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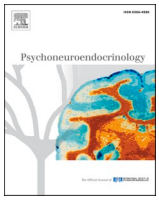

Oxytocin and cortisol concentrations in urine and saliva in response to physical exercise in humans

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ABSTRACT

Background: While peripheral markers of endogenous oxytocin and glucocorticoid release are widely employed in psychological and behavioural research, there remains uncertainty regarding the effectiveness of saliva and urine samples in accurately capturing fluctuating hormone levels in response to relevant stimuli. In addition, it is unclear whether and under which conditions, urinary concentrations correlate with salivary levels of oxytocin and cortisol.

Methods: In the present study, two groups of healthy adult male and female participants (N=43) provided heart rate, saliva, and urine samples before and after exercising at different durations and intensities (3 ×10 min of running vs. 60 min of running). Effects of age, gender, cycle phase, and previous running experience were considered in the statistical analyses. Concentrations of oxytocin and cortisol were analysed in both saliva, and urine using validated assays.

Results: Runners of both groups had significantly increased oxytocin concentrations in urine and saliva after running than before. Oxytocin in saliva was elevated after 10 min and peaked after 30 min of running. Only participants of the long-running group showed an increase in urinary cortisol concentrations following exercise (and only after 90 min of stimulus onset), and neither group had a significant increase in salivary cortisol levels. Oxytocin rise in urine and saliva from basal to post-run was strongly and significantly correlated, as was cortisol rise from basal to post-rest, but no correlations between absolute hormone concentrations were found for oxytocin.

Conclusions: Our results show that both urine and saliva are useful body fluids that can provide meaningful results when measuring oxytocin and cortisol concentrations after a physical stimulus. While temporal resolution may be better with salivary sampling as higher sampling frequency is possible, signal strength and robustness were better in urinary samples. Importantly, we report a strong correlation between the magnitude of change in oxytocin and cortisol concentrations in urine and saliva following physical exercise, but no correlations between absolute oxytocin concentrations in the two substrates.

1. Introduction

The nonapeptide oxytocin (OXT) is of major research interest due to its modulating role in a multitude of social and emotional behaviours, such as maternal care (Bosch and Neumann, 2012), bond formation and maintenance (Insel et al., 1998), and anxiety and stress-coping (Neumann and Landgraf, 2012) (for reviews see (Froemke and Young, 2021; Menon and Neumann, 2023). Additionally, OXT also plays important physiological roles in regulating cardiovascular function (Jankowski et al., 2020) and energy intake (McCormack et al., 2020). Various social and non-social stimuli activate the endogenous OXT system as reflected by increased neuropeptide synthesis in the hypothalamic nuclei of origin, release within the brain, and secretion into peripheral circulation (for review see Jurek and Neumann, 2018), where concentrations can be measured in blood (Kirkpatrick et al., 2014), saliva (de Jong et al., 2015, MacLean et al., 2017), urine (Mitsui et al.,

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2011, Crockford et al., 2014; Preis et al., 2018) and, most recently, in sweat samples (Zagoory-Sharon et al., 2023).

In humans, measuring OXT concentrations in the periphery is generally preferable over more invasive approaches, such as cerebrospinal fluid (CSF) puncture, which requires more intense medical care and poses higher risks. However, the extent to which peripheral OXT measures may be used as proxies for central OXT release is highly context-dependent (Valstad et al., 2017), which must be considered when interpreting results. Measuring OXT concentrations in saliva and urine samples is completely non-invasive, non-disruptive and, therefore, feasible for non-clinical experimental settings, but poses its own methodological challenges: First, in contrast to plasma and saliva samples, OXT concentrations in urine reflect an integrated measure of plasma OXT levels over a certain period, as OXT molecules (or their metabolic degradation products) accumulate since the last emptying of the urinary bladder (Gnanadesikan et al., 2022). Consequently, the temporal resolution of urinary measures is relatively low. However, this could even be of advantage for specific research approaches, because OXT levels are likely less affected by acute, e.g., stress-induced, alterations in neurohypophysial secretion patterns compared to plasma and saliva samples. Second, OXT molecules may become fragmented in the process of kidney glomerular filtration (Gnanadesikan et al., 2022, MacLean et al., 2019). Consequently, common analytical approaches, such as enzyme-linked immunoassays (ELISA) or radioimmunoassays (RIA), may produce high values due to the detection of these OXT fragments. A way to counteract this issue is to extract samples before assaying (Schaebs et al., 2019; Gnanadesikan et al., 2022; Tabak et al., 2023). Finally, urine is concentrated in the renal medulla depending on the body's hydration status (Epstein et al., 1957; Perrier et al., 2013). Accordingly, large variations in substance concentration make it necessary to assess urinary creatinine or specific gravity (SG) for each sample (MacPherson et al., 2018).

