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Title: HPA Axis and Cognitive Function: Circadian Cortisol Pattern and Performance on the Countermanding Task

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Abstract

The Hypothalamic-Pituitary-Adrenal (HPA) axis is a basic neuroendocrine system. It includes all the effects of positive and negative feedback that take place between the hypothalamus, the pituitary gland and the adrenal glands having as a result the circadian cortisol pattern. Findings indicate that the diurnal cortisol affects cognitive function. In this study, we attempted to explore the relationship between the diurnal variation in cortisol and cognitive function by employing one experimental task that assessed eye movements and inhibition. We compared the performance of 20 healthy young adults in two task conditions of the Countermanding task (Countermanding 40 and Countermanding 100) and three conditions of Time (Morning, Afternoon, Evening). Participants presented an overall better performance on the Countermanding 40 condition compared to the Countermanding 100. Additionally, they presented an improvement in their performance in the Evening condition compared to the Morning. The diurnal variation of the performance in the Countermanding task was not related with the diurnal variation of cortisol. Results suggest that cortisol levels of healthy young adults do not affect cognitive function, especially inhibition.

Key-words: HPA axis, diurnal cortisol, Countermanding task, Inhibition, Diurnal variation, Cognitive function,

Contents

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The Hypothalamic-Pituitary-Adrenal (HPA) axis is a basic neuroendocrine system that includes all the effects of positive and negative feedback that take place between the hypothalamus, the pituitary gland and the adrenal glands. Its main role is to regulate the biological processes that take place after exposure to endogenous or exogenous stress factors. The homeostatic regulation of these processes in its turn augments the possibilities of survival of a human being or other animal. Additionally, the proper function of the HPA axis is necessary for the regulation of a series of physiological processes, such as the proper function of the immune system (Cocco et al., 2017), digestion (Farzi et al., 2018), sexual function and fertility (Joseph & Whirledge, 2017), emotion and mood (Chong et al., 2017; Doolin et al., 2017; Klimes-Dougan et al. 2018) as well as energy storage and release (Harris, 2015).

The basic regulation of the HPA axis can be briefly described as follows (Figure 1). At first, microcellular neurons (parvocellular neuroendocrine cells) located at the paraventricular nucleus of the hypothalamus (PVN) synthesize and secrete two peptide hormones, the corticotropin-releasing hormone (CRF) and under certain circumstances the arginin-vassopresin (AVP). Then, CRF is carried through the portal system to the pituitary gland where it gets attached to the CRF receptors type I and II, thus triggering the synthesis and release of the adrenocorticotropic hormone (ACTH), from the corticotropic cells in the anterior pituitary. Through the bloodstream, ACTH reaches to its main target which is the Zona fasciculata of the adrenal cortex. There, ACTH's attachment stimulates the synthesis and secretion of glucocorticoids, a group of corticosteroids, of which the most important endogenous hormone is cortisol. The system's homeostasis is maintained via negative feedback, as the blood's glucocorticoids suppress both the synthesis of ACTH, in the anterior pituitary lobe, and CRF in the hypothalamus (Smith & Vale, 2006).

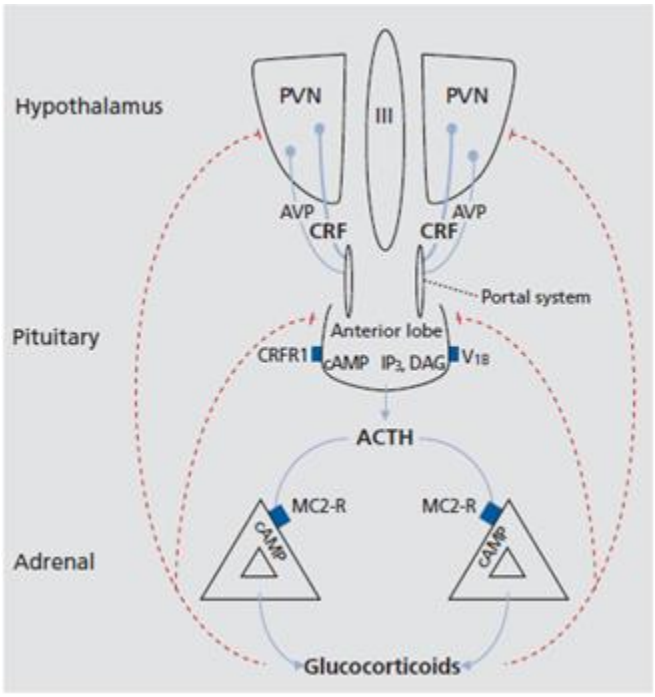

Figure 1: Schematic representation of the hypothalamic-pituitary-adrenal (HPA) axis (Smith & Vale, 2006).

Cortisol secretion follows a very stable circadian rhythm, thus having an endogenous periodic change during one day (Debono, et al., 2009). These rhythms arise from the operation of what is called circadian oscillators. Circadian oscillators organize a series of processes mainly through the autonomic and endocrine systems from which circadian rhythm arises. The central oscillator of the human body rests on the suprachiasmatic nucleus of the hypothalamus (SCN). In the case of cortisol, CRF secretion is under strong scrutiny by the SCN, periodically affecting the secretion through the pathway mentioned earlier, without the free cortisol levels exerting any feedback on the SCN. It should also be noted that there is a second oscillator in the adrenal glands that regulates their sensitivity to ACTH through the visceral nerves. However, this regulation is weak compared to that of SCN and also has a time delay of a few hours (Chan & Debono, 2010).

In addition, ultradian secretion, which is the every 60 to 90 minutes secretion of cortisol, is affected by the interaction of ACTH and cortisol (Russell, Kalafatakis, & Lightman, 2015). The result of this process is a specific pattern of cortisol secretion with levels peaking half an hour after waking up, gradually falling to a minimum before lying down and starting to rise again two to three hours before waking up until they reach the morning peak (Debono et al., 2009). This variation can be shown schematically in the form of a curve, known as the cortisol curve or circadian cortisol

pattern. A healthy HPA axis function is reflected by robust morning cortisol rise followed by an afternoon and evening decline, also known as the Cortisol Awakening Response (CAR) and the Diurnal Cortisol Slope (DCS). Consequently, flattened profiles are considered unhealthy (Adam & Kumari, 2009). The CAR first described by Pruessner et al. (1997), is the dramatic cortisol level increase that happens 30 minutes post-awakening. It is considered as a distinctive part of the circadian cortisol cycle, in which salivary free cortisol concentrations increase between 50 and 160% in healthy adults.

