DIURNAL VARIATION OF CORTISOL, TESTOSTERONE, AND THEIR RATIO IN APPARENTLY HEALTHY MALES

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Preliminary communication

Abstract

The aim of the present study was to investigate whether a certain time of day may be more or less catabolic or anabolic than another and therefore of greater adaptive potential after exercise. Eighteen male university students volunteered to participate in the study. Salivary specimens were collected every 60 min over a 12 h waking period and then measured with the Salimetrics HS-cortisol assay and Salimetrics HS-testosterone assay. Participants remained at rest and were fed identical meals to each other at 08:05, 13:05, and 18:05 h. Blood pressure, heart rate and core temperature were observed every 15 min. Elevated cortisol concentrations were observed in the morning between 08:00 and 09:00 h (mean concentration ± SD = 0.28 ± 0.17 ug/dL) and a nadir in the evening or early night-time, between 17:00 and 20:00 h (mean concentration ± SD = 0.12 ± 0.06 ug/dL). Post hoc analysis revealed that mean 08:00 h cortisol was significantly greater than mean cortisol at 11:00 to 20:00 h ($P < 0.05$) and mean 09:00 h cortisol was significantly greater than mean cortisol from 15:00 to 20:00 h ($P < 0.05$). However, testosterone did not change significantly during the same period. Cortisol, core temperature and systolic blood pressure correlated significantly (although not strongly) with sampling time. In conclusion, salivary sampling to assess the anabolic/catabolic status of a squad or individual or to diagnose overtraining may not be simple or feasible as the variation between individuals is high and therefore setting a threshold value would prove troublesome. Also, the episodic release of cortisol in particular increases the difficulty of deciding a value in healthy or subclinical individuals.

Keywords: *Anabolism; Catabolism; Biological variation; Circadian rhythms*

INTRODUCTION

Almost all biochemical and physiological parameters are circadian rhythmic (Reilly, Atkinson & Waterhouse, 1997; Hayes, Bickerstaff & Baker, 2010). The circadian pacemaker regulates the prominent 24 h variation in biological functions, including the synthesis and release of testosterone and cortisol (Mrosovsky, 2003). Testosterone has both anabolic and anticatabolic effects on muscle tissue (Hayes et al., 2010) as well as associated effects on sexual maturation. Cortisol is a steroid hormone released by the adrenocortical glands under hypothalamic and pituitary control defining the hypothalamo-pituitary-adrenal (HPA) axis. The HPA axis plays a vital role in chronic adaptation to endurance training and acute response to exercise. Cortisol exerts catabolic effects on muscle tissue (Florini, 1987) and has important metabolic functions, such as influencing the metabolism of lipids, proteins, and glucose. Intense physical exercise increases cortisol (Timon et al., 2009), which may inhibit protein synthesis with consequent decrease in muscle mass by its catabolic effect (de Souza Vale et al., 2009). The balance between these anabolic/catabolic hormones is often used as an indicator of overtraining (Duclos, 2008).

Both testosterone and cortisol exhibit circadian rhythmicity with peak concentrations in the morning, around the commencement of diurnal activity, and reduced concentrations in the evening and overnight (Touitou & Haus, 2000). The morning rise in cortisol accelerates metabolism (Florini, 1987) and stimulates gluconeogenesis and proteolytic activity, resulting in increased skeletal protein turnover (Dinneen, Alzaid, Miles & Rizza, 1993). The increase in testosterone at this time may be an attempt to counteract the stimulatory effect of cortisol on skeletal protein degradation (Kraemer, 1988).

