Salivary testosterone and cortisol measurement in sports medicine: A ~~methodological~~ narrative review and user's guide for researchers and practitioners.

Short title: Guidelines for measurement of salivary steroids in exercise studies.

ABSTRACT

Since the initial reporting of salivary hormone measurements in marathon runners in the early 1980s, the practice of utilizing salivary testosterone (T) and cortisol (C) to reflect acute and rhythmic changes to their systemic counterparts has gained considerable momentum. However, substantial variability exists between studies with respect to methodological protocols, laboratory techniques, and interpretation of study findings. These differences can directly influence the salivary hormone values, thus hampering interpretation, limiting cross-study comparison, and constraining the generalizability of individual study findings. This article examines the current body of saliva derived hormone literature before proposing a sequence of practical guidelines to minimize sample variability in salivary hormone research. The guidelines are grouped into three major categories which ultimately coalesce to limit comparison between studies; A) study design, B) sample acquisition and biological variation, and C) technical and analytical error. To achieve this, the present article critically appraises research employing salivary T and C measurements, identifies potential sources of error before proposing appropriate methodological considerations for researchers and practitioners wishing to obtain T and C measurement from saliva.

KEY WORDS: Androgens · Exercise · Sex hormone · Sport · Steroid

GLOSSARY OF TERMS

C: Cortisol (general term whereby the binding status of the hormone is undefined).

T: Testosterone (general term whereby the binding status of the hormone is undefined).

Sal-C: Salivary cortisol.

Sal-T: Salivary testosterone.

Bio-C: Bioavailable cortisol in serum. i.e. cortisol bound loosely to albumin or unbound.

Bio-T: Bioavailable testosterone in serum. i.e. testosterone bound loosely to albumin or unbound.

Free-C: Free cortisol in serum. i.e. unbound cortisol.

Free-T: Free testosterone in serum. i.e. unbound testosterone.

TC: Total cortisol. i.e. serum cortisol concentration which encompasses serum bioavailable cortisol, serum free cortisol, and serum cortisol which is bound to corticosteroid binding globulin.

TT: Total testosterone. i.e. serum testosterone concentration which encompasses serum bioavailable testosterone, serum free testosterone, and serum testosterone which is bound to sex hormone binding globulin.

INTRODUCTION

Salivary testosterone (sal-T) and cortisol (sal-C) measurements are widely used in clinical, psychobiological, and exercise sciences as a surrogate for blood sampling. Sal-C was initially detected in the early 1960's [14,62] whilst sal-T was first reported in 1976 by Landman and colleagues [93]. In clinical settings salivary measurements are rarely used, except for the most extreme cases, such as use of sal-C for initial screening of Cushing's syndrome [114] whilst sal-T is not used at all, having recently been ruled out of diagnosing hypogonadism [53,78]. In contrast, sports medicine and psychobiology research have widely adopted both sal-C and sal-T [11,29,35,71-73] promoting salivary measurement from novel surrogate to pervasive biomarker largely due to the widely held assumption that they mirror their respective systemic (blood) concentrations. This has resulted in the exponential growth in popularity of sal-T and sal-C in these fields, depicted by Fig 1., which represents search results using salivary search terms in PubMed, performed in all fields. Whilst these data depict all returned results, this still underestimates the total number of studies due to authors frequently omitting the term 'salivary' and describing the measure as simply 'free testosterone', or merely 'testosterone', or 'cortisol' in their manuscript title.

Despite increased prevalence in applied research, there are multiple methodological considerations that can hinder sound interpretation of resulting sal-T and sal-C data [74,75]. Recently, the smallest observable change required before alternations in sal-T and sal-C that can be considered biologically significant was determined (termed the critical difference [79]) as 90% and 148% respectively, when determined by enzyme-linked immunosorbent assay (ELISA). Moreover, Valero-Politi and Fuentes-Arderiu [145] reported the critical difference of sal-T measured at monthly intervals to be 78%, using radioimmunoassay (RIA).

In both cases, investigations were conducted in controlled laboratory environments, while conversely, investigations conducted in naturalistic settings undoubtedly exhibit a higher critical difference and are therefore at greater risk of classic type I error. In addition, a recent meta-analysis described a profound effect of study design and sampling regimen on sal-T and sal-C reactivity in response to exercise [69]. Further to the observed heterogeneity in research design, limitations with salivary hormone interpretation in psychobiology [74], behavior [75], and endocrinology [60] have been previously described.