Free cortisol in the central nervous system (CNS) binds on two different types of receptors: Mineralocorticoid (MR) receptors and Glucocorticoid (GR) receptors. MR receptors are five to ten times more closely related to cortisol than GR receptors, so they are activated even at low cortisol levels, participating in the maintenance of cortisol homeostasis (Mifsud & Reul, 2018). In addition, MR receptors have a limited distribution in the CNS, located mainly in peripheral structures, such as the hippocampus and the medial amygdala. GR receptors, on the other hand, are activated when cortisol levels rise, as in stressful situations or during awakening, having as an ultimate goal the propitiation of the body's responsiveness to such stimuli (Kloet, Meijer, Nicola, Rijk, & Joëls, 2018). GR receptors are also widely distributed in the brain in structures such as the hypothalamus, spinal cord, hippocampus and especially in the prefrontal cortex (Koning, Buurstede, Lisa T C M Van Weert, & Meijer, 2019).

From the onset of the discovery of the cortisol regulation mechanism, researchers have shown interest in the possible ways that cortisol levels can affect cognitive function. Most studies in this field focus on the changes that occur on cognitive function after acute alterations in cortisol levels, such as during the administration of exogenous glucocorticoids that imitate cortisol action. One of the most consistent findings in this field is that high and increasing cortisol levels are associated with deteriorating mental function especially in processes that require the proper functioning of the hippocampus such as episodic memory (Het et al., 2005; Lupien et al., 2005). Studies with exogenous corticosteroid administration have disadvantages, the main of them being that they study an experimentally manipulated variable that cannot recur in everyday life, as cortisol levels are never that high as part of the natural cortisol cycle in healthy individuals.

Acute changes in cortisol levels also appear to have a direct effect on executive functions that depend on the functionality of the prefrontal cortex. In the

meta-analysis of Shields et al. (2015) it was found that increased cortisol levels through the induction of acute stress lead to the deterioration of working memory. Clear reinforcement was also found in inhibition, i.e. the ability of the individual to discard information that is useless and irrelevant for the task they perform and to suppress a possible dominant form of action (proponent response), scilicet the action most likely to occur under current conditions (Shields, Sazma, & Yonelinas, 2016). At the same time, the administration of exogenous glucocorticoids compared to the increase of cortisol levels due to the inducement of acute stress to the participants had different effects in the participants' cognitive performance. Specifically, the direct effects of the exogenous administration of glucocorticoids were similar to the effects of acute stress, while their delayed effects (those that are presented a few hours after administration) appear to have the opposite effect (enhancing working memory function and deteriorating inhibition capacity) (Shields, Sazma, & Yonelinas, 2016).

Fewer studies examine the effects of endogenous cortisol variation on cognitive functioning. The majority of these studies tend to focus on episodic memory and hippocampal function and they indicate an association between high morning cortisol levels and episodic memory impairment, especially in the elderly (Lupien et al., 1994; Kuningas et al., 2007). Regarding executive functions, in the study of Evans et al. (2012), 50 older participants performed the TMT test (Trail Making Test), a widely used tool for the assessment of executive functions. In this test, the subject is instructed to connect a set of 25 numbered dots in sequential order. Salivary cortisol samples were taken eight times throughout the day and the test was conducted in the noon. Subjects with more rapid and higher cortisol peak in the morning performed better in the task. Similar results were presented by other studies, where less steep drop in cortisol levels from the peak was associated with reduced performance in TMTB (Beluche et al., 2010) and that healthier cortisol profiles were associated with better executive function (Stawski, et al., 2011). Lee et al. (2007) found that in healthy elderly elevated cortisol levels measured by taking saliva samples before, during and after cognitive assessment were related to poorer cognitive performance in 7 cognitive domains, including executive function. In this study executive function was assessed with the Purdue Pegboard Test, the Trail Making Test (TMT) -part B and the Stroop test. Li et al. (2006) suggested that higher mean cortisol in non-demented elderly is associated with poorer performance on the TMT-A & B and the Stroop Test. In this study saliva samples were collected three times a day, at 8 a.m., 3

p.m., and 11 p.m., but cortisol measurement and neuropsychological assessment were executed on different days. Most of the studies (Beluche et al., 2010; Evans et al., 2012; Kumingas et al., 2007 Lee et al., 2007; Li et al., 2006; Stawski, et al., 2011) examined healthy elderly, a population in which circadian cortisol rhythm and brain-neuroendocrine function relationship are notably different than in healthy young adults (Clow et al., 2004; Lupien, McEwen, Gunnar & Heim, 2009), thus making such results difficult to generalize in younger populations.

Additionally a lot of interest presents the study by McCormick et al. (2007) performed on a sample of 120 young people who were assessed with the Wisconsin Card Sorting Test. This test is used as a measure of executive functioning and requires attention, visual processing, and working memory. Although cortisol levels were measured over time, the time period was short: 4 samples were taken over a period of 35 minutes, with the test administered immediately after the first sampling. The results showed that higher levels of cortisol before the test in females were associated with an increase in errors, while in males there was a reverse trend. However, it should be noted that the average cortisol, per group, before the test was higher in women than in men. On the other hand, there are studies that have not found any relationship between the daily fluctuation of cortisol and cognitive function (Singh-Manoux, et al., 2014; Korten, Penninx, Rhebergen, Deeg, & Comijs, 2018) so there is no absolute agreement on the effect of endogenous cortisol on cognitive function.

Amongst the tools that are available today for the study of executive functions are the oculomotor tasks. These tasks require the execution of a saccade movement based on the instructions given for each test. A saccade is a fast, ballistic, simultaneous movement of both eyes that brings an object onto the fovea where visual acuity is at the highest. Saccades are divided into two major categories: The first category consists of involuntary saccades, that is, movements that occur automatically when a visual stimulus is presented to the peripheral vision. The second category concerns voluntary saccades which are voluntary programmed movements and come from internal processes, without necessarily having any environmental stimulus as a goal (Munoz, 2002). Examples of this category are scanning saccades used to detect something in space, reading movements and movements resulting from instructions.

A widely used test for the assessment of cognitive inhibition is the Countermanding saccade task (CMN). In this test, a central target appears and after a short period of time a peripheral target appears, with a simultaneous disappearance of

the central target. The subject is instructed to make a saccadic move towards the target as quickly as possible only in the absence of a stop signal. The stop signal in this case is the reappearance of the central target after a certain period of time. There are two categories of trials, stop trial and no-stop trial. The test yields two types of saccadic movements: the correct saccades (in the absence of inhibition) and the incorrect saccades (inhibition failure) with each category having the corresponding reaction times (Cutsuridis, 2017). The absence of movement in a stop signal task is considered as correct answer (successful inhibition). The ability of the subject to suppress a response depends on the time between the appearance of the peripheral target and the appearance of the inhibition signal (stop signal delay-SSD) (Pouget, et al., 2011). The longest the period between the two signals is, the more difficult the inhibition becomes (Hanes and Carpenter, 1999). A simple interpretation of this process is through its notion as a speed race between a go process and a stop process to a finish line. The go process designs and generates the saccadic movement while the stop process prevents the start of this movement; whichever of the two "terminates" first determines whether the saccade movement will be performed (Salinas & Stanford, 2013; Verbruggen, et al., 2019).