Despite the broad use of peripheral markers of endogenous OXT release, there is still uncertainty associated with the sensitivity and reliability of saliva and urine samples. Previous studies have shown elevated salivary OXT concentrations as a function of lactation and massage (Carter et al., 2007, White-Traut et al., 2009) thereby providing biological validity. More recently, physiologically standardized stimuli, such as physical exercise, sexual self-stimulation, or exposure to psychosocial stress (Trier Social Stress Test; TSST) have been demonstrated to increase OXT concentrations in saliva in healthy human adults (de Jong et al., 2015). Moreover, Mitsui et al. (2011) showed that 15 min of physical exercise significantly increased urinary OXT concentrations in dogs. Yet to date, similar studies for OXT in human urine are largely lacking, but essential to further validate OXT measurements in urine samples (Tabak et al., 2023). Further, it is unclear, whether and under which conditions urinary OXT correlate with salivary OXT concentrations. Previous studies have shown salivary and plasma OXT (Grewen et al., 2010; Hoffman et al., 2012), and urinary and plasma OXT (Hoffman et al., 2012; Francis et al., 2016) to be moderately correlated in humans. Melton et al. (2022) assessed OXT concentrations in urine and saliva samples of couples before and after a shared leisure activity (board game or painting class) and found that only urine (total OXT per sample but not the concentration) was sensitive enough to detect significant differences between the activities and timepoints. The only study to date investigating a possible direct link between salivary and urinary OXT measures, reported no significant correlation under basal conditions, but did not evaluate potential correlations between post-stimulus measures (Feldman et al., 2011).

To fill this gap, the present study builds on existing evidence that physical exercise (running) robustly increases OXT concentrations in saliva in healthy humans (de Jong et al., 2015), and expands this paradigm to include the assessment of both OXT and cortisol (CORT) in urine samples. First, we predicted an increase in OXT levels in saliva and urine samples in response to physical exercise. Further, we predicted elevated CORT concentrations in saliva and urine as a marker of hypothalamo-pituitary-adrenal (HPA) axis activation following exercising (Cook et al., 1987; Kuoppasalmi et al., 1980). We also predicted a positive correlation of OXT (and CORT) in saliva and urine after exercising, but not necessarily of baseline measures, based on findings of a previous meta-analysis (Brown et al., 2016). Importantly, we also predicted that the magnitude of exercise-induced increase in OXT (and CORT) concentrations would correlate positively in urine and saliva.

2. Methods

2.1. Participants

Healthy adult volunteers were recruited from the environment of the researchers in Regensburg, Germany. Exclusion criteria were heart or renal disease, chronic or acute pain, current Covid infection, pregnancy or lactation, period, hormonal contraceptives, and glucocorticoid medication.

Participants received detailed oral and written information on the procedures and gave their informed consent before participation. Ethical approval for this study was obtained from the ethical commission of the University of Regensburg (approval number 20–1737–101). The study was conducted in accordance with the principles originating from the Code of Ethics of the World Medical Association (Declaration of Helsinki) and in line with the Good Clinical Practice (GCP) guidelines of the International Conference on Harmonization (ICH).

To increase the temporal solution of our sampling regime, we included two runner groups: One group ran loops and stopped in between to deliver additional samples, whereas the other group ran continuously and provided samples only before and after they finished the run as well as after a post-run resting period. The first group (G1) consisted of 20 participants (males: n=10, average age: 31 yrs, range: 20–54 yrs; females: n=10, average age: 25 yrs, range: 20–31 yrs). They were asked to run three times for 10 min with short breaks to provide saliva samples, pulse rates, and to drink a fixed amount of water (total of 400 ml) (see Fig. 1a for the timeline). They were running individually and at their own suitable speed. The second group (G2) consisted of 23 participants (males: n=8, average age: 55 yrs, range: 22–65 yrs; females: n=15, average age in years: 58 yrs, range: 41–78 yrs). This group consisted of experienced runners and ran continuously for 60 min at a certain speed (6.30 min/km). They provided samples only at the beginning and end of the test (see Fig. 1b for the timeline). Due to substantial differences in the age and gender distribution of the two groups, as well as differences in running durations and running expertise, the factor "group" was included as a test predictor interacting with timepoint (basal, post-run, post-rest) into the statistical models, as were other control predictors for age, gender, cycle phase, and running experience (see below for details).

