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Validating the salivary testosterone and cortisol concentration measures in response to short high-intensity exercise

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***Aim*. To validate the testosterone (T) and cortisol (C) concentration measures in saliva in response to short high-intensity exercise.**

***Methods*. Nine healthy males provided matching saliva and plasma samples before and after a 30-second Wingate cycle test. Saliva was assayed for T (Sal-T) and C (Sal-C) concentrations, and plasma for total T and total C, sex hormone-binding globulin, corticosteroid-binding globulin (CBG) and albumin concentrations. The plasma free and bioavailable hormones were calculated.**

***Results*. The Sal-T and plasma T correlations were weak to moderate (r=0.57-0.61) when examined between individuals (pooled data for all participants), but these relationships improved (r = 0.71-0.73) within individuals (data for each participant on average). The Sal-C and plasma C correlations were strong both between individuals (r=0.81-0.84) and within individuals (r=0.83-0.84). The peak relative increases in Sal-T (35±9%) and Sal-C (63±29%) concentrations exceeded the plasma total and/or free hormones, but not the bioavailable hormones. Albumin (10±3%) and CBG (16±4%) also increased with exercise, along with blood lactate (943±119%).**

***Conclusions*. The Sal-T and Sal-C concentration measures were validated in response to short high-intensity exercise, especially for individuals. The hormonal changes in saliva were also more sensitive to exercise (i.e. greater relative responses) than the plasma total and/or free hormones, potentially arising from changes in the binding proteins and blood lactate. These findings support the use of saliva as a medium for steroid determination in sport.**

KEY WORDS: Adrenal glands - Gonads - Plasma.

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The monitoring of testosterone (T) and cortisol (C) in saliva is gaining acceptance for assessing gonadal and adrenal function in sport. Compared with other fluids (*e.g.* blood, urine, sweat), saliva is non-invasive, easy to collect and readily available.[1] Saliva collection also eliminates the confounding effects of stress and enables rapid and frequent sampling. Validation studies have demonstrated moderate to strong correlations (r=0.70-0.92) between salivary T (Sal-T) and C (Sal-C) concentrations and the corresponding blood total hormones (Tot-T and Tot-C) at rest.[2-5] The saliva measures more accurately reflect (r = 0.92-0.97) blood free T (Free-T) or C (Free-C) concentrations to provide valuable data on the biologically active portion (1-5% of total hormones) that is available to tissue.

The saliva-blood correlations are less consistent with exercise. For instance, the Sal-C and Tot-C measures were correlated before, but not after, a sub-max-

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TABLE I.—*Subject characteristics and Wingate test performance (mean± SD).*

Subject details (N=9)	
Age (years)	29.8±5.8
Height (cm)	174.7±4.8
Body mass (kg)	81.2±11.7
Body fat (%)	17.5±6.0
Peak power (W)	921.8±161.4
Mean power (W)	716.7±90.8
Fatigue index (%)	51.4±9.0

imal or maximal rowing test".[6] Similarly, a strong correlation was found between these measures when cycling with submaximal and maximal loads, but a breakpoint was noted with the maximal load.[7] This breakpoint may be explained by the greater increases in Free-C (and Sal-C) concentrations after corticosteroid-binding globulin (CBG) saturation [8] and supported by the Sal-C correlations with Free-C (r=0.89) and Tot-C (r=0.59) with intense running.[9] Most exercise studies have used Tot-C as the only criterion measure. Little investigation of the T responses in saliva and blood has also occurred using the exercise model.

The term bioavailable has been used to describe the steroid pool (*i.e.* free and albumin-bound) that arguably represents the bioactive hormone.[10] That is, the hormone portion bound to albumin (6% for C, 50% for T) is readily available for tissue uptake, subsequent to changes in the hormone-binding protein dissociation rate.[10] This concept is relevant for sport since exercise can not only induce haemoconcentration,[11, 12] effectively increasing binding protein concentrations, but also increase the dissociation rates of the steroid-binding protein complex by altering the metabolic environment.[13, 14] Correlations (r=0.66-0.86) between Sal-T and bioavailable T (Bio-T) at rest suggests that saliva provides an easily accessible marker for bioavailable steroids.[3, 15] However, no validation studies have compared the exercise responses of Bio-T and bioavailable C (Bio-C).