Rose, Sulak, Johnson, Holaday and Kruez (1972) were some of the initial investigators to document the episodic release of cortisol and testosterone over the day. Although cortisol samples were only collected every 90 min, peaks in the early morning and at 12:00 and 16:00 h were clearly evident in diurnally active subjects. These observations are consistent with those of Krieger, Allen, Rizzo and Krieger (1971), who reported similar patterns only 12 months prior. Concentrations of testosterone were shown to be less erratic than those of cortisol, with no "peaks" documented as such. Although consistent, the magnitude of the diurnal change in testosterone levels was significantly less than cortisol. On average, diurnally active men's testosterone concentration declined 42% from 06:00 h (awakening) to 23:00 h, compared with 92% for cortisol during this span. Slag, Ahmed, Gannon and Nuttall (1981) reported similar diurnal variations in cortisol, suggesting peaks

around 12:00 and 16:00 h. However, these times coincided with meals, and a fasted group in the Slag et al. (1981) paper showed a blunted cortisol response at these times.

Interestingly, the aforementioned observations have all been made when blood sampling rather than salivary sampling. Salivary cortisol is an excellent indicator of plasma-free cortisol (Arafah, Nushiyama, Tlaygeh and Hejal, 2007) increasingly used to assess hypothalamic–pituitary–adrenal axis secretory activity and rhythm (Casals, Foj & Jesus Martinez de Osaba, 2011). For example, it is widely accepted that late-night salivary cortisol measurement is a simple and reliable way to screen patients for Cushing's syndrome (Casals et al., 2011). In fact, the Clinical Guideline Committee of the Endocrine Society recommends the use of nocturnal salivary cortisol as a first step procedure in the diagnosis of Cushing's syndrome (Nieman et al., 2008). It is preferable to blood sampling since it can be easily performed on an outpatient basis without disrupting a normal routine. In addition, the saliva collection is a non-invasive sampling procedure that avoids the stress-induced rise in adrenal secretion associated with blood sampling. Therefore, salivary cortisol measurements are increasingly used on a routine basis. However, there is a lack of knowledge regarding significant data required for correctly interpreting salivary cortisol laboratory results, such as the degree of day-to-day intra-individual variation or the degree of inter-individual variation.

The use of salivary testosterone has been reported to be reliable in comparison to serum for reflecting gonadal function and circadian patterns (Dabbs, 1990). Khan-Dawood, Choe and Dawood (1984) have shown the composition of salivary testosterone to be 78% free testosterone, while serum free testosterone was reported to be at 4%. Wang, Plymate, Nieschlag and Paulsen (1981) reported that increases in serum testosterone concentrations relate to a concomitant increase in salivary testosterone concentrations within 1 h. Vittek, Lhommedieu, Gordon, Rappaport and Southren (1985) examined the relationship between salivary and serum free testosterone versus salivary and serum total testosterone and reported significant correlations of $r = 0.97$ and $r = 0.70$-0.87 for free and total testosterone, respectively. These data indicate that salivary testosterone provides a good indication of the fluctuations in free testosterone (Vittek et al., 1985). The aim of the study was to establish baseline salivary testosterone and cortisol concentrations, and their ratio, throughout a waking diurnal cycle. These results can be compared to previously published serum testosterone and cortisol results in order to establish the most opportune training times in terms of work tolerance, recovery, and adaptation.

MATERIALS AND METHODS

Participants

Eighteen male university students age, stature, body mass and BMI of 23.2 ± 3.0 years of age, 180.9 ± 4.3 cm in height, 84.4 ± 15.9 kg in body mass and, a 25.7 ± 4.5 BMI volunteered to participate in the study. Experimental procedures were approved by the University of the West of Scotland Research Ethics Committee. The protocol was explained and subjects gave informed consent to participate in this study. All subjects were habitually physically active, and had abstained from alcohol, caffeine and exercise for 24 h preceding the investigation. Exclusion criteria included poor sleep quality, recent shift work, extreme chronotype according to the Horne-Ostberg Morningness-Eveningness Questionnaire (Horne & Ostberg, 1976) or travel across multiple time zones.

Experimental Design

Fasted participants reported to the laboratory at ~07:45 h approximately 40 min after waking. Laboratory observations were conducted in the University of the West of Scotland's Clinical Exercise Research Laboratory. The ~40m^2 room was cleared of time reference devices and blinded, ensuring no natural light entered the room. Artificial lighting and a constant temperature was maintained.