Given these concerns regarding salivary measurement, there has been a recent call for standardized method for measurement of sal-T and sal-C and the use of standardized control conditions, to facilitate progress in hormone related research [69]. Furthermore, whilst guidelines for detection of T and C in serum are well described [103,124,125,150], there are currently a lack of guidelines for detection of T and C in saliva. As such, we propose that, in the spirit of good scientific practice, it is necessary to outline a series of procedures for researchers and practitioners to adhere to when conducting salivary hormone research. These guidelines can be grouped into three major categories; A) Study design, B) Sample acquisition and biological variation, and C) Technical and analytical error. Each of these aspects (A, B and C) coalesce to limit comparison between studies. Therefore, the aim of this article is to provide a critical appraisal of research using salivary measurements within the field of exercise science and sports medicine, identify possible sources of error and propose appropriate methodological procedures for researchers wishing to obtain T and C measurement from saliva samples. Due to the significant heterogeneity of study design in studies that examine behavioral or psychological stimuli, this article is restricted to studies involving physiological outcome measures. However, the proposed guidelines can be applied across all studies using single or serial salivary hormone measurements.

*******INSERT FIGURE 1:A AND 1:B NEAR HERE*******

Fig 1. PubMed search results from 1st January 1976 to 9th November 2015. Search terms were performed within all fields. Whilst these data depict all returned results, this underestimates the number of studies performed due to authors describing the measure as 'free testosterone', or 'testosterone', or 'cortisol' in their article title.

A: STUDY DESIGN

A recent meta-analysis concerning exercise-induced sal-T and sal-C reactivity to acute exercise identified that effect sizes were dependent upon study design [69]. For example, pooled analysis of power exercise interventions that collected their baseline sample on a control day exhibited a negative (-1.128) standard difference in means (SDM), whereas investigations that collected their baseline sample immediately prior to exercise exhibited a positive SDM (0.486) [69], meaning that study design is a confounding factor in salivary hormone determination. Here we have discussed causes of some of the limitations to appropriate design and interpretation of investigations, and how these may be improved in an applied research environment.

Study Title

Whilst the majority of studies correctly report the use of *salivary* hormone methodology within titles, this is not always the case [29,30,92]. It has been suggested that sal-T and sal-C ostensibly measure the free fraction of the hormone [117] which is considered to diffuse passively across the salivary glands [122]. Khan-Dawood [86] reported free testosterone (free-T) to account for 78% of sal-T, compared with approximately 4% of TT in

adult plasma, although the variability around this value is yet to be determined. Furthermore, 'free testosterone' is traditionally interpreted as the unbound component of serum concentrations. However, despite these issues a number of authors describe sal-T as 'free testosterone' [36,38] within study titles. Such descriptions are both confusing and premature, at least until studies using gold-standard ultrafiltration measures of serum T confirm the T:sal-T ratio *and* the reliability of sal-T to reflect serum derived free-T [53]. Therefore, in the interest of good practice, we believe it necessary to state 'salivary' in the title of research articles for clarity and indexing purposes, and throughout manuscripts for transparency.

Study Design

A recent meta-analysis of randomized control trials (RCTs) and uncontrolled trials (UCTs) resulted in different magnitudes of sal-T and sal-C response to exercise [69]. It was suggested that the anticipatory rise (or fall) prior to exercise was the underlying mechanism for this outcome. To further elucidate this point, two investigations that collected baseline samples on a rest day observed a relative decrease in sal-T following a power-based exercise intervention [48,105], whereas several other studies that collected a sample immediately prior to exercise observed an increase in sal-T [141,152]. Without comparison to a control condition, it is difficult to discern the true effect of an intervention on sal-T and sal-C. Therefore, we recommend utilizing an RCT design where possible. Furthermore, if RCT designs are not feasible, and where participant data is to be compared with their own baseline data, the timing of salivary sampling becomes of paramount importance.

