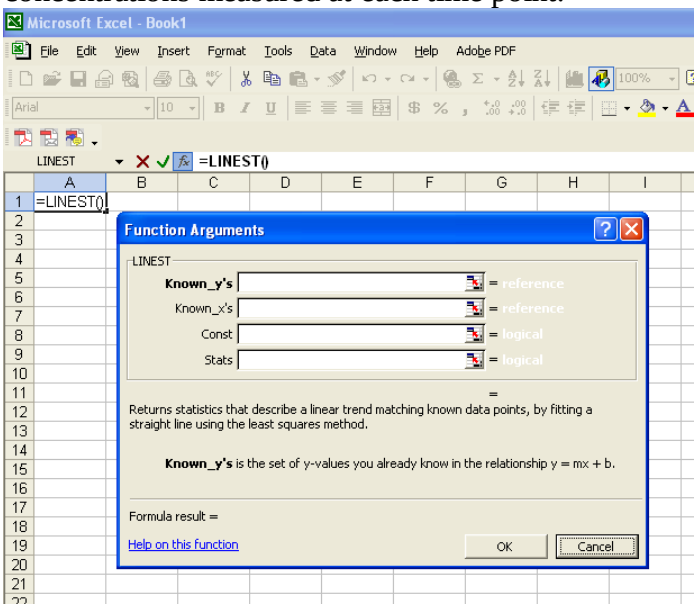
First, select “functions” under the “Insert” tab.



Then, select “LINEST” in the “Statistical” category

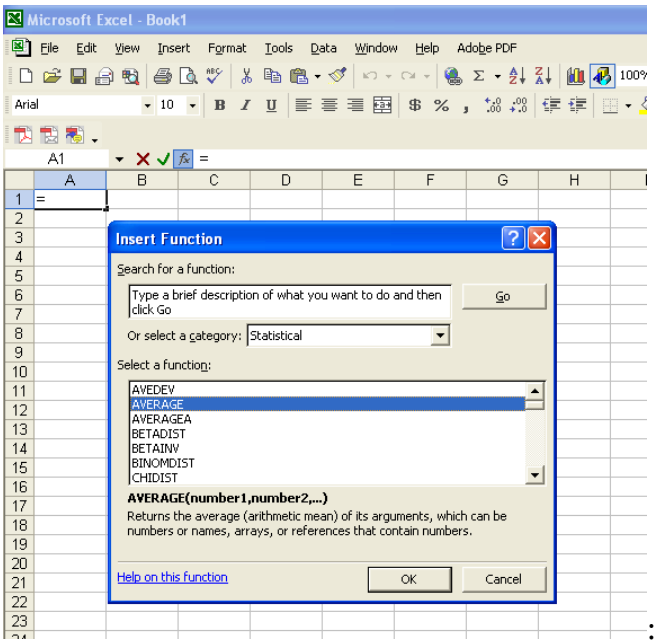


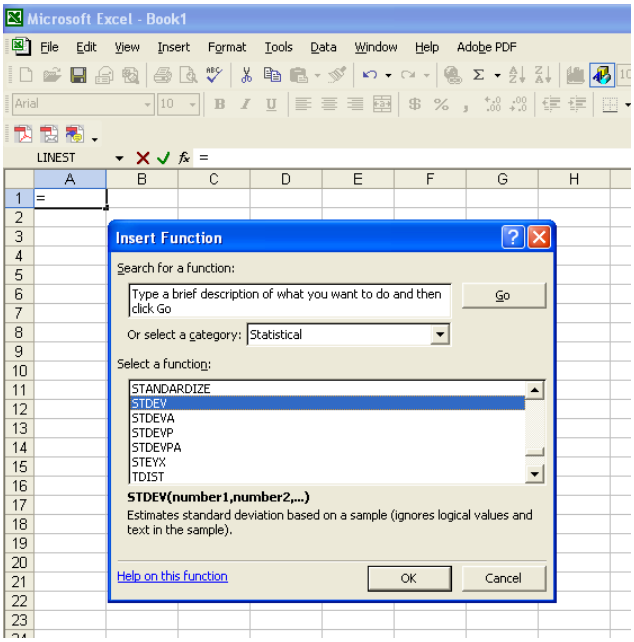
Then, under "Known Y's" label, select the Y values. Those are the log-transformed cortisol concentrations measured at each time point.