Sample collection commenced at 08:00 h. Immediately after the first sampling, participants were provided with a standard breakfast consisting of Weetabix®, milk and orange juice (1,769 kJ, 18% protein, 9% fat, 73% carbohydrate). Participants remained in the study venue until ~20:05 h and provided a saliva sample every 60 min. At 13:05 and 18:05 h participants were provided with a standardised meal that consisted of ham, cheese and tomato wholegrain sandwiches (2,721 kJ, 24% protein, 22% fat, 54% carbohydrate per meal). Participants were permitted to drink water ad libitum and were instructed to rinse their mouths with water after eating. The meal plan had been previously used (Beaven, Ingram, Gill & Hopkins, 2010).

Intra-aural temperature was measured using a clinical-grade infrared ear thermometer (Braun, Germany). Blood pressure and heart rate was measured using an automated

sphygmomanometer (Omron, the Netherlands). Rate pressure product (RPP) was calculated by multiplying systolic blood pressure and heart rate. These parameters were observed every 15 min. Participants were free to engage in sedentary activities of their choice. Physical activity and exercise were not allowed. Subjects left the laboratory only to use the toilet (also light controlled).

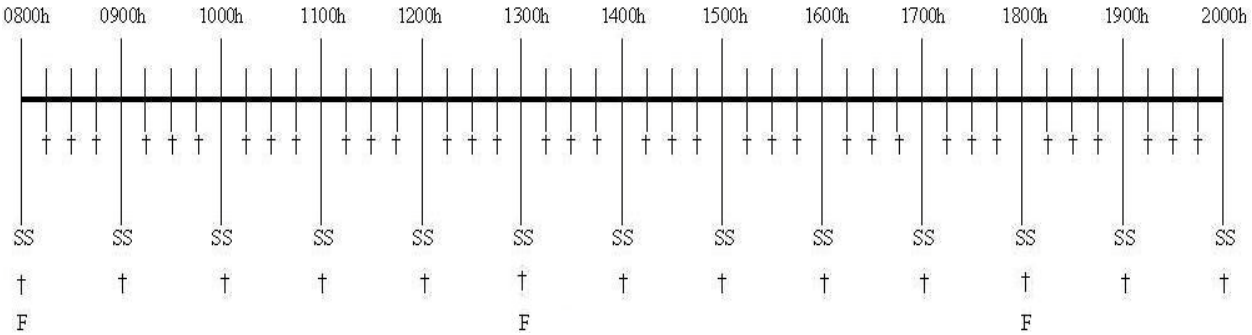

FIGURE 1 Methodological timeline depicting the sequence of events. *†* = battery of tests (blood pressure, core temperature, and heart rate measurement), *SS* = saliva sample, *F* = meal time. Note that when more than one set of measurements was conducted at one time point a constant sequential order was used: *SS*, *†*, *F*.

Saliva collection and analysis

Whole saliva samples of approximately 1.8 mL were collected every 60 min via expectoration into graduated 2 ml cryovials. To prevent blood contamination of saliva, which may result in overestimation of hormone concentrations, subjects were advised to avoid brushing their teeth and drinking hot fluids for >2 h prior to reporting to the study venue. Saliva samples were collected and transported to a freezer immediately where they remained at -80şC until assay. Saliva samples were assayed in duplicate (without separation or extraction) for cortisol and testosterone using commercially available immunoassay kits and protocols (Salimetrics, State College, PA). All samples were assayed for cortisol using a high-sensitivity enzyme-linked immunosorbent assay (ELISA) with a lower limit of sensitivity of <.007 ug/dL, and average intra- and inter-assay coefficients of variation 4.13% and 8.89%, respectively. Testosterone was measured using an ELISA with a lower limit of sensitivity of 1.5 pg/ml, and average intra- and inter-assay coefficients of variation less than 10% and 15%, respectively. The units of measurement for salivary testosterone and cortisol obtained are in picograms per milliliter (pg/mL) and micrograms per deciliter (ug/dL) respectively. All samples from an individual subject were tested within the same assay run.