Timing of Pre- and Post-Intervention Samples

Since the first account of salivary hormone collection around the time of exercise [32], an anticipatory effect of the forthcoming activity has been observed. Cook and colleagues [32] reported increased sal-C and sal-T prior to a marathon run when compared to rest days. Therefore, we suggest that baseline samples are collected on a control day (without the anticipatory effect of intervention), as well as immediately pre-intervention, to elucidate the true effect of; A) the anticipatory effect on sal-T and sal-C compared to a control day, B) the intervention effect on sal-T and sal-C compared to pre-intervention, and C) the combined effect of anticipation and intervention on sal-T and sal-C. With regards to post-intervention sample timing, Crewther et al. [34] observed a negative sal-T response immediately after completion of an exercise intervention, which when reassessed 15 min later, resulted in a positive change from baseline. We therefore propose post-intervention samples are taken between 15-30 min post-intervention as sal-T has been shown to peak 15 min post- exercise intervention and return to baseline values 30 min post-exercise [153].

Data Reporting

There are a number of issues in data reporting which, if standardized, should improve the field of salivary hormone research. For example, reporting raw mean data in combination with either standard deviation (SD), standard error of mean (SEM) or confidence intervals (CI) rather than percentage change from baseline would add value and clarity to the data. Although reporting delta changes may be useful to contextualize findings, it may underestimate the absolute change in individuals with high baseline values. For example, an individual whose sal-T rises from 300 to 400 pmol·L^{-1} following intervention experiences an absolute increase of 100 pmol·L^{-1} and relative change of 33% yet an individual whose sal-T rises from 200 to 280 pmol·L^{-1} following intervention experiences an absolute increase of 80

pmol·L^{-1} but a relative change of 40%. We therefore suggest reporting mean ± SD/SE as a standard *minimum*. Relative changes may be reported, to further contextualise findings in relation to a minimum meaningful change from baseline [91]. Regarding the minimum meaningful change, it may be useful to refer to the critical difference of sa-T and sal-C to determine whether changes observed are biologically meaningful [79,145]. This can be conducted for interventions within [79], or between days [145]. Furthermore, authors should adhere to the Système International d'Unités (SI Units) when reporting concentrations of sal-T and sal-C for continuity. Currently, a mix between pmol·L^{-1} and pg·ml^{-1} is used for sal-T whilst a mix between nmol·L^{-1} and ug·dl^{-1} is used for sal-C.

Strength of Evidence and Recommendations

There is considerable evidence that identifies study design and timing of pre- and post-intervention sample to adversely influence study outcomes from sal-T and sal-C research. Furthermore, discrepancies in data reporting and terminology compound inconsistencies within the literature.

B: SAMPLE ACQUISITION AND BIOLOGICAL VARIATION

Variability of sal-T and sal-C have been previously reported [70,79,137,145]. Recent evidence has described wide variation in the measurement of sal-T and sal-C within a highly controlled laboratory environment [79]. Therefore, in research outside of a laboratory, variability of sal-T and sal-C will likely be greater. In this section we discuss causes of some of the major sources of biological variation and how these may be controlled in an applied research environment.

Chronobiological Variation

Circadian rhythmicity has been established in serum C [129] and T [96,156], as well as in sal-C [111,112] and sal-T [70,90,139]. As a result of these diurnal variations, it is imperative to employ similar sampling times during repeated measures research design. Furthermore, in prospective cohort studies where a baseline measure will be used as control data (i.e. without a control group), it may be difficult to determine true sal-T and sal-C response to exercise if the exercise intervention is protracted. For example, if examining sal-T and sal-C response to a marathon run, recreational athletes would take >3 hrs to complete the course. If using a baseline-as-control design, it would prove difficult to determine whether hormone alterations observed were a result of running, or simply a product of chronological advancement. Therefore, we suggest the use of dual baseline sampling for protracted interventions. i.e. Samples should be collected pre-intervention, and time-matched post-intervention on a control day to eliminate the effect of diurnal variation.

Chronological Age

Longitudinal analysis has revealed that for each decade from the age of 30 years there is a downward trend of TT [67]. This precipitous decline, coupled with increasing SHBG with age, further reduces bioavailable testosterone (bio-T) [7,13,17], thought to constitute the majority of sal-T. Morley and colleagues [109] reported sal-T in 1454 males, with mean basal sal-T ranging from ~100 $pg \cdot ml^{-1}$ (347 $pmol \cdot L^{-1}$) to ~50 $pg \cdot ml^{-1}$ (173 $pmol \cdot L^{-1}$) for ages 30-39 and 80-89 respectively. Recently, we have observed that sal-T demonstrated poor agreement with TT and calculated bio-T and free-T in older men [76,77]. Additionally, older males are

more likely to exhibit hypogonadism [67], yet sal-T is incapable of diagnosing hypogonadism [53,78]. Although older males have lower resting sal-T, they may have the potential to increase sal-T to a greater *relative* extent following exercise [22] further supporting the need to report absolute as well as relative changes suggested earlier. However, more data are required to confirm this phenomenon. Whilst TT decreases with age, a coincident age-related increase in TC [157] and sal-C [21] occur which would therefore lower the T:C ratio, more so in saliva, where SHBG further compromises this relationship. However, this is yet unconfirmed. It is therefore suggested that between group designs are closely age-matched. Moreover, a narrow spread of ages is suggested to prevent large standard deviation in absolute and delta change values.