Under the "Known X's" label, select the time of each sampling (9, 12, 15 and 18). This will ensure that the intervals between sampling are taken into account in the calculation of the slope.

Clicking OK will provide you with value of the slope of the linear regression model fitting your data.





Thus, we now have the cortisol slopes for Day 1 and Day 2 (Table 2)

Table 2: Calculation of slope. Calculation of Difference between slope 1 and slope

Day 1 Slope	Day 2 Slope	Difference	Inconsistent / Consistent?	Average Slope	Typical/Flat?	Subgroup
-0.0617	-0.0503	-0.0113	Consistent	-0.056	Typical	Typical
-0.05	-0.0183	-0.0317	Inconsistent	-0.03415		Inconsistent
-0.0217	-0.063	0.0413	Inconsistent	-0.04235		Inconsistent
-0.0417	-0.05	0.0083	Consistent	-0.04585	Typical	Typical
-0.0523	-0.063	0.0107	Consistent	-0.05765	Typical	Typical
-0.064	-0.07	0.006	Consistent	-0.067	Typical	Typical
-0.0543	-0.0447	-0.0097	Consistent	-0.0495	Typical	Typical
-0.0153	-0.0133	-0.002	Consistent	-0.0143	Flat	Flat
-0.0273	-0.055	0.0277	Inconsistent	-0.04115		Inconsistent
-0.012	-0.0183	0.0063	Consistent	-0.01515	Flat	Flat
		SD= 0.020				

Once you have the slopes for Day 1 and Day 2, calculate the difference score (3rd column Table 2). Then, calculate the SD of the difference scores (=0.020). If the absolute value of the difference score is greater than the SD, then the person is labeled as Inconsistent (4th column Table 2).

Now that you know who is Inconsistent, you want to find those who are Flat or Typical. Therefore, calculate the average of the Day 1 and Day 2 slopes (5th column Table 2). Individuals who have a more negative (average) slope are labeled Typical and those who have a more positive (average) slope are labeled Flat (6th column Table 2)

Note: Slope calculation is a function of the interval between each time point. For the sake of better comparison within and between participants, it is recommended to maintain comparable intervals between each saliva collection, on both days, in all participants. For example, one cannot compare the diurnal slope of salivary cortisol secretion in Participant A, who collected saliva samples at 9:00, 10:00, 11:00 and 19:00, to that of Participant B, who collected saliva samples at 9:00, 12:00, 15:00 and 18:00. Even though both participants collect their morning and evening samples at comparable hours (9:00 for both and 18:00 vs. 19:00 in the evening), and both participants collected 4 samples during the day, the researcher clearly lacks information regarding the course of saliva cortisol between 11:00 and 19:00 in Participant A. Therefore, it is not only recommended to collect several saliva samples to provide more accurate slope calculations, but also, sample distribution throughout the day should be as evenly spread out as possible. We believe that collecting less than four samples would not yield accurate results of the diurnal cortisol pattern.

3. Awakening cortisol response

In the past few years, an increasing number of authors have shown interest in the awakening cortisol response (ACR), a distinct feature of the HPA axis that responds to the endogenous stimulation of waking up by a peak occurring at 30-45 minutes after awakening (for a review, see Clow, Thorn, Evans, & Hucklebridge, 2004). ACR, which is believed to reflect psychological and physical wellness, is known to be relatively stable across days and is blunted under situations of high emotion and stress (Pruessner et al., 1997; Pruessner, Hellhammer, & Kirschbaum, 1999; Clow et al., 2004).