Addressing the limitations of research would improve the interpretation of the Sal-T and Sal-C measures under exercising conditions and, in doing so, may improve the use of saliva as a medium for steroid determination in sport. Thus, the aim of this study was to validate the Sal-T and Sal-C concentration measures in response to short high-intensity exercise. We hypothesized that the saliva-blood correlations would be stronger for the blood free and bioavailable hormones than the total hormones. It was also hypothesized that the hormonal changes in saliva would exceed the blood total hormones.

Materials and methods

Participants

Nine healthy males volunteered for this study. Participants were considered healthy and active, with each engaged in various forms of physical activity (*e.g.* martial arts, weightlifting, cycling) on a regular basis (30-60 minutes duration, 3-6 times per week) for at least 12 months before the study commenced. A standard questionnaire was used to assess the health status of participants. Body composition was assessed using bioelectrical impedance (InBody 3.0, Biospace, Seoul), according to the manufacturer's guidelines. Subject characteristics are summarized in Table I. Each participant had the risks of the investigation explained to them and signed an informed consent before participation. Ethical approval for this study was granted by the Northern Y Regional Ethics Committee of New Zealand, and the Southern Cross University Human Research Ethics Committee.

Experimental procedures

This research compared the salivary and plasma hormones before and after a 30-second Wingate test on friction-braked cycle ergometer (Monark Ergomedic 834E, Sweden).[12, 16] Participants visited the test facility on three occasions, separated by 3-5 days, with the first two occasions serving as familiarization for the exercise procedures and equipment. On the third occasion, matching saliva and blood samples were collected in tandem with the exercise test. Participants were assessed at a similar time of day (2 p.m.±1 hour) to account for diurnal variation and were asked to refrain from any physical activity for 48 hours before each test. The experimental procedures are outlined in Figure 1.

Test procedures

The test procedures were similar to those described elsewhere.[12, 16] Following a standardised warm-up, participants began unloaded pedalling for 60 seconds at 60 rpm and, once the predetermined load was applied, were instructed to pedal at maximum effort for the test duration. A higher than normal resistance (10%

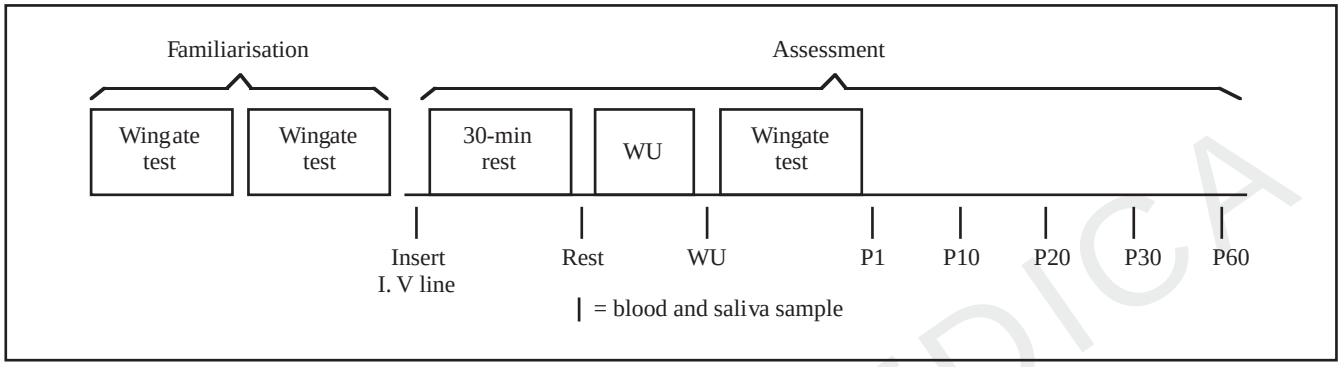

Figure 1.—Schematic representation of the experimental procedures. WU = warm-up, P1-P60 = post-exercise in minutes.

of individual bodymass) was employed with the intention of stimulating dynamic hormonal changes in saliva and plasma. Participants remained seated throughout the test and were given strong verbal encouragement. Seat height was adjusted for each individual and the feet fixed to the pedals with toe-clips. The cycle ergometer was interfaced with a computer processor to record power data every 0.5 seconds, based on the flywheel revolutions and the cycle resistance. Peak power was determined as the highest power recording across the 30-second test and mean power as the average power sustained. A fatigue index (%) was calculated from the difference between the highest and lowest power recordings. The performance results are shown in Table I.