To produce a saccadic movement, different areas of the brain are involved, with the frontal eye field (FEF) being prominent for preparing and producing all the voluntary saccadic movements, the supplementary eye field (SEF) (Pierrot-Deseilligny, Milea, & Müri, 2004) for the production of many successive saccadic movements and the superior colliculus (SC) in the brainstem for performing the saccadic movements (Hall & Colby, 2016). As for the process of inhibition of the saccadic movements, the main areas that appear to be involved are the dorsolateral prefrontal cortex (DLPFC), which plays a critical role in the decision-making process that characterizes the behavior of the eye movements (Curtis, Cole, Rao, & Desposito, 2004), the medial prefrontal cortex (Scangos & Stuphorn, 2010) with mainly the cingulate cortex (CG) for estimating errors (Emeric, Leslie, Pouget, & Schall, 2010), the supplementary motor cortex and especially the FEF and SEF regions (Stuphorn & Schall, 2006) and finally the right inferior frontal gyrus (Aron, 2011). All these areas are characterized by the presence of a large number of glucocorticoid receptors, making the countermanding test suitable for the study of the effect of cortisol on executive functions.

Research objectives and hypotheses

The relationship between executive function and cortisol's fluctuation through the day hasn't been studied sufficiently. In the occasion of oculomotor model there hasn't been any study that considers this relationship under any circumstance.

The main purpose of the study was to investigate the effect of endogenous fluctuation within the day of cortisol in the Countermanding task. Our hypothesis was that the normal variation of endogenous cortisol during the day will affect cognitive function and especially the executive function as it was measured with the Countermanding task, an oculomotor task with increased mental load.

Additionally, we expected that there would be a diurnal variation of performance in the Countermanding task. We hypothesized that this variation in the oculomotor tasks performance would correlate with the diurnal variation of cortisol suggesting, a role of normal cortisol variation in cognitive function.

Method

Participants

The participants for this study were 20 young individuals, 11 females and 9 males, age 18 to 33 (Table 1). Exclusion criteria from the study were the following: neurological problems, cortisol related diseases or HPA axis related diseases, psychotropic abuse, benzodiazepine use and antihistamine use.

Table 1

Demographic of participants (N=20)

	M	*SD*
Age(years)	24,25	4,03
Education(years)	16,3	2,49

Materials

Measurements of salivary cortisol. The cortisol sampling was performed with Sarstedt Company's salivettes Salivette. They consist of a typical centrifuge tube that contains absorbent cotton material. The participants chewing this cotton material stimulated the saliva production and in this manner the sampling was completed in a few minutes and with a completely non-invasive procedure. Subsequently, the samples were centrifuged for the removal of solid materials and they were deep frozen until the analysis. The analysis was put through with the immunological

method of electrochemiluminescence into Cobas e 411 analyser (Roche Diagnostics, Manheim, Germany) at the First Pediatric clinic of children's hospital "Agia Sofia" under Assistant Professor Panagiota Parvanidou's supervision. Before each sampling all the necessary restrictions were respected, such as no food consumption 30 minutes before the sampling, no teeth brushing 30 minutes before the sampling, no use of cosmetic's products for the lips. Mild mouth wash for the removal of any food residues was allowed and had to be finished 10 minutes before sampling.

Oculomotor tasks. All oculomotor tasks were performed with the Saccadometer sp. Z o.o of Ober Consulting Sp zo.o (Poznar,Poland). This device was chosen because it's portable and can be easily used outside the laboratory environment. The device detects ocular movements at 1 kHz with infrared reflection technology (Direct Infra-Red Oculography). The infrared sensors and transmitters are located on a frame that resembles eyeglasses and the measurements are completely non-invasive and comfortable for the subject. Targets of the task are projected by the device itself, to any surface in front of the subject by means of forced light-induced light emission (laser). For pictures of the device see Appendix. Each task and condition was performed in groups of 100 trial blocks. There were a few breaks between each task. The order in which the tests were conducted and the different conditions were in a pseudo-random basis formed by protocol. The inter-trial interval was 1000-2000 ms and random at each task. The following characteristics extracted for all saccadic eye movements: Duration, degree in degrees, maximum and average speed, maximum and average acceleration, maximum and mean deceleration, as well as reaction time. With regard to the countermanding task there were two conditions, depending on the time elapsed between the appearance of the central target and its reappearance as inhibition stimulus (inhibition time-tCM or SSD). The two times were set to 40 and 100 milliseconds (CMN 40 and CMN 100) with the order of conditions changing in each measurement. The most likely occurrence of the inhibition stimulus was set at 50% of the tests and remained the same for both conditions. The whole procedure lasted approximately 40 minutes. The subjects were also given the instruction to try to blink only after completing the eye movement.

Procedure

A total of two different measurements were performed with the first measurement being the cortisol sampling to create the daily profile of cortisol's circadian pattern. The second measurement had to do with the participant's

perfomance in the oculomotor tasks, with the performance in the countermanding task being related to the present research work. All procedures took place in a quiet and familiar environment for the participant.

Cortisol sampling took place over the course of one day for each participant during which oculomotor tasks were performed. A non-working day was selected, ideally with a natural wake and a total of five cortisol samples were collected (at the morning awakening, within 30 minutes of early morning awakening, at 4, 8 and 12 hours after awakening, respectively). This sampling frequency and timing allowed for the best possible representation of the cortisol curve within the day according to the known variations in circadian rhythms.

Oculomotor task measurements were made in areas with common lighting conditions, with the targets projected at a distance of 1,5 - 3 meters and at eye level. A total of three measurements were made per person per day and were performed immediately after cortisol sampling. Specifically, the morning measurements were accomplished after the second cortisol sampling, the afternoon measurements after the third and the evening measurements after the fifth cortisol sampling in order to have a range of variation in cortisol levels. Furthermore, in addition to the measurements, demographic data were collected for possible use in statistical analysis.

Ethical considerations

This study is in accordance with the Helsinki Declaration and has been approved by the Research Ethics Committee of the Eginition General University Hospital. The participants read informative forms regarding the purpose and duration of the study, as well as the nature of the experimental tasks. Also, the participants were informed with a written consent form about the personal data that would be collected and would be treated anonymously and confidentially, according to the legislation and the relevant provisions of the regulation 2016/679 and the instruction 95/46/EK, regarding personal data. The participants signed consent forms that informed them of the voluntary nature of their participation and their right to withdraw at any point during the study.