4. Cortisol Reactivity

Reactivity of the HPA axis can mainly be assessed either pharmacologically, through exposure to real-life stressful events, or using laboratory-induced stressors.

a. Pharmacological challenges:

Pharmacological challenges are often used in stress studies to test the ability of the HPA axis to trigger a feed-forward response. Depending on the level of reactivity that one wishes to assess, CRF (anterior pituitary level) or ACTH (adrenal level) challenges are used. These tests require the help of careful monitoring and highly skilled personnel for the administration of the triggering agents (CRF and ACTH).

b. Induced by real-life stressors:

In the current literature, many authors have studied responses to real-life stressful events such as bereavement, academic examinations, everyday work and parachute jumping. These stressors have been reviewed by Biondi and Picardi (1999). Although these real-life events reflect naturally occurring daily life challenges, the context surrounding the experimental conditions might be difficult to control for.

c. Laboratory-induced stressors

A recent meta–analytical review was performed using 208 stress studies in children, adults and older participants from different laboratories. This review concluded that a stressor that involves social-evaluative threat (e.g. when performance can be evaluated by others) is the most powerful way to induce stress in laboratory facilities (Dickerson & Kemeny, 2002; Kirschbaum et al., 1993). The Trier Social Stress Test (TSST) is one of the best standardized tools to evoke a cortisol stress responses in a laboratory setting. Salivary cortisol levels after the TSST reliably show a 2 to 4-fold elevation in cortisol above baseline within 30 minutes. The TSST consists of delivering a public speech in front of a 'team of experts', following a period of five minutes of preparation. At the end of the speech, participants are asked to serially subtract numbers as fast and as accurately as possible. If a mistake occurs, the participant is stopped and asked to start over from the initial number. Altogether, the TSST lasts approximately 10 minutes. Saliva samples are usually collected prior to the TSST (baseline, 0 min), after the speech preparation,

immediately after the speech and arithmetic performance, and several times afterwards. Cortisol should show a peak in concentrations within 10-30 minutes after the end of the TSST.

d. Feedback regulation of the HPA axis

It is also possible to evaluate the negative feedback of the HPA axis using the Dexamethasone Suppression Test (DST). Dexamethasone is a very powerful synthetic glucocorticoid that acts centrally to inhibit secretion of CRH, ACTH and subsequently, cortisol. Therefore, within hours, cortisol secretion should be interrupted, leading to very low levels of circulating cortisol levels. Typically, dexamethasone is given orally (usually 1 mg), at bedtime (normally set at 23:00) and the HPA hormones are measured the following morning, e.g. at 8:00, 9:00, 10:00, 11:00 and 12:00. Individuals who have poor HPA regulation fail to blunt HPA hormone secretion after dexamethasone intake.

3. What types of population can we study?

You can study just about any type of population you wish to. Salivary cortisol has been measured in various age groups, from infants, to the elderly population, and has been measured in both healthy and clinical populations (e.g. (Cleare, 2003; Gunnar, Bruce, & Hickman, 2001; Gunnar & Vazquez, 2001; Kajantie & Phillips, 2006; Kirschbaum & Hellhammer, 1994; Kirschbaum, Kudielka, Gaab, Schommer, & Hellhammer, 1999; Lundberg, 2005; Lupien et al., 2005; Pruessner et al., 1997; Tu, Lupien, & Walker, 2005; Yehuda, 2002).

4. How should we collect saliva samples?

The most common technique used to collect saliva samples for cortisol assay requires the participant to pass saliva by spitting or drooling directly into a sterilized tube, for a few minutes until sufficient volume (approximately 2 ml) has been collected. To reduce bacterial growth, it is recommended to cool the sample in the refrigerator or the freezer, until the research team retrieves it. Using this device, saliva samples can be collected at home, by the participant, and then mailed back to the laboratory setting without significantly affecting the quality of its assay (Clements & Parker, 1998). It is recommended, as much as possible, to avoid using agents to stimulate saliva flow, such a Kool-Aid crystals, as these have been associated with aberrant levels following cortisol assay.

5. What can bias the quality of measure of salivary cortisol?

a. Salivary cortisol measures

A broad range of events including concurrent stress, illnesses, and minor daily life activities occurring prior to saliva collection can interfere with the concentrations of salivary cortisol. The influence of some factors might occur without our control, such as sudden illnesses and stress. Other factors, e.g. time of testing, intake of food and beverages, or minor psychological and physical stressors (e.g. driving in traffic, performing academic or work-related stressful tasks, recent physical exercise), can be moderated by carefully monitoring these events before saliva

collection.

b. Sex hormone measures

Saliva has become the medium of choice for some analyses of sex hormones as it is less challenging to collect than blood. However, certain guidelines need to be imposed to ensure not only a clean and clear sample, but one with its constituents intact, as these factors will affect the reliability of the final results.