Statistical analysis

Mean, standard deviation, analysis of variance, and Pearson's correlation were used to analyze subject descriptive and experimental data. The effect of sampling time upon salivary hormone concentrations was examined with repeated-measures ANOVA and Tukey's hsd.

RESULTS

Figure 2 shows the diurnal variation of salivary cortisol. Elevated concentrations were observed in the morning between 08:00 and 09:00 h (0.28 ± 0.17 ug/dL) and a nadir in the evening or early night-time, between 17:00 and 20:00 h (0.12 ± 0.06 ug/dL). Post hoc analysis revealed that mean 08:00 h cortisol was significantly greater than mean cortisol at 11:00 to 20:00 h ($P < 0.05$) and mean 09:00 h cortisol was significantly greater than mean cortisol from 15:00 to 20:00 h ($P < 0.05$).

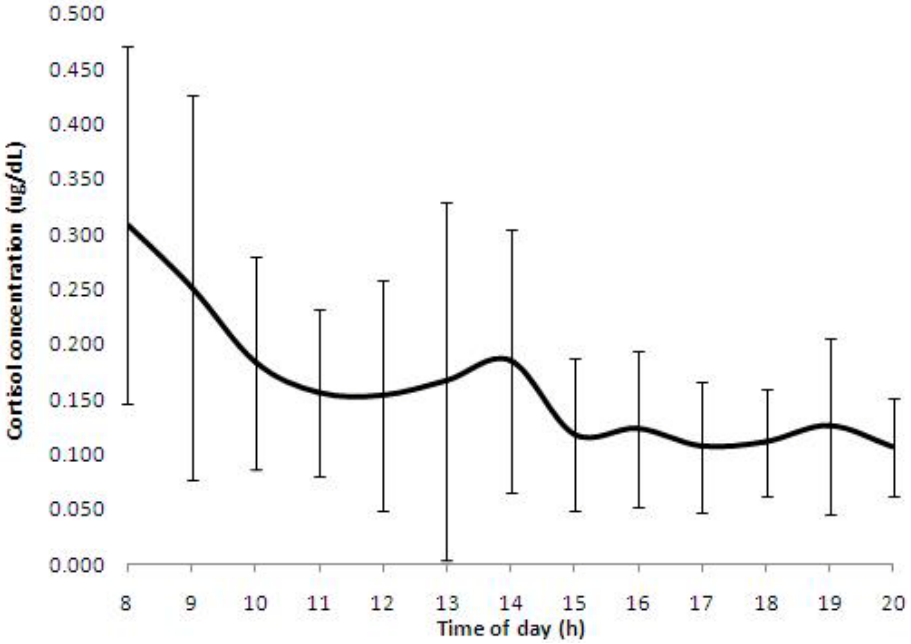

FIGURE 2 Diurnal variations of salivary cortisol.

Figure 3 shows the diurnal variation of salivary testosterone. Despite a trend for increased values at 08:00, 13:00 and 16:00 – 18:00 h, none of these sampling times demonstrated significant increases above other sampling times.