Sex

Until the onset of puberty, males and females exhibit little difference in their resting hormonal profiles. Once puberty is reached, females demonstrate the characteristic pulsatile release of gonatrotrophin and sex steroid hormones throughout menarch and males demonstrate increased androgen steroid hormone production [149]. Differences that manifest at puberty tend to persist through adulthood until women become postmenopausal [63]. Moreover, gender specific syndromes may further increase biological variation. For example, incidence of hypogonadism (clinically low T [6,109]) would increase biological variation within a participant sample, with incidence possibly elevated in athletes [42,43,132]. Polycystic ovary syndrome (PCOS) increases TT [83], and sal-T [138] in females and would therefore confound direct comparisons between symptomatic and asymptomatic females. It is also well reported that differences between men and women exist for both sal-T and sal-C [86,121,151]. We therefore suggest that mixed gender experiments should be avoided where

possible as large standard deviation may increase type II error risk. Gender specific syndromes which may influence androgen status (hypogonadism, PCOS, etc.) should, where possible, be screened for in order to prevent confounding results further.

Long Haul Travel

Long haul travel across multiple time zones causes a depression in sal-C the day following travel [18]. Whilst there is no information from sal-T data, data from serum indicate that after a simulated long-haul flight, TT circadian rhythm was not altered [33]. However, simulation of hypoxia and hypobaria was 12,000 ft, whereas commercial airplanes fly at over 30,000 ft. In addition, as a result of circadian disruption due to crossing multiple time zones, long haul travel may influence serum TC and TT release. Therefore, recent long haul travel should be used as exclusion criteria where possible when obtaining sal-T and sal-C measurements.

Dietary Intake

There are a wide variety of dietary factors that have been shown to influence sal-T and sal-C. Consuming an evening meal causes an increase in sal-T compared to fasting [143], whilst three weeks of daytime fasting, reduces sal-C [146]. High alcohol consumption [110], low zinc intake [2], and a low carbohydrate diet [4] all decrease TT. Volek and colleagues [148] have demonstrated that the relative contribution to energy intake from protein, fat, saturated fatty acids, monounsaturated fatty acids, the polyunsaturated fat-to-saturated fat ratio, and the protein-to-carbohydrate ratio influenced TT but not TC concentrations, using a

17-day dietary recall. Heikkonen and coworkers [80] reported that alcohol consumption depressed TT, but increased TC concentrations.

Controlling for diet may further reduce biological error in sal-T and sal-C research. This can be achieved by providing standardised meals. Dietary recall may be more cost-effective, if the known problems with dietary recall in children [107] and adults [54] can be reconciled.

Postural Changes

Changes in plasma volume with movement between supine and standing positions are now well established though sometimes overlooked. Movement from an initial standing position to a supine position has demonstrated significant haemodilution [64,140] although a method for plasma volume correction has been outlined by Dill and Costill [44]. Failing to standardize body position or failing to correct for any changes in plasma volume increases error and has important interpretative implications [52]. In cases where plasma hormones are measured, correcting for plasma volume changes becomes an important consideration. Sal-C has been shown to increase following 20 min of standing, compared to sitting and lying [81]. Hucklebridge et al. [84] proposed the orthostatic challenge, adjusting from sitting to standing, stimulated the hypothalamic-pituitary-adrenal (HPA) axis as a result of hypotension, increasing sal-C. However, these same authors [84] observed no alteration in the sal-C awakening response when participants remained supine for 45 min or standing immediately upon wakening. Whilst no articles exist to our knowledge, concerning postural change and sal-T, hydration status [100], and salivary flow rate [6] did not influence sal-T concentrations. However, this area requires further confirmatory data as few studies exist addressing postural change and salivary hormone concentrations, particularly sal-T. As there are few data to

confirm the influence postural change has on salivary hormones, we propose that repeated measures designs utilize the same posture for sampling at all sampling points, and we advise samples are collected seated or supine.