It has been shown that sex hormones in saliva are unstable if left at room temperature and need to be handled in a more meticulous fashion than cortisol, during collection.
The most fragile hormones seem to be the estrogens (of which we generally use estradiol) and progesterone, closely followed by DHEA and testosterone.

The company Salimetrics (1), that manufactures enzyme immune assay kits, released a document illustrating the reaction of different molecules to 3 different temperatures over a period of 96 hours. Below in fig.1. are the results for the progesterone and the estradiol from that document (Salimetrics 'The spit report', Vol 5- Issue 2- march 2012-09-14).

Fig.1 : Temperature stability for 96 hours (Salimetrics)

	Progesterone Pg/ml		Control		Estradiol Pg/ml		
Sample	Room temp 21 C	Fridge 4C	Freezer 60 C	Sample	Room temp 21 C	Fridge 4C	Freezer 60 C
1	15,55	20,43	29,95	1	9,37	10,51	13,10
2	43,49	86,09	165,78	2	0,00	0,00	10,37
3	15,13	26,95	42,75	3	0,00	4,87	11,33
4	277,52	364,47	438,26	4	7,48	12,43	18,44
5	51,03	124,19	153,03	5	9,12	22,65	25,98
6	18,67	36,52	56,95	6	0,00	6,67	13,19
7	41,52	44,04	41,99	7	11,36	12,29	10,91
8	27,94	38,67	49,41	8	6,27	9,94	17,69
9	45,78	57,63	108,75	9	9,33	11,54	18,21
10	12,94	36,1	84,23	10	3,63	10,82	17,71
Mean	54,957	83,509	117,11	Mean	5,66	10,17	15,69

Figure 1 shows that there is marked degradation over the 96 hours at room temperature and to a lesser degree at 4C (refrigerated), though still consequential. This might seem to be irrelevant when you consider that it is unlikely that a sample would be left out for four days.

Our goal was to test if the deterioration of samples would show in a shorter timeframe (ie. 24 hours). Using a 24-hour time frame would demonstrate a more realistic scenario whereby samples became defrosted on the way to the research centre, or were kept in the participant's bag at work before being taken home in the evening and placed in a freezer. To demonstrate this, an analysis using 10 samples of progesterone and estradiol was carried out

Analyses:
The 10 samples of progesterone and estradiol were defrosted, brought to room temperature, and then assayed using a Salimetrics EIA kit for the relevant hormone (control). The same 10 samples were then left on the lab bench for a period of 24 hours before performing a second assay (See Fig 2). Figure 2 shows that even in a period of 24 hours, degradation of the hormones in the saliva sample has taken place.

Fig.2: CRFS- 24 hrs at room temperature.

Progesterone pg/ml		
Sample	Control	24hrs later
1	29,763	21,308
2	52,616*	27,341
3	51,652	15,838
4	66,243	21,145
5	14,072	13,790
6	29,371	14,909
7	135,291	52,377
8	4,101	0,000
9	8,092	0,096*
10	10,945	1,286
Mean	38,84	18,67

Estradiol pg/ml		
Sample	Control	24hrs later
1	3,955	2,246
2	4,712	2,969*
3	4,167	2,454
4	4,731	2,501
5	3,418	2,768
6	5,572*	2,403
7	6,058	3,754
8	0,963	0,401
9	0,843	0,512
10	1,041	0,367
Mean	3,321	1,934

Single sample *

Due to this instability it is important to ensure that sex hormone samples are taken at a time point where the samples can be frozen immediately after collection. (Salimetrics recommend no longer than 30 minutess at room temperature and no more than 3 hours refrigerated before freezing.)(1) As studies often require participants to provide diurnal cortisol samples at various time points during the day, it is suggested that the sex hormone samples are among those taken first thing in the morning. This will ensure that the participant can place the saliva immediately into the freezer before they leave home for work or start their normal daily routine.