Collection of saliva and plasma samples

On the experimental day, an intravenous catheter was inserted into the antecubital vein to allow rapid repeat blood sampling (Figure 1). Following a 30-minute equilibrium period, whole blood samples (8 mL) were drawn at rest (Rest) and after the warm-up (WU). At the completion of exercise, post-exercise samples were drawn after 1- (P1), 10- (P10), 20- (P20), 30- (P30) and 60-minutes (P60). Blood samples were collected into heparinised vacutainers (Vacuette, Greiner, Germany) and the plasma portion separated by centrifugation (1900×g, 15-minutes). Participants provided time-matched saliva samples (2 mL) in sterile containers (LabServe, New Zealand) using sugar-free gum (Extra – peppermint, Wrigley's, New Zealand) to increase saliva flow, since steroid hormones are independent of flow rate.[1] This method of specimen collection has been used.[17, 18] To prevent saliva contamination, participants were asked to not eat, brush their teeth or drink hot fluids for one hour before their assessment.[17] Each participant remained seated after exercise until all the fluid samples were collected. The saliva and plasma samples were stored at -80°C until assay. Pilot data indicated no changes in the assessed hormonal measures, taken at a similar time of day, in a group of non-exercising healthy males.

Plasma hormone assays

Plasma Tot-T and Tot-C concentrations were determined in duplicate by radioimmunoassay (RIA) using diagnostic kits (DSL Inc., Texas, USA), following the manufacturer's guidelines. The minimum detection limit for the Tot-T assay was 0.35 nmol·L^{-1} with intra- and inter-assay coefficients of variation (CV) of <4.2% and <4.8%, respectively. The minimum detection limit for the Tot-C assay was 1.38 nmol·L^{-1} with respective intra- and inter-assay CVs of <4.9% and <6.0%.

Plasma binding protein assays

Plasma sex hormone-binding globulin (SHBG) and CBG concentrations were respectively determined using RIA and immunoradiometric kits (Biosource, Belgium), using the manufacturer's protocols. The sensitivity of the CBG assay was 0.46 µg·mL^{-1} and 10 nmol·L^{-1} for the SHBG assay, with intra-assay CVs of <5.4% and <5.2% for the respective protein kits. Plasma albumin was measured in a single assay on the Roche Modular (Hitachi, Japan) using the bromocresol green method.[19] The sample was added

TABLE II.—*Absolute changes in salivary hormone, plasma hormone and plasma binding protein concentrations in response to the Wingate test (mean ± SE).*

	Saliva		Plasma						Plasma		
	Sal-T ($pmol \cdot L^{-1}$)	Sal-C ($nmol \cdot L^{-1}$)	Tot-T ($nmol \cdot L^{-1}$)	Free-T ($pmol \cdot L^{-1}$)	Bio-T ($nmol \cdot L^{-1}$)	Tot-C ($nmol \cdot L^{-1}$)	Free-C ($nmol \cdot L^{-1}$)	Bio-C ($nmol \cdot L^{-1}$)	Albumin ($g \cdot L^{-1}$)	CBG ($\mu m \cdot mL^{-1}$)	SHBG ($nm \cdot mL^{-1}$)
Rest	233±35	8.18±0.62	14.0±1.6	249±36	7.03±1.11	303±33	14.5±2.7	43.5±8.1	46.3±1.0	49.5±2.7	15.3±3.5
WU	265±39	8.62±0.97	14.6±1.4	260 ±33	7.91±1.10	332±35	14.1±2.3	44.9±7.4	50.1±0.7	55.5±2.9	16.3±3.5
P1	280±39	8.50±1.23	15.0±1.4	267±33	8.22±1.09	354±43	15.3±3.0	49.3±9.9	50.9±1.2	57.3±3.5	17.0±3.6
P10	313±45	11.1±1.77	15.2±2.1	269±45	8.19±1.45	381±37	16.6±2.2	52.9±7.0	50.2±0.8	54.8±3.5	17.7±3.4
P20	278±42	14.4±2.21	15.1±2.2	264±47	7.87±1.48	399±55	19.3±3.6	60.8±11.8	49.1±0.8	54.1±4.0	17.1±3.3
P30	256±41	11.6±2.09	13.5±1.7	236±38	6.82±1.19	332±48	16.0±3.7	49.2±11.0	47.7±0.8	51.7±2.8	16.8±3.4
P60	213±46	7.23±1.57	12.4±1.8	212±40	6.07±1.15	266±41	12.3±3.3	37.6±9.8	47.7±0.6	51.1±3.0	16.4±3.4
Between-individual correlations with corresponding saliva			r=0.57*** (0.37-0.71)	r=0.60*** (0.42-0.74)	r=0.61*** (0.42-0.74)	r=0.81*** (0.71-0.88)	r=0.84*** (0.74-0.90)	r=0.84*** (0.74-0.90)			
Within-individual correlations with corresponding saliva			r=0.72** (0.36-0.89)	r=0.71** (0.32-0.90)	r=0.73** (0.39-0.90)	r=0.83*** (0.79-0.87)	r=0.84*** (0.78-0.89)	r=0.84*** (0.78-0.88)			