Acute and Habitual Physical Activity

Exercise has been shown to increase levels of sal-T and sal-C acutely and chronically [19,31,38,120,147], however there is some controversy surrounding chronic elevations in TT [99]; as some reports suggest depressed TT levels as a result of chronic endurance training [8]. The adaptation of the HPA axis to training is mediated by decreased sensitivity to TC [45,95] and altered tissue sensitivity to glucocorticoids [46,47]. However, during a rest day, endurance athletes' serum TC concentrations have been reported as normal [47]. As the T:C ratio has been implicated as a marker of overtraining and training status [3,102], prior physical activity may influence findings of sal-T and sal-C investigations when examining individuals with a high training volume. It is common practice to exclude physical activity for 24 hr prior to study commencement, which is preferential. However, in an applied setting, athletes may not have 24 hr without training. In these instances, duration, and programme variables of last training session should be matched for repeated-measures designs.

Smoking

Basally, smokers demonstrate altered sal-T and sal-C compared to controls [9,106,155]. Furthermore, English and colleagues [49] reported elevated levels of total, free, and bioavailable T in smokers compared with non-smokers. Cigarette smoking is further associated with acutely elevated TC levels. However, the results of TC comparison in

smokers and non-smokers have been inconsistent, and the significance of TC responses in smoking cessation is unclear [136,144]. Therefore, for precaution, we advise smoking status to be used as exclusion criteria when not considered the independent variable.

Sexual Activity

Mohammed [41] reported that, in both males and females, sal-T increased during an evening when there was intercourse and decreased when there was not. Moreover, sal-T has been shown to increase during a visit to a sex club in men [50], but no increase in sal-T or sal-C was observed following sexual thoughts [58]. Sexual activity influences TT concentrations in males and females [119]. Dabbs and Hamilton [65] demonstrated TC response to sexual arousal was highly individualized in females and Exton et al. [51] reported no significant influence of sexual arousal of TC levels in males. We are unaware of any studies to date that report the influence of sexual activity on sal-C or sal-T response to intervention. As such, abstinence should be requested 24 hr prior to salivary collection, similar to alcohol, and physical activity.

Illness

Shattuck and colleagues [130] reported reduced sal-T in response to immune activation. Critical illness is often accompanied by hypercortisolemia, which has been attributed to stress-induced activation of the HPA axis, observed in both sera and saliva [115]. However, low corticotropin levels have been reported in critically ill patients, which may be due to reduced TC metabolism [15]. In men, serum TT decreases during sepsis, burns, myocardial infarction, and surgery [133-135]. Spratt and colleagues [134] reported

patients admitted to critical care units displayed decreased TT that varied according to severity of illness. We are unaware of any study to date that evaluates the influence of low-grade illness (i.e. cold or flu) on sal-C or sal-T. This is likely due to difficulty recruiting and opportunity of sampling yet this may be an area for further exploration. Illness should be controlled for during sal-T and sal-C research to the best of researchers' abilities (i.e. pre-participation questionnaire or verbal screening).

Psychological Influence

Cortisol is the primary stress hormone and therefore is elevated in physiologically and psychologically stressful situations [126]. An increasing number of psychophysiology studies use sal-T and sal-C to reflect differences or changes in mood state [20,113,158], though there are a number of contradictory overlaps and methodological issues [74,75]. Bernhardt and colleagues [12] investigated the sal-T response of football fans watching their teams win or lose. The study found that elation the winning team's fans felt was accompanied by a rise in post-game sal-T levels compared to pre-game. The despondency felt by the losing team's fans was accompanied by a decrease in sal-T. Increased sal-T in response to, or anticipation of, competition and sports performance has previously been observed [127]. This phenomenon has also been observed in the non-physical competition of chess [16,68], reporting increased pre-competition sal-T in eventual winners. Territorial aggression of home teams is also well documented [5] linked to agonistic animalistic behavior defending a home territory [113].