Samples taken at work or while out of the house are less likely to reach a freezer in a timely manner, therefore, it is not recommended that sex hormone samples are taken at the workplace. As well, sex hormone samples should be taken in separate tubes from the cortisol samples to ensure that there is enough samples to analyse the requested hormones. This way the sex hormone sample will remain frozen until it is possible to analyse it.

If it is unavoidable to use one saliva sample for multiple biomarkers then have the lab run them in order of frailty, for example; Estradiol-Progesterone-DHEA-CRP-Testosterone-Cortisol-Alpha-Amylase.

To ensure that these samples are not completely defrosted on the journey to the research center or hospital, a freezer block and insulated bag should be provided to the participant.
The bag and freezer block should be accompanied by specific directions for transporting the samples to the research facility, in as short a time as possible.

6. Time of testing

As we have mentioned before, basal cortisol secretion fluctuates throughout the day. Typically, morning levels are higher than late afternoon levels. Therefore, one should use caution to avoid large individual differences in baseline concentrations by combining cortisol measurements at comparable times during the day. Recently, a few studies have reported that cortisol reactivity to stressful situations can differ according to the time of the day (Kudielka, Schommer, Hellhammer, & Kirschbaum, 2004; Maheu, Collicutt, Kornik, Moszkowski, & Lupien, 2005).

7. Factors that can modify stress hormone measures

Cortisol is reactive to stimulants of the nervous system such as caffeine and nicotine. These agents have been reported to enhance basal and reactive secretion of salivary cortisol (Lane, Pieper, Phillips-Bute, Bryant, & Kuhn, 2002; Lovallo, Farag, Vincent, Thomas, & Wilson, 2006; Lovallo et al., 2005; Rohleder & Kirschbaum, 2006; Steptoe & Ussher, 2006).

Large intake of food in the hour prior to saliva collection can also lead to a sharp artefactual increase in cortisol concentrations. Glucose intake affects the cortisol response to stress in a more significant way compared to protein or fat intake (Gonzalez-Bono, Rohleder, Hellhammer, Salvador, & Kirschbaum, 2002).

Finally, acidic or sweet food and beverage intake shortly prior to saliva collection can alter the pH of saliva during collection. Given that salivary cortisol assay is performed in a constant pH environment, for optimal bindings of reagents, it is recommended to avoid intake of such food and beverages and to have the participant rinse his/her mouth prior to saliva collection. It is also important to ensure that there is no remaining water in the participants mouth following rising, as this may dilute the saliva.

8. How to assess compliance of participants?

In study designs when participants are instructed to collect saliva samples at home, it is important to remind the participants to respect the prescribed timing and conditions of saliva collection. As shown previously, the timing of sampling and the events leading to sampling are crucial in the quality of data collection at the participant's home. The importance of verifying compliance has been reviewed recently (Kudielka, Broderick, & Kirschbaum, 2003). Although monitoring techniques, such as palm-pilots that beep the participant to remind to take a saliva sample, have been successfully used in some studies, this method does not ensure that at the time of the beep, the participant actually takes the saliva sample (Kudielka et al., 2003). To this day, the only validated method available to analyze and monitor participant's compliance with saliva sampling is the Medication Event Monitoring System (MEMS®). This is a tool that measures and analyzes participant's compliance to the prescribed time of saliva sampling (for analysis of stress hormone levels in the natural environment). The MEMS® is the only device, to this day, that can ensure a reliable analysis of participant's compliance with saliva sampling in the home environment. The MEMS® is an electronic monitoring system designed to compile the participants' dosing history. The system is comprised of two parts: a standard plastic vial with

threaded opening and a closure for the vial that contains a micro-electronic circuit that registers times when the closure is opened and when it is closed. Once the sampling is done, the events stored in the MEMS® can be transferred at any time through the MEMS® communicator to a Windows-based computer. The software then analyzes and displays or prints the computed parameters of the participant's compliance. The results obtained with the MEMS® are now widely regarded as the gold standard measure of patient compliance.