Statistical Analysis

For all the analyses we conducted we used the IBM SPSS Statistics 21 with a level of significance set at $\alpha = 0,05$ and the StatSoft Statistica 12.5. We performed a repeated-measures analysis of variance with Task (CMN 40, CMN 100) and Time (Morning, Afternoon, Evening) as within-subject factors for the Countermanding

task; and a repeated-measures analysis of variance with Time (Awake, 30min later, 4hrs later, 8hrs later and 12hrs later) as within-subject factor for the circadian cortisol pattern.

Additionally, we performed various correlation analyses between the significant differences in the performance on the Countermanding task and the corresponding changes in cortisol and between the diurnal cortisol slope and the diurnal performance on the Countermanding task slope. For the correlation analysis of the slopes, due to the fact that cortisol forms a curve, we used a log transformation of the 30min, 4hrs, 8hrs and 12hrs values of cortisol so that the curve becomes more linear. This log transformation made it easier and more precise to calculate the slope of the cortisol for each participant. The first sampling of cortisol, the one corresponding to the awakening, was excluded for the same reason. Furthermore, we calculated the slope for each participant for the reaction time, the percentage of correct responses and the intra-subject variability of the reaction time. Afterwards the slopes of cortisol were correlated with the slopes of the reaction time, the percentage of correct responses and the intra-subject variability. This analysis has the advantage that the total change in cortisol levels as depicted with the calculation of cortisol's slope is compared with the total change in the performance on the Countermanding task.

Results

Countermanding Task

Reaction time. We conducted a repeated-measures analysis of variance with task (CMN 40, CMN 100) and time (Morning, Afternoon, Evening) as within-subject factors; in this analysis the dependent variable was the reaction time scores (Tables 2 & 3). Results showed a main effect of the task $F(1,19) = 38.23$, $p = <0.001$, $\eta^2p = 0.67$ where regardless of the time, all participants were 23,49 milliseconds slower in the CMN 100 task. There was also a main effect of time $F(2,38) = 7.17$, $p = 0.002$, $\eta^2p = 0.20$ where regardless of the task all participants were 5,51 milliseconds faster in the Afternoon in comparison with the Morning and 18,65 milliseconds faster in the Evening in comparison with the Morning. Post-hoc comparisons with the Fisher LSD test revealed that the Evening condition differ significantly from the Morning ($p = <0.001$) and the Afternoon ($p = 0.01$) condition. Finally, there was no significant task x time interaction $F(2,38) = 0.24$, $p = 0.792$, $\eta^2p = 0.01$ (Figure 2).

Table 2

Reaction time, Percentage of correct responses and Intra-subject variability of reaction time for each condition of the countermanding task regardless of the time

	Task	*M*	*SD*
Reaction time (in milliseconds)	CMN 40	278,75	6,83
	CMN 100	302,23	7,71
Percentage of correct responses	CMN 40	0,83	0,03
	CMN 100	0,72	0,04
Intra-subject variability of reaction time	CMN 40	89,74	5,47
	CMN 100	101,22	5,11

Table 3

Reaction time, Percentage of correct responses and Intra-subject variability of reaction time for each time condition regardless of the task

	Time	*M*	*SD*
Reaction time (in milliseconds)	Morning	298,54	9,01
	Afternoon	293,03	6,28
	Evening	279,90	7,31
Percentage of correct responses	Morning	0,71	0,04
	Afternoon	0,81	0,03
	Evening	0,82	0,03
Intra-subject variability of reaction time	Morning	110,02	6,60
	Afternoon	93,95	5,39
	Evening	82,48	5,20

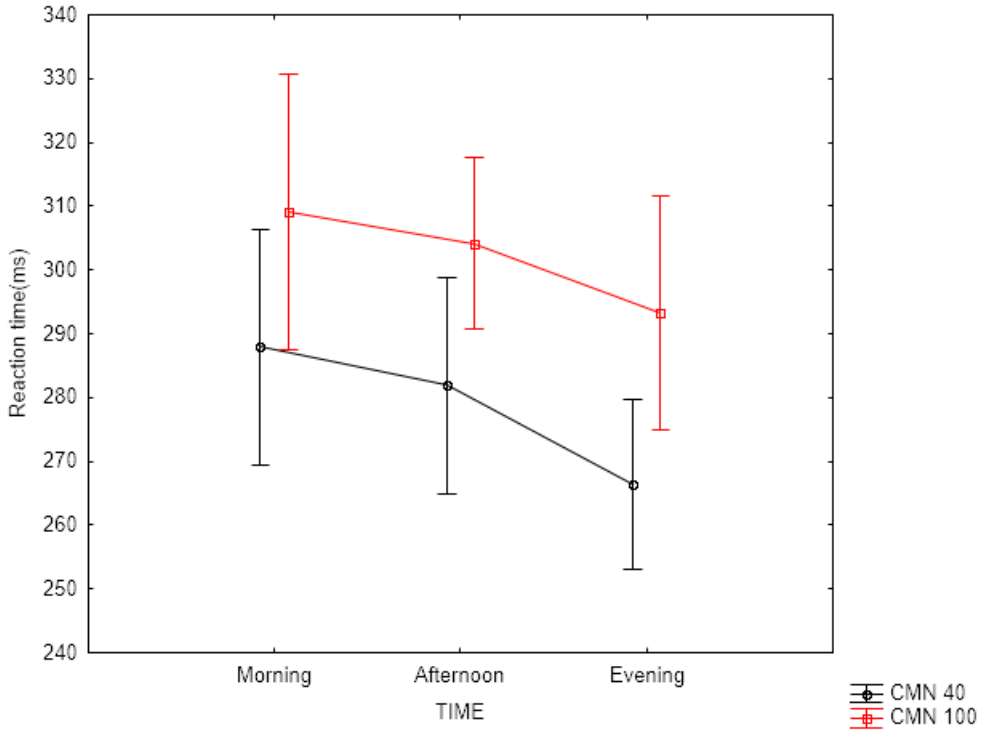

Figure 2. Participants' reaction time in the Countermanding task.

Percentage of correct responses. We conducted a repeated-measures analysis of variance with task (CMN 40, CMN 100) and time (Morning, Afternoon, Evening) as within-subject factors; in this analysis the dependent variable was the percentage of correct responses (Tables 2 & 3). Results showed a main effect of the task $F(1,19) = 15.85$, $p = <0.001$, $\eta^2p = 0.45$ where regardless of the time, all participants had 11% more correct responses in the CMN 40 task in comparison with the CMN 100 task. There was also a main effect of time $F(2,38) = 12.26$, $p = <0.001$, $\eta^2p = 0.41$ where regardless of the task all participants had 10,5% more correct responses in the Evening in comparison with the Morning and 9,8% more correct responses in the Afternoon in comparison with the Morning. Post-hoc comparisons with the Fisher LSD test divulged that the Morning condition differed significantly from the Evening ($p = <0.001$) and the Afternoon ($p = <0.001$) condition. Finally, there was no significant task x time interaction $F(2,38) = 0.13$, $p = 0.883$, $\eta^2p = 0.007$ (Figure 3).