Chronic stress can decrease TT [1,104]. It has been suggested that the suppression of steroidogenesis in the testes is due to reduced synthesis of testicular androgens, caused by the inhibitory effect of high adrenocorticotropic hormone (ACTH) levels that accompany chronic

stress [24]. Conversely, acute stress can increase TT [123], which may be a consequence of increased sensitivity to LH [24] which is supported by documented sympathetic stimulation by catecholamine release in males and females [26,27]. The use of saliva sampling as opposed to venous blood draws may reduce stress in some instances. However, familiarization with passive drool is still advised to avoid apprehension. Fingerprick sampling and capillary tubes may be a viable alternative due to the relatively small volume of serum required for analysis and therefore less apprehension than during venous blood collection. Capillary TC accurately reflects samples from venous blood [55], yet further investigation is required as to whether this is a viable technique for testosterone measurement.

An often cited relationship exists between T and aggression, which is further demonstrated by the reported increased aggression or 'roid-rage' following consumption of supraphysiological dose T in anabolic androgenic steroid users (AAS). However, in individuals within normal physiological ranges of TT, only a weak positive relationship between TT and aggression exists in humans [5]. We propose that aggressive or stressful stimuli should be used as inclusion/exclusion criteria, preceding saliva collection. Where this may not be possible, practitioners may wish to control for stress by administering a stress questionnaire. E.g. perceived stress questionnaire (PSQ) [89].

Ethnographical Differences

Martin et al. [101] described racial differences in diurnal sal-C rhythms whereby African Americans exhibited dampened morning-to-evening sal-C slopes than Caucasions. Christiansen [25] observed that sal-T from men in !Kung San was lower in comparison to published normal mean values. Panizzon et al. [116] described significant heritability in sal-T measures (~.42 and ~.47 for at-home and in-lab values respectively). Serum TT levels are

higher in adult male Bangladeshi migrants to the United Kingdom compared to residents of Bangladesh [107], and in native Anymara men in urban versus rural Bolivia [10], as well as Chinese men living in Pennsylvania versus Beijing [128]. However, this may be as a consequence of dietary intake. Winter and colleagues [154] have reported higher TT concentrations in African-American men compared to Caucasian men, whereas Litman and coworkers [98] observed no such difference. Therefore, in between-group investigations, it may be useful to control for ethnic differences or at least acknowledge the influence ethnicity or geographical location may exert.

Strength of Evidence and Recommendations

In summary, a number of our recommendations are based on serum data, which we have extrapolated to the determination of salivary hormones. This in itself is problematic due to some authors reporting weak, or no relationships between salivary and serum values [61,76,78,87,131]. There is however, evidence that chronological variation, chronological age, sex, dietary intake, acute and habitual physical activity, smoking, illness, mood state and psychology, and geographical differences influence sal-T and sal-C. Conversely, whilst there is evidence of long haul travel influencing sal-C, currently there is no investigation to our knowledge concerning the influence of travel on sal-T. Whilst TT was reportedly unaltered following hypoxic hypobaria, conditions investigated did not closely match those of long haul flights and thus we cannot declare for certain whether sal-T is influenced by long haul travel. Sal-C has been shown to vary following postural change. However, there are no data addressing postural changes and sal-T. Extrapolating from serum data, Hoffman and colleagues [82] observed no change in TT following reduced plasma volume and therefore

sal-T may remain unchanged. However, this is purely speculation until further confirmatory data.

C: TECHNICAL AND ANALYTICAL ERROR

As previously described [79], the critical difference includes both biological error, and technical and analytical error. As with the biological error discussed above, steps should be taken to minimize the technical and analytical error when assessing samples. Lazarou and colleagues [94] reported that in a study examining reference values for hypogonadism in 25 laboratories, there were 17 different threshold values for TT. Indeed, the threshold for hypogonadism diagnosis varied by 350% (130 $ng \cdot dl^{-1}$ to 450 $ng \cdot dl^{-1}$). Whilst there is some discrepancy concerning clinical thresholds in serum testosterone, the innate variability of sal-T means it is unsuitable for clinical diagnosis [53,78]. To exemplify this point, Jensen et al. [85] compared analysis of sal-T and sal-C samples amongst four and three laboratories respectively. These authors reported recovery of spiked material for testosterone and cortisol was 80-94% and 83-100% respectively. Moreover, substantial differences existed between laboratories, as a result of traceability, clean-up procedures, or issues with calibration.