MEMS® supplies can be purchased from the AARDEX GROUP (www.aardexgroup.com). The following products are necessary when using the MEMS® caps to measure patient compliance:

- MEMS® Power View software to analyze the data (US $473 per license per workstation)
- MEMS® Reader/Communicator (US $122)
- MEMS® Caps (number of caps to be determined according to the research study) ($115 per cap). The battery for each cap lasts for 36 months, at which time the entire cap has to be discarded.
- Vial for each MEMS® Cap – (US $ 4.70) Item: 250cc HDPE screw-top white vial 45-400.

(Prices quoted above are from 2012. Please contact AARDEX GROUP for prices as they are subject to change)

9. Various statistical methods to analyze cortisol

Many assay techniques are available to quantify free cortisol from saliva samples. The most common assays are radioimmunoassay (RIA), time-resolved immunoassay with fluorometric detection (DELFIA) and enzyme immunoassay (EIA). These techniques rely on the principle of competitive binding between free cortisol and reagents. Correlations betweens concentrations yielded from these techniques depend on the type of population tested (clinical vs. healthy) and on the range in concentrations assayed (Addison vs. Cushing) (Raff, Homar, & Burns, 2002; Raff, Homar, & Skoner, 2003). Therefore, one should use some caution and consider the type of assay used when comparing values obtained from one study to another study. The choice of one technique over another depends not only on the prices of the chemical kits, and availability at laboratories, but also on the percentage of inter and intra-assay coefficient of variations. Briefly, inter-assay variations refer to the variability related to the assay BETWEEN runs, while intra-assay variations refer to the variability WITHIN runs. Further information regarding assay techniques can be obtained from the Stress Hormones Analysis Laboratory.

a. How to organize the data?

To organize your data prior to statistical analysis, it is preferable to assign each participant with a number or code, and to enter these data in one line per participant. Each column represents a variable measure (e.g. age, sex, education, time of testing). For the sake of confidentiality, we advice researchers to keep personal information separately from the corresponding code. Below is an example of a spreadsheet organized in Excel.

Table 2: Example of the organization of a spreadsheet

CODE	Age	Sex	Education	Time of testing
F01	25	1	15	14:00
F02	24	1	14	13:45
F03	27	2	17	14:04
F04	28	1	13	14:17
F05	21	2	16	13:59
F06	23	2	12	14:01
F07	26	2	14	13:54
F08	25	1	15	14:05
F09	24	2	16	14:08
F10	23	1	14	13:52

Once these spreadsheets are prepared, they can be imported in popular statistical packages, e.g. SPSS, which can carry most of the analyses required to test your hypotheses.

b. How do we analyze our findings?

The type of statistical analyses used to test the research questions depend on the hypotheses that are addressed in the study. Several statistic textbooks are available as guides for performing analyses on the collected data.

Cortisol reactivity can be computed many different ways, e.g. as increments, percentage, ratio, area under the curve (AUC). Below is an example of a group of 10 participants, who were exposed to a 10-minute stressor (between 0 and 10 min). Saliva samples for cortisol assay were collected at baseline (0 min), right after the stressor (10 min), and at +20, +35 and +50 min after the end of the stressor.

Table 3: Increment, percentage, ratio and AUC cortisol response to a 10-minute stressor in 10 participants

	Minutes					Increment	%	Ratio	AUC
CODE	0	10	20	35	50				
F01	0.30	0.45	0.60	0.50	0.40	0.30	100.0	2.0	24.00
F02	0.25	0.56	0.70	0.57	0.38	0.45	180.0	2.8	27.00
F03	0.22	0.47	0.65	0.43	0.34	0.43	195.5	3.0	22.93
F04	0.34	0.76	0.80	0.57	0.40	0.46	135.3	2.4	30.85
F05	0.26	0.34	0.50	0.47	0.23	0.24	92.3	1.9	19.73
F06	0.29	0.56	0.64	0.43	0.24	0.35	120.7	2.2	23.30
F07	0.31	0.44	0.56	0.46	0.28	0.25	80.6	1.8	21.95
F08	0.27	0.22	0.76	0.60	0.37	0.49	181.5	2.8	24.83
F09	0.24	0.29	0.58	0.42	0.30	0.34	141.7	2.4	19.90