**Significant correlations ,5P<0.01
***Significant correlations P<0.001
( ) indicates 95% confidence intervals

at pH 4.1 and the coloured albumin bromocresol green complex measured at 570 nm. The CV for this assay was <1%.

Calculation of plasma free and bioavailable hormones

The plasma results were used to calculate Free-T and Free-C concentrations from published formulae (see below).[20, 21] All concentrations in the Free-T formula are expressed in $nmol \cdot L^{-1}$. To convert Free-T from $nmol \cdot L^{-1}$ to $pmol \cdot L^{-1}$, multiply by 1000.

$$\text{Free-T}=-52.65 + 24.4\ (\text{Tot-T}) - 0.704\ (\text{SHBG}) - 0.0782\ (\text{Tot-T} \times \text{SHBG}) - 0.0584\ (\text{Tot-T}^2)$$

All concentrations in the C formula are expressed in $\mu mol \cdot L^{-1}$. To convert Tot-C from $nmol \cdot L^{-1}$ to $\mu mol \cdot L^{-1}$, divide by 1000. To convert CBG from $\mu g \cdot mL^{-1}$ to $\mu mol \cdot L^{-1}$, multiply by 0.01923. To convert Free-C from $\mu mol \cdot L^{-1}$ to $nmol \cdot L^{-1}$, multiply by 1000.

$$\text{Free-C}=\sqrt{Z^2+0.0122\ (\text{Tot-C})} - Z,$$
$$\text{Wherein } Z=0.0167+0.182\ (\text{CBG}-\text{Tot-C})$$

The Bio-T and Bio-C results were estimated as the sum of the free and albumin-bound hormones,[10] based on the binding affinity of T (4×10^{-4}) and C (0.3×10^{-4}) to albumin,[22] at a molar weight of 69 000 Daltons.

Salivary testosterone and cortisol assays

The saliva samples were thawed and centrifuged (3000×g, 5-minutes) to assist with the separation of foam and other contaminates.[17] The salivary hormones were determined in triplicate using diagnostic kits (DSL, Inc. Texas) and RIA modifications.[18, 23] Briefly, standards from DSL kits were diluted in phosphate buffered saline (PBS) to cover the concentration range of 0-1730 $pmol \cdot L^{-1}$ for Sal-T and 0-140 $nmol \cdot L^{-1}$ for Sal-C. The DSL standards were also diluted in PBS to obtain low and high values for each assay with the antibodies diluted in a PBS solution containing 0.05% bovine serum albumin. The performance characteristics (*e.g.* linearity, sensitivity, precision) of these assays are discussed elsewhere.[18, 23] Saliva aliquots of 100 µL and 50 µL were used for the respective Sal-T and Sal-C assays. The minimum detection limit for the Sal-T assay was 3.5 $pmol \cdot L^{-1}$ with intra- and inter-assay CVs of <9.0% and <8.7%, respectively. The Sal-C assay had a detection limit of 0.14 $nmol \cdot L^{-1}$ with an intra- and inter-assay CVs of <7.4% and <8.7%, respectively. The saliva and plasma samples for each individual were analysed within the same assay run for each hormone assessed.

Blood lactate

Blood lactate was measured at rest and approximately 5 minutes after exercise, to coincide with the

peak lactate responses to maximal cycling exercise,[12] using a portable blood analyser (Lactate Pro, Arkray, USA).

Statistical analysis

Data were log transformed before analysis to normalise the distribution and reduce non-uniformity of error. The relative changes in the hormonal and protein variables were examined using analysis of variance (ANOVA) with repeated measures and Fisher's t-test as the post hoc procedure. The peak relative changes in these variables were also compared using repeated measures ANOVA and t-tests. Relationships between the salivary and plasma hormones were assessed using Pearson product moment correlation coefficients (*r*). The saliva-plasma hormonal relationships were examined between individuals (pooled data for all participants) and within individuals (data for each participant on average), after Fisher's transformation. This approach was taken to address possible inter-subject differences in the hormonal correlations.[24] The relative changes in blood lactate were examined using a t-test. The significance level was set at P≤0.05.