All 43 participants completed the running test and provided all samples, however, samples of n=3 female participants (n=2 of G1 and n=1 of G2) had to be excluded due to either using hormonal contraception or being on their period on the testing day, resulting in a total sample size of n=40 participants used in statistical analysis. The sample size was chosen based on a previous study using a similar paradigm (10 min of running) to elicit OXT release into saliva in healthy humans (*n=17*; de Jong et al., 2015). Assuming a similar effect size, we could therefore expect sufficient statistical power with our sample being at least as large as de Jong et al.'s.

2.2. Procedures

Testing was carried out in June, July, and August 2021. All tests were conducted in the late afternoon, between 4 and 7 pm, to avoid daytime effects on hormone measurements. Participants were asked to refrain from eating for two hours before the start of the test. Saliva and urine samples were collected by the participants themselves. Specifically, the participants arrived at the designated meeting point 30 min prior to the

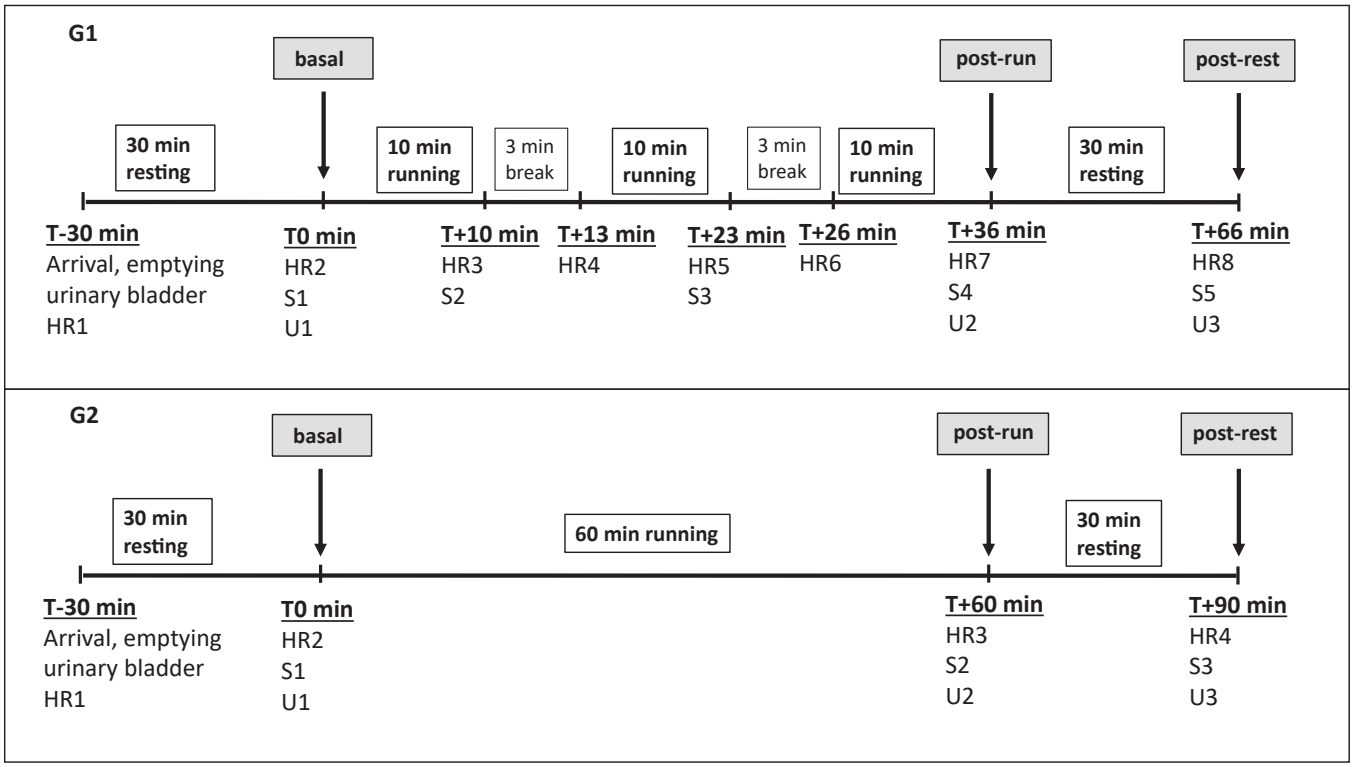

Fig. 1. Experimental timeline for group 1 (G1, upper panel) and group 2 (G2, lower panel). Pulse rate (HR), saliva samples (S) and urine samples (U) were taken as indicated.