Crewther and colleagues [37] reported power trained males had mean basal sal-T concentrations of 107 $pg \cdot ml^{-1}$ (371 $pmol \cdot L^{-1}$) whereas Ghigiarelli et al. [57] reported basal levels of as high as 180 $pg \cdot ml^{-1}$ (624 $pmol \cdot L^{-1}$). In a multicentre study [40], mean sal-T concentrations from the same 100 males ranged from 240 ± 95 to 410 ± 191 $pmol \cdot L^{-1}$ (69-118 $pg \cdot ml^{-1}$) suggesting considerable heterogeneity between laboratories. Fiers et al. [53] recently provided evidence for T binding to salivary proteins, therefore limiting the agreement with free-T in serum, determined by equilibrium dialysis. These taken together, suggest large variability in sal-T, so precautions are needed in order to reduce sampling error.

In this section, we detail some of the major analytical variation and provide advice for researchers using sal-T and sal-C.

Collection Method

Flavored beverage crystals and lemon juice have been used to stimulate flow of saliva. Flavored crystals may cause an increase in measured sal-C concentrations whereas lemon juice may compromise sal-C determination as a result of decreased pH [59]. With regards to sample acquisition, Granger et al. [61] observed chewing sugar free dental gum resulted in increased sal-T levels after 1 min (mean 168 $pmol \cdot L^{-1}$, SEM = 58 $pmol \cdot L^{-1}$ compared to 138 $pmol \cdot L^{-1}$, SEM = 53 $pmol \cdot L^{-1}$) but thereafter no significant differences were observed. Granger et al. [61] investigated the effects of different sample collection techniques on the measurement of sal-T. In this study, sal-T collected using cotton dental roll, cotton swab and sugar free gum were compared to un-stimulated saliva collection. Compared to un-stimulated saliva (mean=10.1 $pg \cdot ml^{-1}$ [34.9 $pmol \cdot L^{-1}$]), sal-T levels were twofold higher (mean=20.7 $pg \cdot ml^{-1}$ [71.4 $pmol \cdot L^{-1}$]) using dental cotton roll, and almost threefold higher (28.3 $pg \cdot ml^{-1}$ [98.1 $pmol \cdot L^{-1}$]) after using a cotton swab. Moreover, Shirtcliff and coworkers [131] reported compromised assay results when using cotton materials to absorb saliva. It was shown that the cotton interference effect was of sufficient magnitude to attenuate the association between serum and saliva levels. Therefore, unstimulated, mid-flow saliva is recommended for analysis. It may also be advised to wash out the mouth with distilled water approximately ten minutes before sample collection to clear the oral cavity of debris.

Blood Contamination

Hormones in saliva are present at far lower concentrations than in circulation. As a result, blood leakage into the oral mucosa can compromise the validity of salivary hormone analysis [61,88]. Blood contamination may be caused by teeth brushing, drinking hot fluid, or the use of swabs for sample collection [96]. Blood can leak into saliva as a result of micro-injuries such as burns, cuts or abrasions or gum disease/poor oral hygiene. In sport, both the abrasion to the gums from the use of gum shields and facial injuries can lead to the presence of blood in saliva. Kivlighan et al. [88] investigated the effect of blood leakage on sal-T and sal-C concentrations. Saliva samples were taken before, immediately after, and every 15 min for 1 hr following vigorous tooth brushing and the same protocol without tooth brushing was performed by a control group. There was a significant increase in sal-T at 15, 30 and 45 min post micro-injury compared to control. Paradoxically, there was no difference in sal-C levels between the micro-injury and the control condition. This suggests that sal-C is unaffected by blood contamination in saliva although given the appreciable concentration gradient between serum and saliva C, this requires further investigation. Whilst few studies report the acceptable transferrin level in saliva, this could be used as exclusion criteria [39,88]. We propose that investigators ensure avoidance of hot food and drinks, and teeth brushing prior to sample collection. Furthermore, we suggest that following gum shield use, samples are visually inspected for blood contamination and then leakage confirmed by transferring analysis.

Storage

Steroid hormones such as C have generally been considered to be stable in saliva even when stored at room temperature for a number of days [23,56]. However, more recent studies have raised concern with respect to sample treatment and storage prior to analysis

[61,117,142]. Concentrations of sal-C were found to decrease by 9.2% per month in samples stored at room temperature [56] compared to baseline. Toone et al. [142] observed that following seven days of storage at 4 ºC, sal-T decreased by 26 ± 15% whereas sal-C remained unchained compared to baseline.