Results

Salivary and plasma hormone concentrations

The hormonal concentrations in saliva and plasma were found to lie within the expected physiological range for males (Table II). In terms of the relative hormonal concentrations, Sal-T represented only a small portion of plasma Tot-T (1.7-2.1%) and Bio-T (3.3-3.8%), and was similar to Free-T (94-117%). Likewise, Sal-C represented only a small fraction of plasma Tot C (2.4-3.6%), but a greater portion of Bio-C (1724%) and a smaller portion of Free-C (55-75%) when compared to the T measures.

Salivary and plasma hormone correlations

Significant (P<0.001) weak to moderate correlations (*r* = 0.57-0.61) were demonstrated between the Sal-T and plasma T concentration measures between individuals (Table II). Moderate correlations (*r*=0.71-0.73, P<0.01) were identified between these T measures when analysed within individuals. Significant (P<0.001) strong correlations were demonstrated between the Sal-C and plasma C concentration mea-

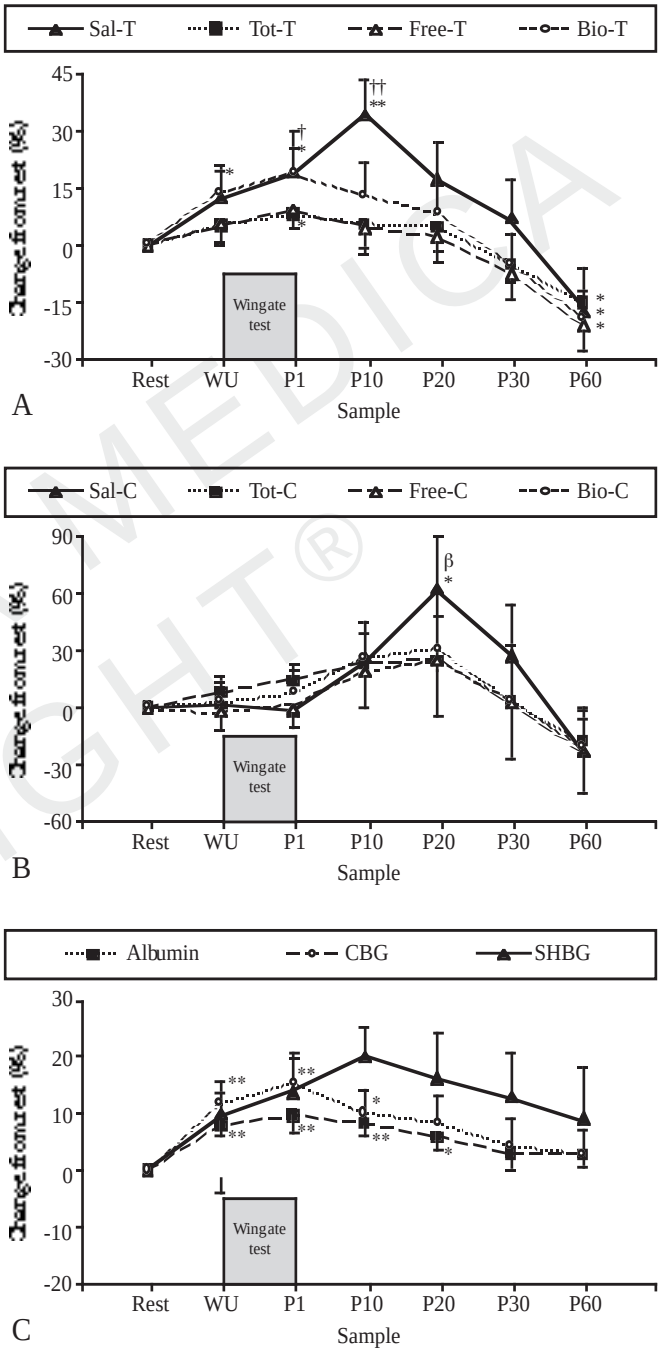

Figure 2.—Relative changes in salivary hormone, plasma hormone and plasma binding protein concentrations with the Wingate test (mean±SE).
*Significant difference from rest P£0.05
**Significant difference from rest P<0.01
†Significant difference from the peak increase in Tot-T and Free-T P<0.05
††Significant difference from the peak increase in Tot-T and Free-T P<0.01
bSignificant difference from the peak increase in Tot-C P<0.05.

sures both between individuals (r=0.81-0.84) and within individuals (r=0.83-0.84).