F10	0.28	0.32	0.63	0.54	0.31	0.35	125.0	2.3	22.90

Below are examples of calculations of increment, percentage, ratio and area under the curve using the data presented above in Table 1.

i. Increment:

In participant F06, the increment or change in cortisol from baseline (0 min) to 10 minutes after the end of the stressor (20 min) would be:

Increment = concentration at 20min – concentration at 0min
= 0.64 μg/dL – 0.29 μg/dL = 0.35 μg/dL

The participant F06 showed an increase of 0.35 μg/dL in cortisol, 10 minutes after exposure to the stressor.

ii. Percentage

For the same participant (F06), the percentage of increase from baseline (0 min) to 10 min after the stressor (20 min) would be:

Percentage = 100 x (concentration at 20min – concentration at 0min)/concentration at 0min
= 100 x (0.64 μg/dL – 0.29 μg/dL)/ 0.29 μg/dL = 120.7%

Participant F06 showed a 120.7% increase from baseline to 10 minutes after the end of the stressor.

Note: 0% increase would mean that there was no change in cortisol between these two time points.

8.3. Ratio:

The increment and the percentage of increase should be distinguished from the ratio, which is calculated using the following formula:

Ratio = concentration 0 min / concentration 20 min
For instance, for the same participant F06,

Ratio = 0.64 μg/dL / 0.29 μg/dL
= 2.2-fold increase

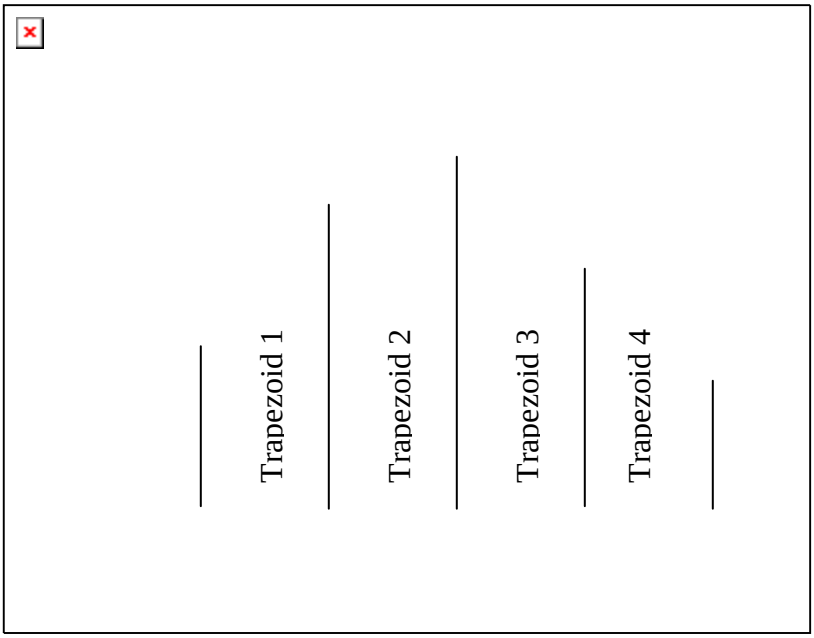
Thus, while participant F06 showed an increase of 0.35 μg/dL or an increase of 120.7% in free salivary cortisol from baseline to 10 min after the end of the stressor, he showed a 2.2-fold

increase in cortisol during that period.

Note: a 1-fold increase would mean that there was no change in cortisol concentrations between these two time points.

iii. Area under the curve:

We can also obtain a global measure of the cortisol response by calculating the area under the curve (AUC) of the response. This technique has been well described by Pruessner et al. (2003) (Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003). Briefly, the calculation of AUC is based on breaking down the AUC in many trapezoids, then, calculating and adding the area of each trapezoid to yield one overall value.