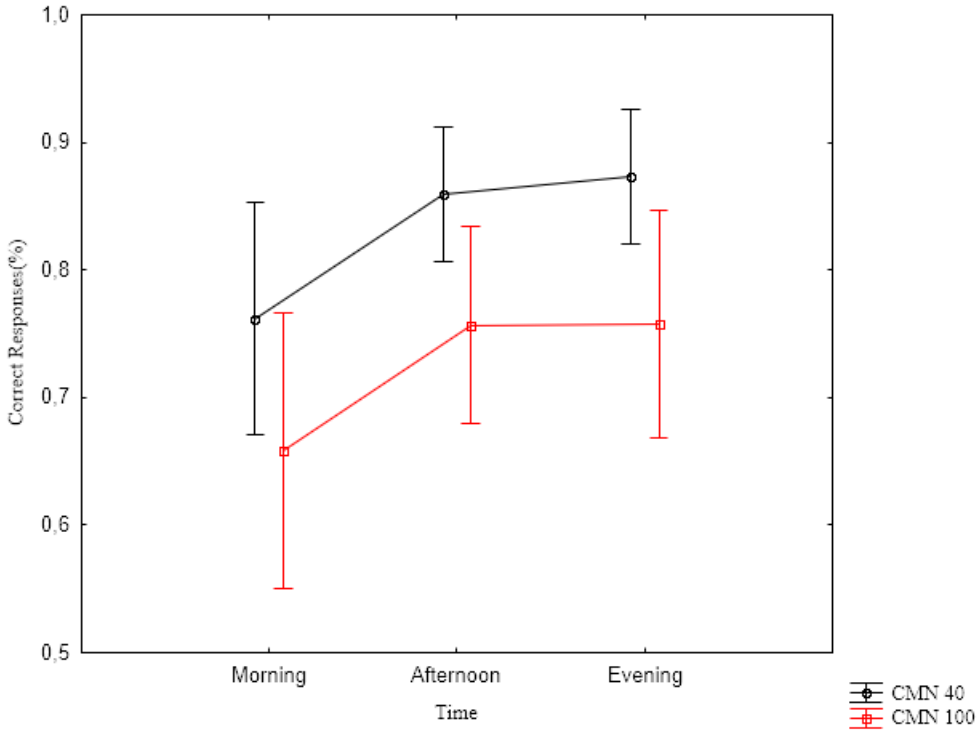

Figure 3. Participants' percentage of correct responses in the Countermanding task.

Intra-subject variability of correct responses' reaction time. We conducted a repeated-measures analysis of variance with task (CMN 40, CMN 100) and time (Morning, Afternoon, Evening) as within-subject factors; in this analysis the dependent variable was the standard deviation of the correct responses' reaction time (Tables 2 & 3). Results showed a main effect of the task $F(1,19) = 12.74$, $p = 0.002$, $\eta^2p = 0.40$ where regardless of the time, all participants had 11,48 bigger fluctuation in their standard deviation in the CMN 100 task in comparison with the CMN 40 task. There was also a main effect of time $F(2,38) = 16.44$, $p < 0.001$, $\eta^2p = 0.46$ where regardless of the task all participants had 16,07 smaller fluctuation of their standard deviation in the Afternoon in comparison with the Morning; 27,54 smaller in the Evening in comparison with the Morning; and 11,47 smaller fluctuation in the Evening in comparison with the Afternoon. Post-hoc comparisons with the Fisher LSD test unveiled that the Morning condition differed significantly from the Afternoon condition ($p = 0.002$); the Morning condition differed significantly from the Evening condition ($p < 0.001$); and the Afternoon condition differed significantly from the Evening condition ($p = 0.02$). Finally there was no significant task x time interaction $F(2,38) = 0.06$, $p = 0.94$, $\eta^2p = 0.003$ (Figure 4).

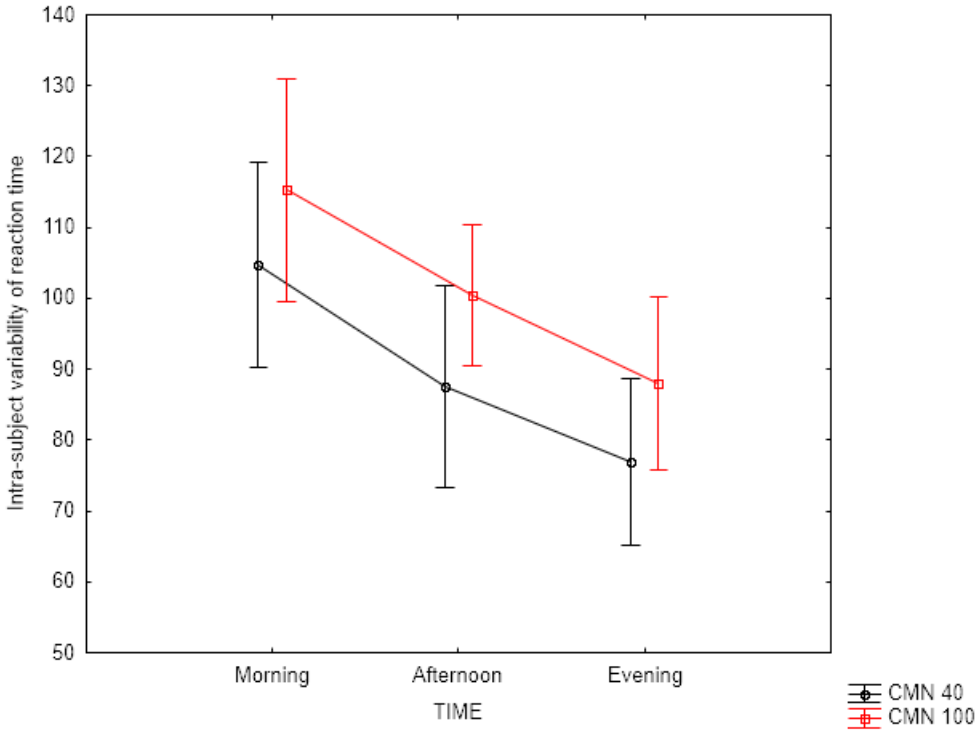

Figure 4. Participants' intra-subject of the reaction time in the Countermanding task.