Salivary and plasma hormone responses

A significant time effect was identified for the relative changes in Sal-T (F (6, 48)=7.09, P<0.001), Tot-T (F (6, 48) = 4.03, P<0.01), Free-T (F (6, 48)=4.12, P<0.01) and Bio-T concentrations (F (6, 48)=5.35, P<0.001). Post hoc analysis revealed a significant increase in Sal-T at P10 (35±9%) from rest (Figure 2A). Compared to rest, plasma Tot-T increased at P1 (8±4%) and decreased at P60 (-15±5%), with Free-T also decreasing at P60 (-21±7%). Similarly, Bio-T increased at WU (14±7%) and P1 (19±6%), before decreasing at P60 (-19±7%). The peak increases in these variables were significantly different (F (3, 24) =7.08, P<0.001) with the relative changes in Sal-T and Bio-T both greater than Tot-T and Free-T, but no different from each other. There were no differences in the peak decreases between these variables (F (3, 24)=0.46, P>0.05).

A significant time effect was identified for the relative changes in Sal-C concentrations (F (6, 48)=4.70, P<0.001), but not for Tot-C (F (6, 48)=2.25, P>0.05), Free-C (F (6, 48)=1.32, P>0.05) and Bio-C concentrations (F (6, 48)=1.55, P>0.05). Post-hoc results identified a significant increase in Sal-C at P20 (63±29%) from resting values (Figure 2B). A comparison of the peak increases in these measures also revealed a significant effect (F (3, 24)=3.74, P<0.05) with the relative changes in Sal-C found to be greater than Tot-C, but no different from Free-C and Bio-C. The peak decreases in these variables were not significantly different (F (3, 24)=0.19, P>0.05).

Plasma binding protein responses

A significant time effect was found for the relative changes in albumin (F (6, 48)=8.75, P<0.001) and CBG concentrations (F (6, 48)=5.85, P<0.001), but not for SHBG (F (6, 48)=2.15, P>0.05). Post-hoc analysis identified a significant increase in albumin concentrations at WU (8±2%), P1 (10±3%), P10 (8±2%) and P20 (6±2%) from rest (Figure 2C). Compared with rest, the CBG responses were also elevated at the WU (12±4%), P1 (16±4%) and P10 (10±4%) samples. There was no significant differences between the peak increases in the plasma binding proteins (F (2, 16)=0.74, P>0.05).

Blood lactate responses

Lactate concentrations post-exercise (11.8±0.8 mmol.L^{-1}) were significantly different from rest (1.2±0.1 mmol.L^{-1}), representing a relative increase of 943±119% (P< 0.001).

Discussion

The exercise-induced changes in the salivary and plasma T measures were correlated between individuals and these relationships improved when examined within individuals. The correlations between the salivary and plasma C measures were of similar magnitude between individuals and within individuals. The relative hormonal increases in saliva exceeded the plasma total and/or free hormones, but not the bioavailable hormones. Albumin and CBG also increased with exercise, along with blood lactate.

The Sal-T and plasma T concentration measures were correlated (weak to moderate) between individuals. Similar correlations (r≥0.65) were demonstrated between various hormones (*i.e.* T, C, oestradiol) in saliva and blood before, during and after rowing exercise.[25] However, this paper did not examine these results within individuals.[25] The stronger relationships within individuals in this study might be attributed to subject variation in the blood hormone responses to exercise [16, 26] and their subsequent manifestation in saliva. Other possible sources of subject variation include binding protein responses and dissociation rates, as well as peripheral hormone kinetics (*e.g.* steroid metabolism). These factors could explain the non-significant correlations reported elsewhere with exercise.[27] Thus, consideration of blood-saliva hormone dynamics from both a group (between individuals) and subject (within individuals) perspective may improve validation research.

The Wingate test increased T concentrations in both fluids, but the relative increase in Sal-T exceeded plasma Tot-T and Free-T. One exercise study reported a greater relative increase in Tot-T (v. Sal-T), although this result was derived from only those subjects with elevated blood concentrations.[25] Recent findings suggest that Free-T (v. Sal-T) increases more after resistance exercise,[27] but only a single post-exercise sample was collected. Our observations might be explained by increased hormone bioavailability in saliva, as indicated by the similar peak responses of Sal-T and Bio-

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