start of the test. They were then asked to empty their bladder before measuring their pulse rate as a proxy of heart rate (HR1) by manually feeling the pulse on their wrist (radial pulse) or neck (carotid pulse), counting the beats for 15 sec, and multiplying by 4. Pulse rate was subsequently used to confirm that the exercise indeed led to physical agitation in the participants and as a control predictor in the statistical models. In both G1 and G2, the pulse rate was measured at each time-point, when urine or saliva samples were taken, as indicated in Fig. 1.

Then, G1 participants spent 30 min sitting down and reading quietly by themselves. During this period, they drank 100 ml of water. Next, basal saliva (S1) and urine (U1) samples and another pulse measurement (HR2) were taken before starting the first round (10 min) of running. Immediately upon their return, they provided the second saliva (S2) sample, pulse rate (HR3), and drank 100 ml of water while having a 3-minute break. Just before starting to run again, they took another pulse rate measurement (HR4). The procedure was repeated (S3, HR5, drinking 100 ml of water, HR6) after the second 10-min run, before they started to run the last round. Thereafter, pulse rate (HR7), a saliva sample (S4; post-run), and the second (U2; post-run) urine sample were collected. During a final 30-min break of sitting down quietly, they drank 100 ml of water, and took the last pulse rate (HR8), the last saliva sample (S5; post-rest), and the last urine sample (U3; post-rest) (Fig. 1).

G2 runners completed a modified version of the procedure described above: After emptying their bladders and providing the first pulse rate measure, they spent 30 min sitting down or stretching in preparation for the run. During this time, they drank 200 ml of water. Then they provided the basal saliva (S1) and urine (U1) samples, before they ran continuously for 60 min in groups of 4–7 people. Afterwards, they provided the second saliva sample (S2; post-run) and the second urine sample (U2; post-run) and rested for 30 min, while drinking 200 ml of water. Finally, they provided the last saliva (S3; post-rest) and urine sample (U3; post-rest) (Fig. 1).

2.3. Sample collection and hormone analyses

Sample collection and handling: Saliva samples were collected by the participants using commercially available cotton swabs (Sarstedt Salivetten, blue cap, Article-No. 51.1534.500), following the manufacturer's instructions. Urine samples were collected in plastic sampling cups (Boettger, Art.-No. 06–013–0100). Upon collection, all samples were immediately stored on ice and transported to the lab. There, saliva samples were centrifuged (for 10 minutes at 1300 *g* at 4°C), then aliquoted into 0.5 ml portions, and frozen at −80°C until analysis using a sensitive RIA (RIAgnosis, Sinzing, Germany). Urine samples were aliquoted and frozen in the same way. These steps were completed within 3 hours of collection. Additionally, the specific gravity (SG) of each urine sample was measured before freezing with a digital refractometer (model TEC++, serial no. T6017) to account for differences in urine concentration. The SG correction factor was calculated using the formula given by Miller et al. (2004) using the average SG of all collected samples as the population mean. Urinary hormone concentrations were statistically analysed as ng/ml (CORT) or pg/ml (OXT) multiplied by the sample specific SG correction factor and expressed as concentration per ml SG.

Sample preparation: Urine samples were extracted prior to analysis using LiChroprep Si60 heat-activated at 700 °C for 3 hours. First, 20 mg of LiChroprep Si60 in 1 ml of distilled water was added to each 0.5-ml sample, then mixed for 30 min, and then washed twice with distilled water and 0.01 N HCl, all at 4°C. Finally, samples were eluted with 60 % acetone for 30 min at 4°C and vacuum evaporated. The extraction recovery range was 85–90 %. The data were not corrected for recovery. While an extraction step is crucial for urine (and plasma) samples to eliminate potentially interfering molecules and minimize matrix effects, saliva (as well as microdialysate and cerebrospinal fluid) samples can be analyzed for OXT with or without extraction (Tabak et al., 2023). In fact, Salivette-collected saliva samples spiked with 0.5, 1 or 2 pg synthetic OXT provided essentially the same data after evaporation with or

without prior extraction (Landgraf, personal communication). Thus, in contrast to urine, saliva is unlikely to contain substances that interfere with the RIA. Hence, for this study, saliva samples were analysed without prior extraction using the protocol described by de Jong et al. (2015).