Cortisol

Circadian cortisol pattern (cortisol curve). We conducted a repeated-measures analysis with time (Awake, 30min later, 4hrs later, 8hrs later and 12hrs later) as within-subject factor; in this analysis the dependent variable was the levels of cortisol. The purpose of this analysis was to produce the curve that depicts cortisol's variance throughout the day. Results showed a main effect of time $F(4,76) = 41.08$, $p < 0.001$, $\eta^2p = 0.68$ as expected (Figure 5). Cortisol's highest levels were at 30min with 0,97 µg/dL and the lowest were at 12hrs after the awake with 0,14 µg/dL (Table 4). The largest intra-individual volatility was presented at the cortisol levels 4hrs after the awake (Std = 0,08) and the smallest in cortisol levels 12hrs after the awake (Std = 0,016). In conclusion, the circadian cortisol pattern observed is similar to the one expected.

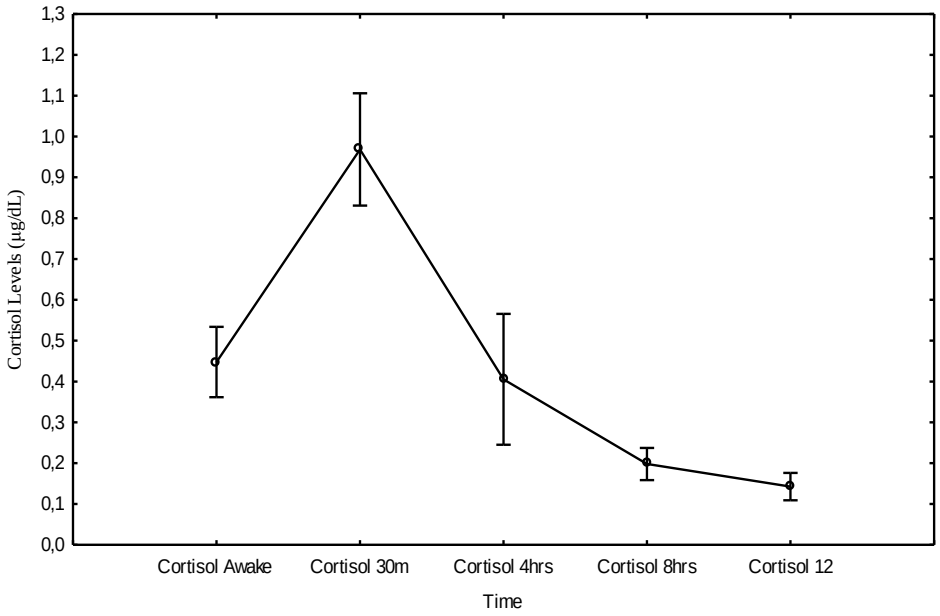

Figure 5. Participants' circadian cortisol pattern.

Table 4

Cortisol's levels for each time condition

	M	*SD*
Cortisol awake	0,45	0,04
Cortisol 30min	0,97	0,07
Cortisol 4hrs	0,41	0,08
Cortisol 8hrs	0,20	0,02
Cortisol 12hrs	0,14	0,02

Cortisol-countermanding task relationship

Multiple comparisons using the Pearson product-moment correlation coefficient (Pearson's r) were performed. The purpose of these analyses were to examine the correlation between the differences in the performance on the countermanding task and the changes in cortisol levels in the same time period. The changes (mean difference) in the performance on the countermanding task as it was measured with reaction time, percentage of correct responses and intra-subject variability of correct responses' reaction time -that in the post-hoc analyses in the previous repeated measures analyses differed significantly in the time condition- were correlated with the changes in cortisol levels in the same time period.

For the reaction time, the post-hoc analyses that differed were the Evening/Morning and the Evening/Afternoon; for the percentage of correct responses,

were the Evening/Morning and the Afternoon/Morning; and for the intra-subject variability of the correct responses' reaction time, were the Evening/Morning, the Evening/Afternoon and the Afternoon/Morning. Because the analyses didn't reveal any interaction between time and task, the mean average of the two tasks (CMN 40 and CMN 100) was used in the correlation analysis. For example, the changes in the performance between Evening and Morning were the mean average of the two tasks in the Evening minus the mean average the two tasks in the Morning. The remaining changes in the performance were calculated accordingly.

Regarding the reaction time, the Evening-Morning and the Evening-Afternoon mean differences regardless of the task were correlated with the corresponding Evening-Morning and Evening-Afternoon differences in cortisol levels. No significant correlation was observed; specifically there was no correlation in the differences in the Evening-Morning $r(18) = 0.171$, $p = 0.471$ and no correlation in the differences in the Evening-Afternoon $r(18) = -0.28$, $p = 0.216$.

Concerning the percentage of correct responses, the Evening-Morning and the Afternoon-Morning mean differences regardless of the task were correlated with the corresponding Evening-Morning and Afternoon-Morning differences in cortisol levels. No significant correlation was observed; specifically there was no correlation in the differences in the Evening-Morning $r(18) = -0.174$, $p = 0.464$ and no correlation in the differences in the Afternoon-Morning $r(18) = -0.313$, $p = 0.179$.

For the intra-subject variability of the correct responses' reaction time, the Evening-Morning, the Evening-Afternoon and the Afternoon-Morning mean differences regardless of the task were correlated with the corresponding differences in cortisol levels. No specific correlation was observed; specifically there was no correlation in the differences in the Evening-Morning $r(18) = 0.22$, $p = 0.35$, no correlation in the differences in the Evening-Afternoon $r(18) = -0.186$, $p = 0.432$ and no correlation in the differences in the Afternoon-Morning $r(18) = 0.038$, $p = 0.163$.

The analysis of the slopes between the diurnal cortisol slope and the diurnal performance on the Countermanding task slope didn't reveal any specific correlations; specifically there was no correlation in the cortisol slope with the slope of reaction time $r(18) = 0.185$, $p = 0.434$, no correlation in the cortisol slope with the slope of the percentage of correct responses $r(18) = 0.074$, $p = 0.757$ and no correlation in the cortisol slope with the slope of the reaction time intra-subject variability $r(18) = 0.037$, $p = 0.877$.

Discussion

The present study aimed to explore the diurnal variation in the cognitive function and especially the inhibition of an already initiated response, as it was assessed with the performance on the Countermanding task. We measured the performance of 20 young adults on two conditions of the Countermanding task throughout a day (Morning, Afternoon, Evening). Additionally, we collected 5 samples of cortisol for the purpose of investigating the relationship between the changes on the performance on the Countermanding task and the changes on the diurnal cortisol as a key HPA axis biomarker in younger adults. In this research, we chose to study a sample of younger participants. This decision was made due to the fact that older adults tend to exhibit a greater CAR (Almeida et al., 2009b) and flatter wake-to-evening slopes compared to middle-aged and young adults (Nater et al., 2013). Other researchers have also reported that age correlates with a flatter diurnal slop (Adam et al., 2006; Dmitrieva et al., 2013; Gaffey, Bergeman, Clark, & Wirth, 2016; Karlamangla et al., 2013; Nater et al., 2013), which could be attributed to either lower morning cortisol and/or higher evening cortisol. Thus, existing evidence support that diurnal cortisol increases with age with a possible dysregulation of the HPA axis. This alteration of the HPA axis regulation makes it difficult to study the effects of cortisol in older adults and underlines the necessity of studying such phenomena in younger healthy populations.