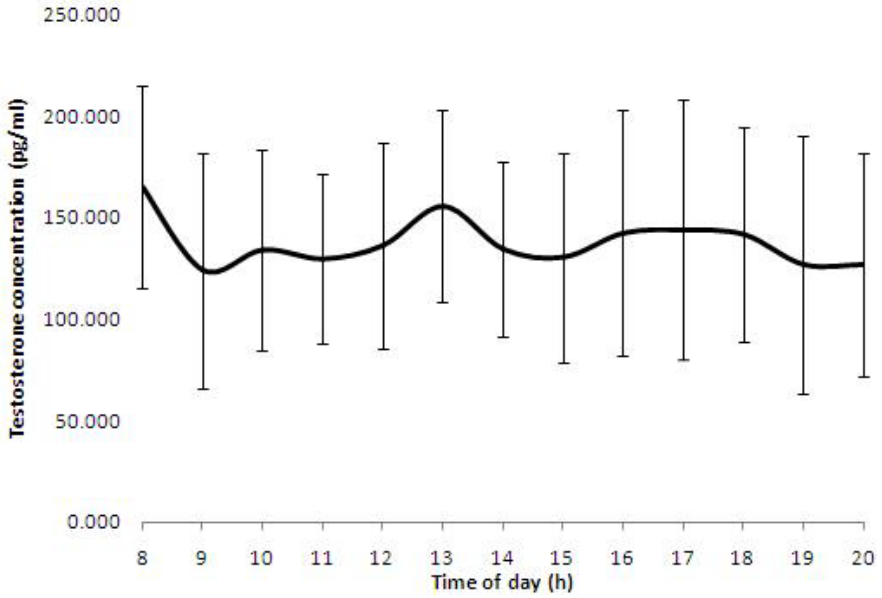

FIGURE 3 Diurnal variations of salivary testosterone.

Figure 4 shows the diurnal variations in salivary testosterone to cortisol ratio. It appears that the ratio increased gradually over the day with a slight drop in the early afternoon with the earliest values (08:00 and 09:00 h) being significantly lower than at 13:00 and 16:00 – 20:00 h ($P < 0.05$). The testosterone:cortisol ratio at 17:00 h was also greater than at 10:00 and 11:00 h ($P < 0.05$). This followed the inverted pattern of cortisol, as testosterone values were relatively constant throughout the day.

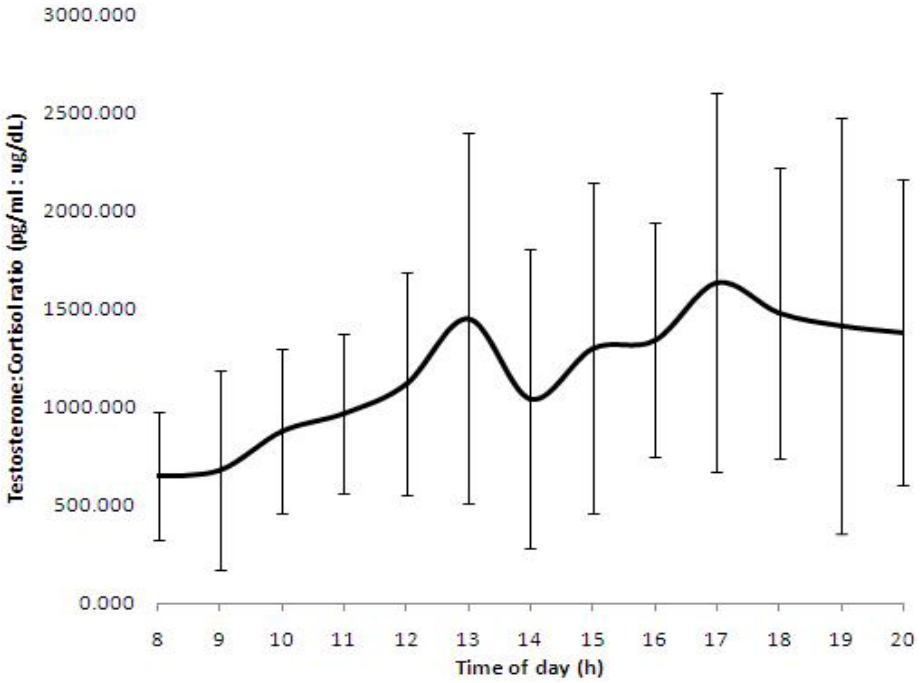

FIGURE 4 Diurnal variations of the salivary testosterone:cortisol ratio.

As with previous researchers, time of day correlated significantly with core temperate, systolic blood pressure, cortisol and therefore testosterone:cortisol ratio. Interestingly, our investigation showed no time of day effect for testosterone, diastolic blood pressure or heart rate. A number of correlations existed between physiological parameters and these are shown in table 1. As RPP showed no significant change over the 12 h and values did not correlate to sampling time, it appears that cardiovascular strain at rest shows no time of day effect.

		Systolic blood pressure	Diastolic blood pressure	Heart rate	Core temperature	Cortisol	Testosterone	Cortisol:testosterone ratio	RPP	Time of day
Systolic blood pressure	R^2		.414**	.357**	.294**	-.049	.009	.086	.723**	.081*
	P		.000	.000	.000	.483	.901	.215	.000	.022
Diastolic blood pressure	R^2	.414**		.477**	.081*	-.172*	-.217**	.023	.555**	.042
	P	.000		.000	.022	.013	.002	.740	.000	.238
Heart rate	R^2	.357**	.477**		.367**	.083	-.162*	-.004	.899**	-.046
	P	.000	.000		.000	.231	.018	.952	.000	.194
Core temperature	R^2	.294**	.081*	.367**		-.028	.123	.087	.408**	.126**
	P	.000	.022	.000		.684	.075	.209	.000	.000
Cortisol	R^2	-.049	-.172*	.083	-.028		.278**	-.525**	.031	-.403**
	P	.483	.013	.231	.684		.000	.000	.654	.000
Testosterone	R^2	.009	-.217**	-.162*	.123	.278**		.203**	-.106	-.057
	P	.901	.002	.018	.075	.000		.003	.125	.404
Cortisol:Testosterone ratio	R^2	.086	.023	-.004	.087	-.525**	.203**		.049	.254**
	P	.215	.740	.952	.209	.000	.003		.479	.000
RPP	R^2	.723**	.555**	.899**	.408**	.031	-.106	.049		.021
	P	.000	.000	.000	.000	.654	.125	.479		.557
Time of day	R^2	.081*	.042	-.046	.126**	-.403**	-.057	.254**	.021	
	P	.022	.238	.194	.000	.000	.404	.000	.557	

TABLE 1 Pearson's correlation for systolic blood pressure (mmHg), diastolic blood pressure (mmHg), heart rate ($b \cdot mins^{-1}$), core temperature (°C), cortisol (ug/dL), testosterone (pg/mL), cortisol:testosterone ratio, RPP and time point. **. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).

DISCUSSION

The presence of "peak" morning salivary cortisol levels seen here are in agreement with previous investigations (Brivio et al., 2010; Touitou & Haus, 2000). The existence of a large SD in the present study may be attributed to variations in the awakening cortisol response (ACR). Previous studies have shown that approximately 20% of

participants show an inverted ACR. Hansen, Gardem Christensen, Eller and Netterstrom (2003) reported that 18% of a study group showed negative ACRs. Wust, Wolf, Hellhammer, Federenko, Schommer and Kirschbaum (2000) found that, over 2 days, a mean of 23.2% of a study group were classified as "non-responders". Non- responders were defined as having a blunted increase from basal level within the first 45 min post-awakening. Eek, Garde, Handen, person, Orbaek and Karlson (2006) found that among 142 participants (75 female and 67 male) monitored during 3 work days and 1 day during the weekend, the daily prevalence of negative ACRs varied between 19% on a work day and 38% on a day off. Most participants exhibited one or more negative ACRs occasionally during the 4 days, and most a mixture of positive and negative responses. The difference between positive and negative responses could not be explained by self reported awakening time, subjective stress or sleep disturbance (Eek et al., 2006). In addition, certain proportions of participants did not follow the expected diurnal rhythm when saliva samples were collected at fixed time-points. In such studies, the expected diurnal pattern is a negative slope from the first sample (not collected immediately upon awakening). Therefore, deviations from this negative slope have been noted as "flat cycles" (Stone et al., 2001). A study covering 2 days of saliva sampling showed that 51% of the participants had the typical decline during both days; 17% did not show the diurnal pattern on both days; and 31% had a different pattern during the 2 days (Stone et al., 2001). A replication study concluded that at least 10% of participants had no significant diurnal cycle (Carlsson et al., 2006). The reason for these inconsistent patterns is not known. Recent findings of Eek et al (2006) suggest that non-responding appears to be related to state rather than trait anxiety, but it cannot be excluded that it is related to adherence to time of sampling.

Interestingly, no significant fluctuations in mean cortisol or testosterone were observed as a result of meal times in the present investigation. An increase of mean cortisol at 14:00 h (the first sample after lunch) was observed, however this was statistically insignificant. The evening meal resulted in no visible cortisol or testosterone fluctuation and a breakfast related change could not be detected possibly due to the already high concentrations.

As cortisol exhibited significantly higher concentrations in the morning than in the evening, and testosterone demonstrated no significant variance during the day, it is suggested that variation in the testosterone:cortisol ratio is due to variation in cortisol. This suggests that individuals are less catabolic in the evening, experiencing an increase in relative anabolism via reduced cortisol rather than an increase in testosterone being responsible for the chance in anabolic/catabolic balance. Since cortisol primarily affects protein degradation (Adlercreutz et al., 1986), a decrease in cortisol is expected to enhance skeletal muscle hypertrophy through reduction in protein degradation (McMurray, Eubank & Hackney, 1995). From our results therefore it is suggested that healthy individuals at rest experience more protein degradation in the morning than in the evening. Higher cortisol levels (and lower testosterone/cortisol) are mildly suggestive that morning hours are less suitable for training activities that are highly catabolic, such as exhaustive distance running or performing repetitions to failure in the weight room. These activities may be more appropriate in the late afternoon-early evening hours when testosterone/cortisol is at its highest ratio of the day.

As there was no significant variance in RPP over the testing period, and it did not correlate with either hormone, it is suggested that cardiac metabolic demand remained constant throughout the day and was not discernibly related to hormone concentration. Establishment of a time-of-day specific response to exercise relative to RPP requires further research. This pursuit may be of more relevance to endurance exercises and sports rather than to those of a power dependent nature.

Ockenfels, Porter, Smyth, Kirschbaum, Hellhammer and Stone (1995) presented between-subject variation in cortisol for unemployed and employed subjects as SDs to values measured at six different times of the day. Between subject SD was reported to be 61% and 86% on awakening and at 18:00 h respectively. Hansen et al. (2003) presented SD at awakening to be 16% and 51% at 18:00 h. The present investigation reports SDs of 53% and 43% at 08:00 h (the first sample taken) and 18:00 h respectively. Despite this favourable comparison, we suggest that the between- and within- subject variation observed in salivary cortisol may be too great to accurately assess the anabolic/catabolic state of a sports person in a single set of assessments. It is likely that a long term data set be produced for each individual to ascertain trends and appropriate times for training and recovery.

When carrying out ordinary tasks and daily routines, changes in hormone concentrations and physiological measures reflect our physiological adaptive accomodations to the environment (Hansen, Garde & Persson 2008). To some degree, this means that measures obtained reflect environmental exposure (e.g. physical activity level, heat, cold and sound) as well as how we perceive our daily tasks and relationships. But the measures obtained will also reflect normal cyclic biological variations (e.g. diurnal and seasonal variations), effects of lifestyle factors, as well as analytical interventions and errors (Hansen et al., 2008). The magnitude of variations, however, can be estimated, statistically modeled and attributed to variations within the individual (intra-individual variation) as well as between individuals (interindividual variation). In the present investigation, the low intra-individual variation (when compared to previous investigations) is most likely due to few physiological stressors and constant environmental conditions during the testing period. However, interindividual variation still existed, likely due to individual lifestyle differences. This may impair our ability to detect normal cyclic biological variations for a group. The need to maximise internal validity (constant rest, maintain environmental temperature, excluding natural light, training state, etc.) when investigating diurnal variations in hormone concentrations renders extrapolation of results from laboratory settings into an occupational, exercise, or sport context difficult.

In conclusion, there were significant changes in cortisol and testosterone/cortisol ratio at specific times of the day. Mornings were the most catabolic (via increased cortisol) and late afternoons were most anabolic (via increased testosterone/cortisol ratio). It is very tempting to suggest that (A) morning training sessions are best suited for lower load training due to the catabolic milieu present and (B) early evening training is suited for larger training loads as disruption of anabolic status may be more difficult due to the elevated testosterone/cortisol ratio. However, saliva sampling of testosterone and cortisol at rest may not be a sensitive enough technique to detect diurnal patterns of interest to athletes and coaches for determination of overtraining or preparedness. Individual variation also affects the application of training loads to an individual based on group means as this may provide an "optimal" window of anabolic peak. In regards to individual variations in testosterone and cortisol over time discussed earlier, Casals et al. (2011) suggested that due to the degree of individuality in biological variation in salivary cortisol secretion, only large changes, greater than 104% between two consecutive measurements, may be useful. This assertion may be skewed as it is based on salivary samples used in diagnosis of Cushing's disease - where extremely high cortisol levels are prevalent.

The findings of the present study are interesting as they confirm previous works in the area in that endocrine markers of catabolism are highest in the morning and lowest in the late afternoon and evening hours. Individual variation may inhibit applications of these principles without chronic measure for each individual trained.

PRACTICAL ASPECTS

The application of findings in the present investigation could affect practice of coaches who target hypertrophy in their athletes. However, this is particularly speculative as an increased anabolic environment may not necessarily result in increased hypertrophy. It is possible that the afternoon may be more favorable for adaptations to resistance exercise as increased testosterone has been shown to be associated with hypertrophy.

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DNEVNE VARIJACIJE NIVOA KORTIZOLA I TESTOSTERONA TE NJIHOV ODNOS KOD ZDRAVIH MUŠKARACA

Prethodno saopštenje

Sažetak

Cilj istraživanja je bio da se utvrdi da li je određeno vrijeme dana više ili manje kataboličko, odnosno anaboličko, nego drugo, i prema tome od većeg adaptivnog potencijala nakon vježbanja. Osamnaest studenata muškog pola dobrovoljno je učestvovalo u studiji. Uzorci pljuvačke su prikupljani svakih 60 minuta unutar 12 sati budnog perioda te su mjereni pomoću Salimetrics HS-kortizol i Salimetrics HS-testosteron mjerenja. Ispitanici su se odmarali te su se identično hranili u 08:05, 13:05 i 18:05 h. Krvni pritisak, srčani ritam te temperature tijela su praćeni svakih 15 minuta. Povišen nivo koncentracije kortizola je primjećen u jutarnjim satima između 08:00 i 09:00 h (srednja koncentracija ± SD = 0.28 ± 0.17 ug/dL) sa najmanjim vrijednostima u večernjim satima između 17:00 i 20:00 h (srednja koncentracija ± SD = 0.12 ± 0.06 ug/dL). Post hoc analizom je otkriveno da je prosječna koncentracija kortizola u 08:00 h bila značajno veća nego u 11:00 do 20:00 h (P < 0.05) te je bila veća u 09:00 h nego od 15:00 do 20:00 h (P < 0.05). Vrijednosti koncentracije testosterona se nisu značajno mijenjale u istom period. Kortizol, temperature tijela i sistolni krvni pritisak su značajno korelirali (mada veza nije bila jaka) sa vremenom uzimanja uzoraka. Da zaključimo, prikupljanje uzoraka pljuvačke s ciljem da se procijene anaboličko/katabolički status grupe ili individue ili da se dijagnostikuje pretreniranost moglo bi biti komplikovano i teško izvodljivo jer su varijacije između individual visoke i prema tome postavljanje graničnih vrijednosti bi moglo biti otežano. Također, episodno otpuštanje kortizola povećava poteškoće u određivanju vrijednosti kod zdravih ili subkliničkih individual.

Ključne riječi: *Anabolizam; Katabolizam; Biološke varijacije; Dnevni bio-ritmovi*

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Received: 23 February 2012
Accepted: 17 April 2012