With regard to storing saliva samples, two freezing options are generally used. Saliva can be stored for a year in a domestic freezer (−20 ºC), and possibly several years in a laboratory-based freezer (−80 ºC [66]). After collection, saliva should be frozen as soon as possible [117] to precipitate mucins; however if unavailable, saliva can be stored at room temperature for up to 6 hr [66]. Storing saliva at >−5 ºC will not freeze the samples and is not generally recommended due to bacterial growth which may degrade salivary components and interfere with antibody binding [61]. Granger et al. [61] analyzed saliva samples stored at 4 ºC which were assayed for sal-T on a weekly basis for 4 consecutive weeks. Contradictory to the data of Toone et al. [142], measured sal-T levels *increased* by 20.6% after one week and 330.8% after four weeks compared to baseline. Therefore, we recommend studies freeze salivary samples as quickly as possible in a freezer as cold as possible. −80 ºC is preferred but −20 ºC is suitable as long as samples are analyzed within 12 months.

Analysis

Numerous techniques have been used to measure sal-C and sal-T concentrations, including enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and liquid chromatography–tandem mass spectrometry (LC–MS/MS). Analytical sensitivity differs between these methods, as discussed elsewhere [53,124]. Despite the ease and speed of analysis compared to LC-MS/MS, results from ELISA and RIA are generally considered less accurate, possibly due to the lack of consistent reference standards. More recently,

handheld detection devices have come to market offering salivary hormone determination [28]. However, whilst there are conference proceedings suggesting acceptable reliability and validity of these devices, no full text articles exist to date confirming suitability of sal-T and sal-C values using this method.

Variability of serum thresholds varies between laboratories [94], and possibly as a result, reference ranges for sal-T have not been agreed to date. As such, sal-T and sal-C assays are limited to interpretation by identifying 'outliers' within a group/team and/or comparison with previously determined measure. In an attempt to minimize error through analytical procedure, we suggest LC-MS/MS be used where possible. When this is not viable, due to the innate laborious methodology, it is imperative all analysis is conducted in the same laboratory, by the same investigator, in duplicate or triplicate. We also advise that direct comparisons are made only with findings from the same methodological technique. Coefficient of variation needs to be reported in all cases.

Strength of Evidence and Recommendations

In summary, there is evidence that sal-T and sal-C is directly influenced by collection method, blood contamination, storage duration and temperature, and analysis method. Whilst saliva collection has obvious advantages over blood, accuracy, variability, and salivary protein binding may limit interpretation of results. As capillary sampling has been validated for analysis of TC, this may be an option for TT determination, however this currently requires validation.

CONCLUSION

Salivary analysis of C and T in sports people will soon enter its fourth decade as a research tool. The benefits of this method are well documented and primarily focus on ease of sample and subsequent analysis. However, there is a general lack of consistency between studies that use salivary measures of T and C as surrogates for their systemic counterparts. These include issues related to A) study design, B) sample acquisition and biological variation, and C) technical and analytical error. We would encourage researchers to consider each of A, B, and C, as outlined above in the formulation of study design, participant information sheets, and in their reporting of study outcomes.

****INSERT TABLE 1 NEAR HERE *****

Table 1: Sources of error in sal-C and sal-T measurement and methodological and technical guidelines recommended to limit error rates. Where evidence exists that disregarding the guideline will influence sal-T and sal-C, a checked box is present. Where evidence exists that disregarding the guideline may influence sal-T or sal-C, based purely on serum data, 'evidence in TT', or 'evidence in TC' is checked.

****INSERT TABLE 2 NEAR HERE *****

Table 2: Conversion between commonly used units for salivary testosterone within physiologically observed ranges. The molecular mass of testosterone is 288.42 $g \cdot mol^{-1}$ and therefore 1 mol = 288.42 g. As such, 2 $pmol \cdot L^{-1}$ x 288.42 = 576.84 $pg \cdot L^{-1}$. 1 L = 1000 mL, so 576.84 $pg \cdot L^{-1}$ = 0.57 $pg \cdot ml^{-1}$.

****INSERT TABLE 3 NEAR HERE *****

Table 3: Conversion between commonly used units for salivary cortisol within physiologically observed ranges. The molecular mass of cortisol is 362.46 $g \cdot mol^{-1}$ and therefore 1 mol = 362.46 g. As such, 2 $nmol \cdot L^{-1}$ x 362.46 = 724.92 $ng \cdot L^{-1}$. 1 L = 10 dl, so 724.92 $ng \cdot L^{-1}$ = 72.5 $ng \cdot dl^{-1}$.

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