The area of a trapezoid is calculated as following:
Height x (average of length of parallel sides)
= interval between two samples x (cortisol concentration at time 1+ concentration at time 2)/2

AUC = area trapezoid 1 + area trapezoid 2 + area trapezoid 3 + area trapezoid 4
= 10 min x (0.29 µg/dL + 0.56 µg/dL)/2 + 10 min x (0.56 µg/dL + 0.64 µg/dL)/2 + 15 min x (0.64 µg/dL + 0.43 µg/dL)/2 + 15min x (0.43 µg/dL + 0.24 µg/dL)/2
= 23.30 µg/dL over the course of 50 minutes

Over the course of the 50-minute experimentation, including baseline, cortisol reactivity and cortisol recovery, Participant F06 secreted 23.30 µg/dL of free salivary cortisol.

10. Frequently Asked Questions (FAQ) regarding saliva collection

Should I collect my sample if I am sick?

No, if you are sick or under unusual stress during this week, please contact the research team so that we can set a different time for the saliva collection.

If I forgot and ate breakfast and/or brushed my teeth before collecting my saliva, what do I do? Can I start over again?

Yes, call the research team so we can arrange for you to start on another day.

I'm having a difficult time collecting enough saliva.

If you are having difficulty collecting saliva we have a few recommendations. You can try:

- press the tip of your tongue against your teeth very gently.
- think about your favorite dessert.

I forgot to rinse my mouth before I collected my saliva, what should I do?

A film develops in your mouth at night; this is why we ask that you rinse out your mouth to remove that film before collection.) But if you've already completed the process of spitting, that's fine.

Can I eat or drink between the awakening and 30 minutes after awakening samples?

No, you should only drink water between the awakening and 30-minutes-after -wakening samples (no coffee or other caffeinated drinks).

Can I take a shower between the awakening and 30 minutes after awakening samples?

Yes

What if I miss my collection time? How much time do I have to collect the sample?

If you accidentally miss your collection time, please complete the sample as soon as possible (within an hour of the specified time).

What if I go to bed before 9 P.M.?

If your bedtime is before the last saliva collection, please collect the last sample collection before you go to bed.

If I miss the awakening sample, can I complete the rest of the day and collect my awakening sample the next day?

It is better to start over and complete all sampling on the same day.

11. References

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12. Appendices

Below are examples of a typical screening questionnaire and a log book that one can use to control for some of the methodological issues.

Demographic Information

Name	
Add	
Phone	
Date of birth	
Height/Weight	
Yrs schooling	
Language	

Medical Screening

Smokers or not? (Exclude if more than 10-15 per day cortisol/nicotine interaction) _____

General health?

Vision ☐
Audition ☐

Do you take any medication? _____

Medical history?

Cardio-vascular diseases ☐
Myocardial infarction ☐
Heart block ☐
Slow cardiac conduction ☐
Heart failure ☐
Hypotension ☐
Hypertension ☐
Other ☐ _____

Neurological diseases ☐
AVC ☐
Parkinson ☐
MS ☐
Head trauma ☐
Other ☐ _____

Diabetes ☐
Cholesterol ☐

Glaucoma ☐
Kidney ☐
Asthma, respiratory disease ☐
Infectious illness ☐
Unstable thyroid dysfunction ☐
Adrenal dysfunction ☐
Lupus ☐
Peptic or gastric ulcer in the past year ☐
Psychiatric disorders in the past or currently ☐
- Depression ☐
- Bipolar disorder ☐
- Anxious disorder ☐
- Schizophrenia ☐
- Alcohol/Drug ☐
- Dementia ☐
- Other ☐_____

General Rx?

Neuroleptics ☐
Anti-depressant ☐
Anxiolytics ☐
GC's ☐
Mineralocorticoids ☐
Anti-convulsivants ☐
Anti-Parkinsonnien ☐
Sedatives ☐
Cholesterol ☐
CNS ☐
Other ☐_____

General anesthesia in the last year? _____

Have there been any major life events in the past year? Ex. a change of residence, death of a close family member within the past 6 months…

_____
_____

Saliva Collection

Directions:

At the times indicated (your watch or pager will help remind you):

1. Rinse your mouth with water and swallow several times to remove excess water and food particles from your mouth. *Do not brush your teeth, this might induce micro-injuries in your mouth and affect the sampling.*
2. Collect saliva sample.
3. Put the sample at cool temperature in the refrigerator or in the freezer.
4. Answer the questions in the journal for the time of each sample.

* *Do not eat between sample 1 & 2*