Oxytocin analysis: First, 50 μL of assay buffer were added to each evaporated sample. This was followed by 50 μL antibody (raised in rabbits against OXT). After a 60-min pre-incubation interval, 10 μL125I-labeled tracer (Hartmann Analytic GmbH, Braunschweig, Germany) were added, and samples were allowed to incubate for 3 days at 4° C. Then, unbound radioactivity was precipitated by activated charcoal/Dextran (Sigma–Aldrich, St Louis, MO, USA). Under these conditions, an average of 50 % of total counts are bound with <5 % non-specific binding. The detection limit was in the 0.1–0.5 pg/sample range, depending on the age of the tracer, with typical displacements of 20–25 % at 2 pg, 60–70 % at 8 pg and 90 % at 32 pg of standard neuropeptide. The linear range of the standard curve was 0.2 – 5 pg with 73 % and 76 % of saliva and urine samples, respectively, falling within the linear range. Cross-reactivities with arginine vasopressin (AVP), ring moieties and terminal tripeptides of both OXT and AVP and a wide variety of peptides comprising 3 (alpha-melanocyte-stimulating hormone) up to 41 (corticotropin-releasing factor) amino acids are <0.7 % throughout. The intra- and inter-assay variabilities were <10 %. Generally, all samples from an individual challenge were assayed in the same batch. Serial dilutions of urine samples containing high levels of endogenous OXT run strictly parallel to the standard curve indicating immuno-identity.

Cortisol analysis: Salivary and urinary CORT concentrations were measured by the laboratory of Prof. Kirschbaum at the Technical University Dresden using a commercially available chemiluminescence immunoassay with high sensitivity (IBL International, Hamburg, Germany). Intra- and inter-assay coefficients of variation were below 9 %. Four urine samples fell above the assay's detection limit (> 200 ng/ml, confirmed in a second run), and were removed from further statistical testing. Two saliva samples did not yield enough volume for both assays, thus only OXT concentrations were measured.

2.4. Statistical analyses

All data were analysed in R statistical software (R version 4.2.1, R Core Team, 2022) with $P \leq 0.05$ considered statistically significant.

To test for the effect of running on OXT and CORT concentrations in saliva and urine and on pulse rate in the two groups, linear mixed models (LMM) with Gaussian error structure were fitted using the function "lmer" of the package "lme4" (version 1.1–31). Hormonal concentrations were log-transformed for fitting the models to obtain homogenous and normally distributed residuals (assumptions of homoscedasticity and normality). To be able to compare salivary concentrations in response to running in the two groups, we used saliva sample S4 (collected after 3 ×10 min running) as the post running sample in G1 (samples collected after 10 (S2) and 20 min (S3), respectively, were excluded from the group comparison as they did not have a matching sample in G2). In addition to the test predictor (two-way interaction between timepoint and group) and their main effects, we included several control predictors: the age of the participants (in years; z-transformed co-variate to facilitate fitting the models and interpretation of model estimates), their running experience (self-report of how many times they run per month for at least 30 min; factor with 4 levels: never, less than 5 times, between 5 and 10 times, more than 10 times), their gender (combined with cycle phase for female participants; factor with 4 levels: male, female in follicular phase, female in luteal phase, female in menopause), and their pulse rate (in beats per minute, bpm; z-transformed co-variate; except for the model fitted to test the effect of running on pulse rate, where it was the response variable). We further included the random intercept effect of participant code to account for repeated sampling of the same participants.

All full models were compared against a null model lacking the test predictor and their main effects but retaining the control predictors and random effect using a likelihood ratio test ("anova" function in R) (Field et al., 2012). If the full-null model comparison did not reveal significance no further tests were performed (Forstmeier and Schielzeth, 2011). If the interaction term was not significant, a reduced model was fitted, lacking the two-way interaction but retaining the two main effect terms and the random effect structure. Diagnostic plots (residuals vs. fitted and qqplot) were visually inspected to examine assumptions of normality and homogeneity of variances for each model. Model stability was assessed by excluding levels of the random effect and comparing estimates derived with those derived for the full data set. