Title: Is it required to abstain from fluid consumption in the 10 min before collection of a saliva sample?

Authors: Diogo V. Leal, PhD[a] and John Hough, PhD[b]

Affiliations:

[a]*Research Center in Sports Sciences, Health Sciences and Human Development, University of Maia, Maia, Portugal*

[b]*School of Science and Technology, Nottingham Trent University, Nottingham, United Kingdom*

Running Head: Water consumption on exercise-induced salivary hormone concentrations

Email address and contact details of corresponding author:

Diogo V. Leal

diogo.leal@umaia.pt

University of Maia, Avenida Carlos Oliveira Campos, 4475-690, Castêlo da Maia, Portugal

X handle: @diogo_luis_leal

Word count: 3,122 words

ABSTRACT

The non-invasive and reliable saliva collection method for biomarkers analysis is increasingly becoming more common in field-based and laboratory-based research. Current guidelines recommend interrupting water consumption 10 min before saliva sampling to avoid inaccurate, diluted concentrations of the biomarkers being examined. However, the impact of saliva dilution on salivary cortisol and testosterone levels has not been examined yet. This was a randomized, crossover experiment. Fourteen active, healthy males completed 3 separate cycle-bout trials, where a set volume of water (250 mL) was consumed 10 min, 5 min or 1 min before saliva sampling. Saliva was collected Pre-, Post- and 30 min Post-Exercise. No differences were observed in resting samples in any of the trials ($p < 0.05$). However, salivary cortisol increased from Pre- to Post-Exercise in the 10MIN trial (+52%, $p = 0.022$) and 5MIN trial (+36%, $p = 0.005$) only. Salivary testosterone also increased in 10MIN trial (+34%, $p = 0.005$), 5MIN trial (+37%, $p < 0.001$) and 1MIN trial (+24%, $p < 0.035$). This study proposes that consuming water up to 1 min before providing a saliva sample will not dilute the sample, allowing for an accurate determination of salivary cortisol and testosterone concentration levels. Practically the 10 min fluid abstinence required before saliva sampling in the previous guidelines could be reduced to 1 min, however if examining the response of salivary cortisol to an exercise trail a fluid abstinence of 5 min may be more appropriate. This conclusion will allow for less restriction on researchers and participants alike.

INTRODUCTION

Salivary biomarkers are commonly measured in field and laboratory-based research in exercise science and beyond (Hough et al., 2021; Leal et al., 2021; Papacosta & Nassis, 2011). The widespread use of this media is likely due to its non-invasive nature which is easy to collect and analyse (Papacosta & Nassis, 2011). Cortisol and testosterone are hormones that can be measured in saliva and are often measured in the sport and exercise research field and in a number of health-related research studies due to their important roles in the maintenance of health (Hayes et al., 2016). Individually salivary and circulatory cortisol and testosterone have been shown to have a moderate to strong positive correlation (Leal et al., 2021; VanBruggen et al., 2011). In addition, the reliability of measuring cortisol and testosterone in saliva has been reported to be strong in both males and females (Dabbs et al., 1995; Hough et al., 2021; Leal et al., 2021). Overall, the good measurement reliability and the correlation with circulatory hormones support the usefulness of salivary measurements of cortisol and testosterone as a non-invasive replacement for circulating levels.

A continuous 30 min, high-intensity cycle bout referred to as the 55/80 has been shown to elicit robust and reproducible elevations in salivary cortisol and testosterone in healthy, physically active male individuals (Hough et al., 2021). Furthermore, these responses were shown to be blunted following short periods of intensified training (Hough et al., 2013, 2015). This suggests that the exercise-induced responses of these salivary cortisol and testosterone to the 55/80 may be useful biological markers of early stage of overtraining, which can have long-term fatigue consequences in individuals.

Many experimental studies measuring salivary cortisol and testosterone concentrations restrict water consumption 10 min before sampling to reduce the risk of diluting the collected saliva sample. This is following the guidance from analytical kit manufactures to measure cortisol and testosterone. The concern with consuming water near to a sampling collection point is the risk of diluting the saliva sample leading to a lowered hormone concentration. No experimental research on the impact of diluting saliva by consuming water on the salivary cortisol and testosterone has been completed to date. This is an important question to consider as to reduce the time constrain placed onto participants particularly in the field. Therefore, the main aim of this study is to examine if consuming water 10 min, 5 min or 1 min before providing a saliva sample would dilute samples and consequently provide an lowered concentration than is truly present. The experimental hypothesis is that water consumption within a 10 min period pre saliva sampling will lead to a lowered saliva sample hormone concentration due to dilution.

METHODS

Participants

A randomized, crossover experiment study was conducted on 14 recreationally active male volunteers (10 were tested at the University of Bedfordshire labs and 4 at the Nottingham Trent University labs). Participants who frequently work night shifts were not allowed to participate, as these require 4 days to adjust their circadian rhythm of cortisol secretion (Niu et al., 2015). All participants were deemed healthy via our health questionnaire and therefore had no specific conditions that might influence our hormonal concentrations. They were not taking any prescribed medications that could alter the hormone measures. Participants were non-smokers as smoking can influence cortisol concentrations. All anthropometric and physiological characteristics of the participants are presented in Table 1. This study was granted ethical

approval by the University of Bedfordshire Research Ethics Committee and by the Nottingham Trent University Ethical Committee and was done in accordance with the Declaration of Helsinki. After comprehensive verbal and written descriptions of the study and of the benefits and risks of partaking in it, participants gave their written informed consent.

****Insert Table 1 near here****

Procedures

All trials were completed at the same time of day (~12:00) to avoid diurnal variation. A standard breakfast chosen by each participant was consumed 4 hours before the exercise testing began and replicated before each main trial. Participants were requested to consume at least 500 mL of water during the hours preceding testing and would not start the exercise trial unless euhydrated (i.e., urine osmolality < 700 mOsmol·kg^{-1}of H_2O) (Sawka et al., 2007) and body mass measurements were collected to determine the rate of sweat loss during and after exercise. Heart rate (HR) and rating of perceived exertion (RPE) were monitored continuously throughout each exercise test and values were recorded at the last 15 s of each stage (Figure 1). Participants were asked to maintain their normal weekly training load in between each main trial.

A food diary was completed the day before each trial. By use of the Dietplan version 6.70.74 software (Forestfield Software, West Sussex, UK) mean energy intake 24 hours prior to each trial was 9439 ± 3954 kJ (2256 ± 945 kcal), and carbohydrate, (58% ± 12%), fat (27% ± 13%), and protein (14% ± 2%) intake were determined.

Preliminary measurements and main experimental trials

On the first visit to the lab, participants completed an incremental maximal oxygen uptake ($\dot{V}O_{2max}$) test on a manually braked cycle ergometer (Monark 824E, Vansbro, Sweden). Following a 3-min warm-up at 75 W, the testing protocol started at 180 W and workload was increased by 30 W every 3 min until volitional exhaustion. Gas samples were collected throughout using a breath-by-breath ergospirometry exercise system (MetaLyzer 3B, Cortex, Leipzig, Germany). Participants' HR and RPE were recorded at the last 15 s of each stage. The $\dot{V}O_{2max}$ test was used to determine maximum workload ($\dot{W}_{max}$), and consequently to calculate the exercise intensities to be used in the main exercise trials (i.e., 55% and 80% $\dot{W}_{max}$). The individuals' $\dot{W}_{max}$ was determined using the equation:

$$\dot{W}_{max} = \dot{W}_{final} + (t/T) \times \dot{W}_{inc} \quad \text{(Kuipers et al., 1985)}$$

where $\dot{W}_{final}$ is the workload at the last completed stage, t is the time in seconds reached in the final stage (completed or uncompleted), T is the duration of each stage (i.e., 180 s), and $\dot{W}_{inc}$ is the workload increment (30 W).

The exercise test used in this study was a continuous, 30-min, high-intensity cycle bout, designed of intercalated blocks of 1 min at 55% $\dot{W}_{max}$ and 4 min at 80% $\dot{W}_{max}$ and is referred to as the 55/80 (Hough et al., 2011). This is an exercise stress test that has been reported to induce reproducible salivary cortisol and testosterone responses in healthy young males (Hough et al., 2021). Secondly, it has also been reported to be able to highlight blunted cortisol

and testosterone exercise induced responses following periods of elevation in exercise training period (Hough et al., 2013, 2015). All participants completed 4 trials in total – three main experimental trials and one $\dot{V}O_{2max}$ test. In addition, 9 participants also completed an additional resting control trial (CTL), during which the same volume of water as for the exercise trials was consumed. The CTL trial was added to the study design to allow for comparison of salivary cortisol and testosterone concentrations at rest and in response to exercise, despite its diurnal variation at this time of day has been reported elsewhere (Hough et al., 2011; Leal et al., 2021).

All participants abstained from exercise, caffeine, and alcohol intake in the 24 hours before each trial. The three main exercise trials were identical apart from the timing of water consumption before providing a saliva sample (Figure 1). During the completion of the 55/80 bout, a set volume of water was provided (100 mL) to be consumed within 30 s every 5 min up to and including 20 min to standardize the consumed volume among all participants. Additionally, depending on the trial being completed, participants were required to also consume 250 mL of water at either 10 min (10MIN), 5 min (5MIN), or 1 min (1MIN) before providing a saliva sample, totalling a water intake of 650 mL each trial. We aimed to provide a quantity of fluid to be consumed in a short period of time before the sample collection. Males have an average oral volume of ~27 mL for each mouthful of fluid (Jones and Cincinnati, 1961). It was deemed that a cupful measure (250 mL) would be appropriate to consume in a short time period. All participants reported to the laboratory at ~11:30 and completed a 76-statement recovery-stress questionnaire (RESTQ-76 Sport). The RESTQ-76 discriminates 48 nonspecific and 28 sport-specific statements of stress and recovery, consisting of 4 main scales of general stress, general recovery, sport stress and sport recovery (Kellmann & Kallus, 2001).

Body mass was measured Pre- and Post-Exercise and HR and RPE were recorded at the last 15 s of each 55/80 stage. Saliva samples were collected at Pre-, Post-, and 30 min Post-Exercise in all exercise trials.

*****Insert Figure 1 near here*****

Saliva collection, handling, treatment, and analysis

Saliva samples were collected into 7 mL polystyrene sterile bijou containers (Sterilin, Thermo Scientific, Loughborough, UK) by unstimulated passive drool, following the 'draining method' (Navazesh, 1993). Samples were collected with eyes closed, head tilted slightly forward and avoiding any orofacial movement. Minimum collection time was 3 min for each participant to allow for collection of sufficient sample volume (~2 mL). Following collection, all samples were kept on crushed ice during the entire period of testing, which did not exceed 1 h 20 min. Saliva samples were then centrifuged at 14,600 *g* for 3 min (Espresso Microcentrifuge, Thermo Scientific, Loughborough, UK) and the supernatant was transferred into 1.5 mL aliquots in Eppendorf tubes (Eppendorf, Hamburg, Germany) to be stored at -80°C until further analysis. Quantitative determination of salivary cortisol and testosterone concentrations were analyzed using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Salimetrics, PA 16803, USA) following the manufacturer's recommended procedures. The intra- and inter-assay coefficients of variation for salivary cortisol and testosterone were 4.6% and 6.0%, and 4.6% and 9.8%, respectively.

Statistical analyses

Descriptive analysis was carried out to observe the central tendency and dispersion of the data. Normality tests were then performed on all data (Q – Q plots and Shapiro-Wilk tests) and homogeneity of variance was examined. A logarithmic transformation to base 10 was performed on non-normally distributed data, and normality was re-checked. Logarithmic data sets were used for the analysis of salivary cortisol across all trials. When logarithmic data sets were still not normally distributed (salivary testosterone) a non-parametric Friedman's analysis of variance (ANOVA) was used on the original data. On finding a main effect, a two-related samples test (Wilcoxon signed-rank test) was used. A factorial (two-way) repeated measures ANOVA (Trial x Time) with Bonferroni adjustments was used to examine the salivary cortisol responses to exercise and *post hoc* tests for multiple comparisons were used. A one-way (Trial) repeated measures ANOVA with Bonferroni adjustments was used to compare the RESTQ-76 Sport questionnaire scores, urine osmolality and body mass, average HR responses and RPE scores during exercise, and hormone responses during CTL. Where Peak Post-Exercise values are presented, the data assume the highest post-exercise values observed. Data are reported as mean ± SD. The level of significance was set at $p < 0.05$.

RESULTS

Hydration status

The mean urine osmolality for all trials was 403 ± 202 (10MIN), 321 ± 195 (5MIN), 283 ± 194 (1MIN) and 473 ± 152 (CTL) $mOsmol \cdot kg^{-1}$of H_2O and did not alter across all trials ($p > 0.05$) (Table 2).

Total weight loss

Total loss in body mass when corrected for fluid intake was not different across the exercise trials ($p > 0.05$) and was 0.6 ± 0.2 kg (10MIN), 0.7 ± 0.2 kg (5MIN), 0.5 ± 0.1 kg (1MIN) and 0.2 ± 0.2 kg (CTL). Total weight loss was lower in CTL compared to all exercise trials ($p < 0.01$) (Table 2).

Recovery-stress questionnaire

There was no trial effect in any of the 4 main scales – Sport Stress, Sport Recovery, General Stress, General Recovery, with the responses within each scale being similar across all trials ($p > 0.05$ in all) (Table 2).

Physiological responses to exercise

Average HR did not alter across trials ($p > 0.05$) and was 161 ± 10 beats·min^{-1} (10MIN), 162 ± 10 beats·min^{-1} (5MIN), and 161 ± 12 beats·min^{-1} (1MIN). Moreover, RPE also remained unchanged ($p > 0.05$), with average values of 15 ± 1 in all three trials (Table 2).

*****Insert Figure 2 near here*****

Hormonal responses to CTL

Salivary cortisol & salivary testosterone

Salivary cortisol or testosterone did not alter during the CTL trial ($p > 0.05$)(Table 3).

Hormonal responses to exercise

Salivary cortisol

No trial effect was found ($p = 0.091$). A time effect was found ($p = 0.036$), with salivary cortisol increasing from Pre- to Post-Exercise in the 10MIN trial ($p = 0.022$), 5MIN trial ($p = 0.005$), but not in 1MIN trial ($p = 0.156$). Salivary cortisol returned to baseline levels in all trials at 30 min post-exercise (all $p > 0.05$). No interaction was observed ($p = 0.593$). Average absolute increases from Pre- to Peak Post-Exercise of 3.25 nmol·L^{-1} (~52%), 2.57 nmol·L^{-1} (~36%), and 2.77 nmol·L^{-1} (~38%) were found in 10MIN, 5MIN, and 1MIN trials, respectively (Figure 2).

Salivary testosterone

The Friedman's analysis highlighted an effect across the testosterone dataset ($p < 0.001$). Post hoc analysis highlights a significant increase from Pre- to Post-Exercise in the 10MIN trial ($p = 0.005$), the 5MIN trial ($p < 0.001$) and the 1MIN trial ($p = 0.035$). The salivary testosterone returned to baseline values at 30 min Post-Exercise in all trials (all $p > 0.05$). Average absolute increases from Pre- to Peak Post-Exercise of 203 pmoL·L^{-1} (~34%), 200 pmol·L^{-1} (~32%), and 146 pmol·L^{-1} (~24%) were shown in 10MIN, 5MIN, and 1MIN trials, respectively (Figure 2).

*****Insert Table 2 near here*****

*****Insert Table 3 near here*****

DISCUSSION

This study examined if consuming water 5 and 1 min before saliva sampling would dilute samples leading to erroneously lower salivary cortisol and testosterone concentration values when compared with the recommended 10 min. No trial effect was shown when comparing the saliva cortisol and testosterone concentrations across all three trials. However, salivary cortisol significantly elevated in response to the 55/80 in 10MIN and 5MIN trials only with an increase in salivary testosterone in all trials, with acute average absolute-changes of 3.25 $nmol \cdot L^{-1}$ (~52% - 10MIN) and 2.57 $nmol \cdot L^{-1}$ (~36% - 5MIN) in salivary cortisol, and 203 $pmol \cdot L^{-1}$ (~34% - 10MIN), 200 $pmol \cdot L^{-1}$ (~32% - 5MIN) and 146 $pmol \cdot L^{-1}$ (~24% - 1MIN) in salivary testosterone. In addition, this present study showed no differences in physiological stress measured by the RESTQ-76 questionnaire, hydration status, HR or RPE when comparing all trials.

Cortisol is known to be elevated during and after emotional and psychological stress (Burke et al., 2005). In this present study, analysis of the RESTQ-76 questionnaire scores indicate no disparities in any of the general or sport-specific stress and recovery scales, which emphasizes the participants were in a similar state of wellbeing and predisposition to undertake physical activity at the start of each trial. Therefore, this indicates that the observed salivary cortisol and testosterone responses have not been influenced by a change in psychological wellbeing in the participants across the trials completed. Additionally, controlling for food consumption (Gibson et al., 1999), alcohol (Badrick et al., 2008) and caffeine (Lovallo et al., 2006) intake,

and hydration status (Maresh et al., 2006) during experimental testing suggests that the observed hormonal responses have also not been influenced by these covariates.

It has been reported that a weight loss above 2% body mass leads to decreased aerobic capacity and mental performance (Cheuvront et al., 2003). The average body mass of the participants in this present study was 85.7 ± 9.4 kg, and each was provided with 650 mL of water to be consumed during each exercise test (30 min in total). This volume was chosen, as guidelines propose that individuals whose body mass is around 90 kg, should drink between 600-800 $mL \cdot h^{-1}$ when running at a relatively low intensity (8.5-10 $km \cdot h^{-1}$), in order to avoid losing >2% body mass (Sawka et al., 2007). Observing a loss of >2% body mass would be indicative of an average weight loss of approximately 1.7 kg. The average weight loss in the main exercise trials when corrected for fluid intake did not change across trials and was 0.6 ± 0.2 kg (10MIN), 0.7 ± 0.2 kg (5MIN), 0.5 ± 0.1 kg (1MIN). These results suggest that the water consumption plan followed in this study may have been appropriate, as it did not induce a weight loss above this threshold. In addition, the body mass loss during exercise has not led to a change in the hormones examined.

Current guidelines propose that fluid consumption should cease at least 10 min before providing a saliva sample (Salimetrics & SalivaBio, 2011). Yet, and despite the short restriction period proposed to avoid saliva dilution, it has been reported that fluid restriction during exercise leads to elevated circulating cortisol levels (Francesconi et al., 1985; Maresh et al., 2006). This present study intended to examine if consuming water within the proposed 10 min period, specifically 5 min and 1 min before providing a saliva sample would dilute saliva and

therefore provide erroneously lowered salivary cortisol and testosterone levels. No effect was observed when comparing the salivary cortisol and testosterone concentrations across all the three main exercise trials. These findings suggest that water consumption 1 minute before saliva sample collection will not alter the salivary cortisol and testosterone concentrations. Although the 1MIN trial did not see exercise induced increases in salivary cortisol, unlike the 10MIN and 5MIN trials. This may suggest a possible impact of consuming water 1 minute before sampling on the exercise induced salivary cortisol responses to a high-intensity exercise bout. Indeed, this could also be due to individual salivary cortisol responses to the exercise bout itself. Acute increases in salivary and plasma cortisol and testosterone has been reported in healthy male individuals by previous research using the 55/80 (Hough et al., 2013, 2015). Indeed, both plasma (Leal et al., 2019) and salivary (Leal et al., 2021) testosterone have been proposed to be more sensitive to exercise stress when compared to cortisol, with the latter eliciting more individualized and variable responses. (Leal et al., 2019; Leal et al., 2021). Therefore, we suggest that the current 10 min barrier for ceasing water consumption can be reduced to be 1 minute. It is still important to acknowledge that in our preliminary data suggest that if measuring exercise-induced cortisol responses, a 5 min fluid restriction period may be appropriate before saliva sampling.

The controls put in place on possible covariates in this study e.g. hydration status and collection time of day, for example, provide strength to the current study. However, it is appropriate to consider the limitations of our study also. The research question in this study is only addressed in a male population and therefore limits the application of the findings to a male cohort only. Therefore, future research should focus on a similar question in a female cohort. Also it is

important to consider the influence that variability in hydration status may have on these findings. The current study controlled for hydration status so we cannot confirm if altered states of hydration influence the findings. Finally, the relatively small sample size in this study may lead to lowered statistical power in some of the analysis.

To the best of our knowledge, no studies have previously examined the influence of water consumption within the recommended 10 min before sample collection on the salivary hormone responses. This study is the first to propose that reducing this period to 1 min will not dilute saliva to a point where hormonal concentrations (certainly salivary cortisol and testosterone) would be substantially and erroneously lower. This current work also suggests that if measuring exercise-induced cortisol responses a 5 min fluid restriction period before sample collection may be more appropriate. Further research to expand on this current work would be to examine the same research question in a female population and to consider different hydration protocols and their impact on salivary hormone concentrations.

Acknowledgements: The authors would like to acknowledge all participants for their time and consideration in participating in this study.

Authorship: Both authors were equally involved in the study design, data collection, data analysis and interpretation. DVL drafted the first version of the manuscript and JH contributed with important intellectual content during the manuscript drafting or revision. Both authors have read and approved the final version.

Conflict of interest: The authors report there are no competing interests to declare.

Funding sources: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Protocol: N/A.

References

Badrick, E., Bobak, M., Britton, A., Kirschbaum, C., Marmot, M., & Kumari, M. (2008). The relationship between alcohol consumption and cortisol secretion in an aging cohort. *Journal of Clinical Endocrinology and Metabolism*, *93*(3), 750–757. https://doi.org/10.1210/jc.2007-0737

Burke, H. M., Davis, M. C., Otte, C., & Mohr, D. C. (2005). Depression and cortisol responses to psychological stress: A meta-analysis. *Psychoneuroendocrinology*, *30*(9), 846–856. https://doi.org/10.1016/j.psyneuen.2005.02.010

Cheuvront, S. N., Carter, R., & Sawka, M. N. (2003). Fluid Balance and Endurance Exercise Performance. *Current Sports Medicine Reports*, *2*(4), 202–208. https://doi.org/10.1249/00149619-200308000-00006

Dabbs, J. M., Campbell, B. C., Gladue, B. A., Midgley, A. R., Navarro, M. A., Read, G. F. I., Susman, E. J., Swinkels, L. M. J. W., & Worthman, C. M. (1995). Reliability of salivary testosterone measurements: A multicenter evaluation. *Clinical Chemistry*, *41*(11), 1581–1584. https://doi.org/10.1093/clinchem/41.11.1581

Francesconi, R. P., Sawka, M. N., Pandolf, K. B., Hubbard, R. W., Young, a J., & Muza, S. (1985). Plasma hormonal responses at graded hypohydration levels during exercise-heat stress. *Journal of Applied Physiology (Bethesda, Md. : 1985)*, *59*(6), 1855–1860.

Gibson, E. L., Checkley, S., Papadopoulos, A., Poon, L., Daley, S., & Wardle, J. (1999). Increased salivary cortisol reliably induced by a protein-rich midday meal. *Psychosomatic Medicine*, *61*(2), 214–224. https://doi.org/10.1097/00006842-199903000-00014

Hayes, L. D., Sculthorpe, N., Cunniffe, B., & Grace, F. (2016). Salivary Testosterone and Cortisol Measurement in Sports Medicine: a Narrative Review and User's Guide for Researchers and Practitioners. *International Journal of Sports Medicine*, *37*(13), 1007–1018. https://doi.org/10.1055/s-0042-105649

Hough, J., Corney, R., Kouris, A., & Gleeson, M. (2013). Salivary cortisol and testosterone responses to high-intensity cycling before and after an 11-day intensified training period. *Journal of Sports Sciences*, *31*(14), 1614–1623. https://doi.org/10.1080/02640414.2013.792952

Hough, J., Leal, D. V., Scott, G., Taylor, L., Townsend, D., & Gleeson, M. (2021). Reliability of salivary cortisol and testosterone to a high-intensity cycling protocol to highlight overtraining. *Journal of Sports Sciences*. https://doi.org/10.1080/02640414.2021.1918362

Hough, J., Papacosta, E., Wraith, E., & Gleeson, M. (2011). Plasma and salivary steroid hormone responses of men to high-intensity cycling and resistance exercise. *Journal of Strength and Conditioning Research*, *25*(1), 23–31. https://doi.org/10.1519/JSC.0b013e3181fef8e7

Hough, J., Robertson, C., & Gleeson, M. (2015b). Blunting of exercise-induced salivary testosterone in elite-level triathletes with a 10-day training camp. *International Journal of Sports Physiology and Performance*, *10*(7), 935–938. https://doi.org/10.1123/ijspp.2014-0360

Jones, D.V., Work, C.E. (1961) Volume of a Swallow. American Journal of Diseases in Children, 102(3):427. doi:10.1001/archpedi.1961.02080010429023.

Kellmann, M., & Kallus, K. W. (2001). Recovery-stress questionnaire for athletes: User manual. In *Recovery-stress questionnaire for athletes: User manual*. Human Kinetics.

Kuipers, H., Verstappen, F. T. J., Keizer, H. A., Geurten, P., & van Kranenburg, G. (1985). Variability of aerobic performance in the laboratory and its physiologic correlates. *International Journal of Sports Medicine*, *6*(4), 197–201. https://doi.org/10.1055/s-2008-1025839

Leal, D. V., Standing, A. S. I., Furmanski, A. L., & Hough, J. (2021). Polymorphonuclear leucocyte phagocytic function, γδ T-lymphocytes and testosterone as separate stress-responsive markers of prolonged, high-intensity training programs. *Brain, Behavior, and Immunity - Health*. https://doi.org/10.1016/j.bbih.2021.100234

Leal, D. V., Taylor, L., & Hough, J. (2019). Reproducibility of Acute Steroid Hormone Responses in Men to Short-Duration Running. *International Journal of Sports Physiology and Performance*, *14*(10), 1430–1437. https://doi.org/10.1123/ijspp.2018-1004

Leal, D. V., Taylor, L., & Hough, J. (2021). Exercise-Induced Salivary Hormone Responses to High-Intensity, Self-Paced Running. *International Journal of Sports Physiology and Performance*. https://doi.org/10.1123/ijspp.2020-0541

Lovallo, W. R., Farag, N. H., Vincent, A. S., Thomas, T. L., & Wilson, M. F. (2006). Cortisol responses to mental stress, exercise, and meals following caffeine intake in men and women. *Pharmacology Biochemistry and Behavior*, *83*(3), 441–447. https://doi.org/10.1016/j.pbb.2006.03.005

Maresh, C. M., Whittlesey, M. J., Armstrong, L. E., Yamamoto, L. M., Judelson, D. A., Fish, K. E., Casa, D. J., Kavouras, S. A., & Castracane, V. D. (2006). Effect of hydration state

on testosterone and cortisol responses to training-intensity exercise in collegiate runners. *International Journal of Sports Medicine*, *27*(10), 765–770. https://doi.org/10.1055/s-2005-872932

Navazesh, M. (1993). Methods for Collecting Saliva. *Annals of the New York Academy of Sciences*, *694*(1), 72–77. https://doi.org/10.1111/j.1749-6632.1993.tb18343.x

Niu, S. F., Chung, M. H., Chu, H., Tsai, J. C., Lin, C. C., Liao, Y. M., Ou, K. L., O'Brien, A. P., & Chou, K. R. (2015). Differences in cortisol profiles and circadian adjustment time between nurses working night shifts and regular day shifts: A prospective longitudinal study. *International Journal of Nursing Studies*, *52*(7), 1193–1201. https://doi.org/10.1016/j.ijnurstu.2015.04.001

Papacosta, E., & Nassis, G. P. (2011). Saliva as a tool for monitoring steroid, peptide and immune markers in sport and exercise science. *Journal of Science and Medicine in Sport*, *14*(5), 424–434. https://doi.org/10.1016/j.jsams.2011.03.004

Salimetrics, L. L. C., & SalivaBio, L. L. C. (2011). Saliva collection and handling advice. *State College, PA: Salimetrics LLC, SalivaBio LLC*, 1–14.

Sawka, M. N., Burke, L. M., Eichner, E. R., Maughan, R. J., Montain, S. J., & Stachenfeld, N. S. (2007). American College of Sports Medicine position statement: Exercise and Fluid Replacement. *Medicine and Science in Sports and Exercise*, *39*(2), 377–390. https://doi.org/10.1249/mss.0b013e31802ca597

VanBruggen, M. D., Hackney, A. C., McMurray, R. G., & Ondrak, K. S. (2011). The relationship between serum and salivary cortisol levels in response to different intensities of exercise. *International Journal of Sports Physiology and Performance*, *6*(3), 396–407. https://doi.org/10.1123/ijspp.6.3.396

428

429

List of Tables

Table 1 Participants' anthropometric and physiological characteristics**,** Mean ± SD (min - max).

Variable	
Age (year)	22 ± 2 (19-25)
Body mass (kg)	84.1 ± 8.5 (71.4 – 101.0)
Height (cm)	180 ± 4 (175 - 186)
Maximum oxygen uptake ($\dot{V}O_{2max}$) (ml·kg^{-1}·min^{-1})	42 ± 7 (30 - 54)
Maximum heart rate (HR_{max}) (beats·min^{-1})	187 ± 10 (164 - 197)
Maximal work rate ($\dot{W}_{max}$) (W)	272 ± 39 (204 - 339)

N = 14

Table 2 Average raw data for urine osmolality, total weight loss and the physiological and RESTQ responses in 10MIN, 5MIN, 1MIN and CTL bouts.

Average Raw Data			
Urine Osmolality (mOsm·kg^{-1} H_2O)			
10MIN	5MIN	1MIN	CTL
403 ± 202 (80 – 610)	321 ± 195 (40 – 600)	283 ± 194 (80 – 530)	473 ± 152 (360 – 600)
Total weight loss (kg)			
10MIN	5MIN	1MIN	CTL
0.6 ± 0.2 (0.4 – 0.9)	0.7 ± 0.2 (0.4 – 1.2)	0.5 ± 0.1 (0.4 – 0.8)	0.2 ± 0.2 (0.1 – 0.6)
Heart rate (beats·min^{-1})			
10MIN	5MIN	1MIN	CTL
161 ± 10 (140 - 171)	162 ± 10 (143 - 178)	161 ± 12 (136 - 184)	N/A
RPE (6-20)			
10MIN	5MIN	1MIN	CTL
15 ± 1 (13-16)	15 ± 1 (12-17)	15 ± 1 (12-17)	N/A
RESTQ			
General Stress			
10MIN	5MIN	1MIN	CTL
6 ± 2 (3-8)	6 ± 2 (4-8)	6 ± 2 (4-8)	7 ± 2 (4-9)
General Recovery			
10MIN	5MIN	1MIN	CTL
15 ± 2 (12-19)	15 ± 2 (12-20)	15 ± 2 (12-20)	14 ± 2 (12-16)
Sport Stress			
10MIN	5MIN	1MIN	CTL
6 ± 5 (3-8)	6 ± 3 (3-8)	7 ± 3 (3-12)	7 ± 3 (3-11)

Sport Recovery			
10MIN	5MIN	1MIN	CTL
15 ± 2 (13-20)	15 ± 1 (13-17)	15 ± 1 (13-16)	14 ± 2 (10–16)

Values are mean ± SD (min – max)

436

Table 3 Average raw data the salivary cortisol and testosterone responses in 10MIN, 5MIN, 1MIN and CTL trials.

Salivary Cortisol (nmol·L^{-1})			
	Pre-Exercise	**Post-Exercise**	**30 Min Post-Exercise**
10MIN	6.2 ± 3.0 (2.2 – 12.8)	8.7 ± 6.2* (3.7 – 25.8)	7.7 ± 6.3 (1.9 – 22.1)
5MIN	7.1 ± 3.3 (2.3 – 12.8)	8.9 ± 5.2* (3.4 – 23.2)	7.4 ± 3.7 (3.9 – 15.6)
1MIN	7.2 ± 3.3 (3.4 – 13.5)	8.9 ± 5.4 (2.4 – 21.1)	9.1 ± 6.6 (3.7 – 24.1)
CTL	7.5 ± 4.5 (3.4 – 16.1)	5.8 ± 2.2 (3.1 – 9.1)	4.3 ± 1.4 (2.4 – 6.6)

Salivary Testosterone (pmol·L^{-1})			
	Pre-Exercise	**Post-Exercise**	**30 Min Post-Exercise**
10MIN	590 ± 176 (290 – 907)	760 ± 280† (339 – 1205)	624 ± 238 (360 – 1334)
5MIN	615 ± 232 (363 – 1126)	814 ± 270† (484 – 1424)	648 ± 251 (406 – 1242)
1MIN	610 ± 154 (373 – 909)	724 ± 293* (476 – 1352)	672 ± 252 (363 – 1173)
CTL	622 ± 154 (383 – 823)	606 ± 163 (383 – 869)	581 ± 185 (379 – 859)

437

438 Values are mean ± SD (min – max)

439 †Different than Pre-Exercise ($p < 0.01$). *Different than Pre-Exercise ($p < 0.05$)

440

441

List of Figures

Figure 1 Schematic presentation of the trial day protocol, with 10MIN, 5MIN and 1MIN representing each experimental trial where water was consumed either 10 min, 5 min and 1 min before providing a saliva sample, respectively. The exercise protocol is referred to as the 55/80 and consists of 12 intercalated stages of 1 min and 4 min; $\dot{W}_{max}$ – maximum power output.

Figure 2 Salivary hormone responses to the 55/80 protocols at Pre-exercise and Peak Post-exercise: a) Salivary cortisol; b) Salivary testosterone.

**Different than Pre-exercise values at 10MIN ($p < 0.05$). ‡Different than Pre-exercise values at 5MIN ($p < 0.01$). #Different than CTL in all trials ($p < 0.05$).