The performance on the countermanding task that assessed the cognitive function revealed a stable performance pattern with participants performing better in the easiest condition (CMN 40) and worst in the most difficult one (CMN 100), as expected. Specifically, participants were faster, had more correct responses and less intra-subject variability in the CMN 40 condition compared to the CMN 100. Furthermore, participants performed better in the Evening condition regardless of the task. Participants were faster in the Evening condition compared to the Morning and Afternoon ones. They also had more correct responses in the Evening and Afternoon conditions compared to the Morning condition. Furthermore, their reaction time intra-subject variability was less in the Evening and the Afternoon compared to the Morning condition, indicating an amelioration in their performance. Our results exhibit a robust improvement in the performance on the Countermanding task that seems to follow a diurnal pattern, videlicet the participants presented an overall improvement in their performance throughout the day.

To our knowledge this is the first time that a diurnal variation in the Countermanding task has been observed. Most of the studies have focused on the test-retest reliability of the Countermanding task or similar stop signal tasks. A high test-retest reliability indicates a high temporal stability. Wöstmann et al., (2013) examined the test-retest reliability of the prosaccade, antisaccade and stop-signal task with a test-retest time interval of 28 days. In line with previous studies (Cornblatt et al., 1988; Ettinger, Kumari, Zachariah, et al., 2003; Kindlon et al., 1995; Klein & Berg, 2001; Klein & Fischer, 2005; Kuntsi et al., 2005; Logan, 1994; Saville et al., 2011; Soreni et al., 2009), most of the inhibition-related variables that were assessed showed good test–retest reliability. Especially for the stop signal task, research has shown a good reliability of the go component of the task and unreliability of the stop signal reaction time (SSRT) (Wöstmann et al., 2013). In contrast to these findings, previous studies (Kindlon et al., 1995; Soreni et al., 2009) have found a good test-retest reliability for both the go component and the SSRT.

One possible explanation for the observed diurnal variation in the performance on the Countermanding task would be the existence of a learning effect. The test-retest studies focusing on the stop signal task (Kindlon et al., 1995; Soreni et al., 2009; Wöstmann et al., 2013) or other oculomotor tasks such as prosaccades and antisaccades (Calkins, Iacono, & Curtis, 2003; Klein & Berg, 2001; Klein & Fischer, 2005; Roy-Byrne, Radant, Wingerson, & Cowley, 1995), indicate that there is a stability on the performance when the tasks are performed in the same time conditions. This stable and robust test-retest performance renders difficult the attribution of the improvement on the performance, observed in our research, to a learning effect. One limitation of these test-rest studies is that they usually have a large time interval between the two testing time points, usually being larger than 1 month. A briefer test-retest time interval would be better for the examination of the existence of a learning effect.

The circadian cortisol pattern or cortisol curve observed in this study was the same as the one depicted in the literature, scilicet highest cortisol levels at 30min after awake and lowest before the bedtime (Debono et al., 2009; Krieger et al., 1971; Weitzman et al., 1971). The cortisol curve differed from person to person, as it was expected, with some of the participants presenting their highest cortisol levels at the third sampling or even the fifth. These differences in the fluctuation of the cortisol

levels throughout the day, for each participant, indicate the differences in stress-response for each person and the perplexity of HPA axis' regulation.

Although we observed a diurnal variation in the participants' performance on the Countermanding task and a diurnal variation in the cortisol levels, these two didn't reveal any significant correlation. The changes in the task performance seem to be independent of the changes in the cortisol levels. Neither the mean differences nor the diurnal slopes revealed any kind of relationship between these two. Ennis, Moffat, & Hertzog (2016) showcased that neither the diurnal cortisol slope nor the total cortisol output was significantly related to working memory or processing speed which are considered as part of the executive function. In contrast with our results, they found that higher waking cortisol was related to working memory (Ennis et al.2016). Additionally, some other studies have not found any evidence of a robust association between any feature of the diurnal cortisol pattern or cortisol levels and cognitive performance (Singh-Manoux, et al., 2014; Korten, Penninx, Rhebergen, Deeg, & Comijs, 2018). It is also important to note that there are no studies investigating the association between the Countermanding task and the cortisol levels.

Some other studies (Beluche et al., 2010; Evans et al., 2012; Kumingas et al., 2007 Lee et al., 2007; Li et al., 2006; Stawski, et al., 2011) have found that the diurnal cortisol levels were related to the cognitive performance and particularly that the flatter diurnal cortisol rhythms were related to worst cognitive performance. These studies used elderly participants who are known to have higher cortisol levels (Karlamangla et al., 2013; Nater et al., 2013) and a possibly deregulated HPA axis. Furthermore, the studies that have shown an impairment on working memory (Arnsten, 2009; Schoofs et al., 2009 ,2008; Shansky & Lipps., 2013) and inhibition (Sänger et al., 2014), or an improvement on inhibition (Schwabe et al.,2013; Shields et al.,2015), are studies that focus on the changes that occur on cognitive function after acute alterations in cortisol levels, such as during the administration of exogenous glucocorticoids or with the inducement of acute stress that imitates cortisol action. The results of these studies might be better attributed to the stress mechanisms rather than the alteration in the cortisol levels. Cortisol, in a stress response, interacts with many biological processes to influence core executive functions. Cortisol interacts with stress-induced increasements in noradrenergic (one type of catecholamine) activity to exert its effects on cognitive processes (Roozendaal et al., 2006; Lupien et al., 2007), perhaps due to effects of noradrenergic activity on

attention (Robbins & Arnsten, 2009). Similarly, stress also increases dopaminergic activity (Robbins & Arnsten, 2009). That is to say, dopamine both interacts with cortisol (Mizoguchi et al., 2004) and follows an inverted U to enhance or impair working memory (Robbins & Arnsten, 2009). Additionally stress alters immune system activity (Segerstrom & Miller, 2004; Steptoe et al., 2007) and upregulates other adrenal hormones such as dehydroepiandrosterone (DHEA) or noradrenaline (Allen et al., 2014; Lennartsson et al., 2012; Shields et al., 2016; Thomas et al., 2012). Many of these hormones and immune system factors reportedly have an effect on cognition (Allen et al., 2014; Mehta & Josephs, 2010; Shields et al., 2016; Sparkman et al., 2006). Finally, stress alters catecholaminergic activity and CRH, both of which exert effects on the executive function (Arnsten, 2009; Shansky & Lipps. 2013; Uribe-Mariño et al., 2016).

Effects of stress on any of the biological processes mentioned earlier may be significant in part for the effects of stress on executive functions. An inability to consider all these different biological processes may result in a fragmented consideration of how stress and especially cortisol influences cognitive function. Indeed, our results make clear that the diurnal variation in cortisol levels does not appear to be responsible for producing effects on executive functions and especially inhibition. The effects of cortisol particularly through an acute stress response or exogenous administration on core executive function may be more complex than those captured in these studies.

This study includes some possible limitations. First of all the sampling of cortisol took place throughout the course of one day. If more sampling days had been used we might have had an even more robust circadian cortisol pattern. Additionally, our participants were young healthy adults and we cannot generalize our Countermanding Task results in other age groups. Possible future research could examine the existence of a diurnal pattern on the Countermanding task in other age groups. Another possible limitation is the fact that participants did not provide their cortisol samples at the same time. Finally, we did not test the participants twice in the same time condition to confidently rule out the existence of a learning effect.

In summary, our results suggest that the performance on the Countermanding task presents a diurnal variation and specifically that there is a significant improvement on the performance throughout the day. Specifically, participants in the Evening condition exhibited a robust improvement compared to the Morning

condition. This improvement on the performance on the Countermanding task is not related with the diurnal variation on cortisol levels.

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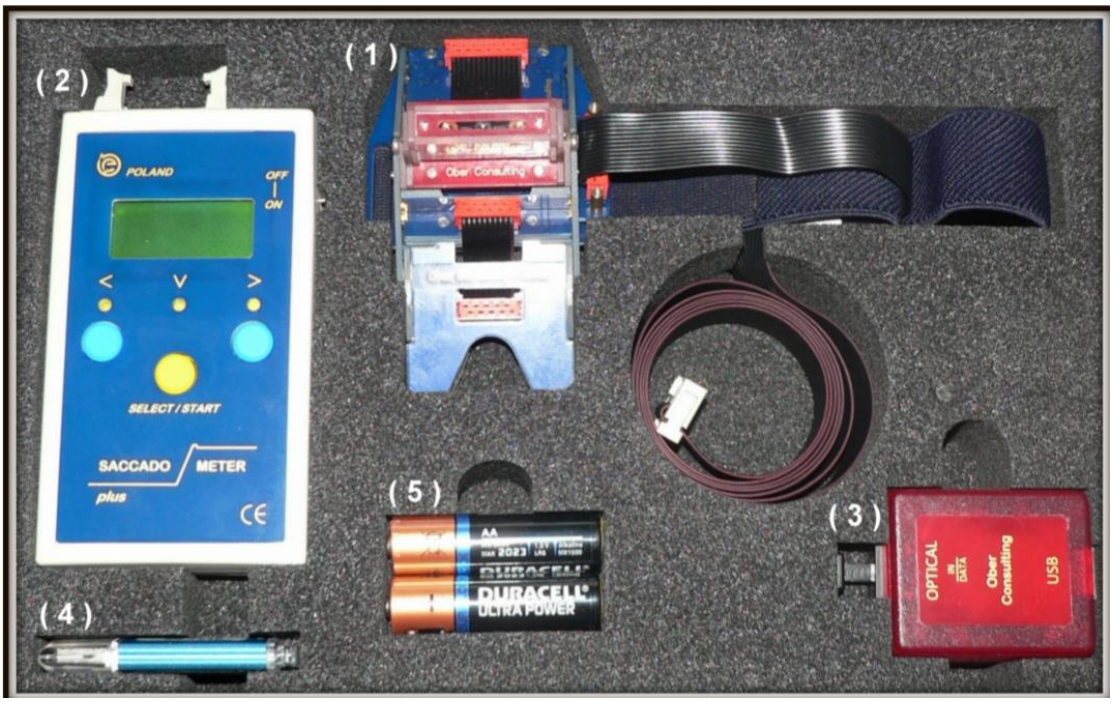
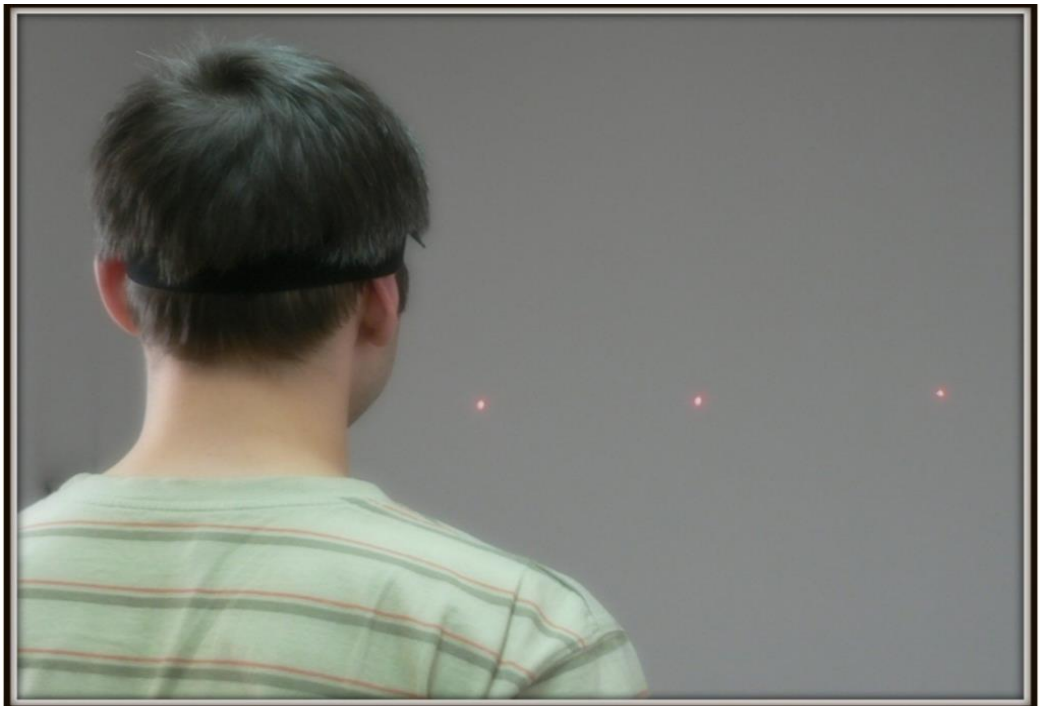
Appendix

Picture 1. Salivettes used for the cortisol sampling.



Picture 2. The device of Saccadometer plus.

Picture 3. Saccadometer Plus and its application on the participants.



Picture 4. Saccadometer Plus and the targets presented on the participants.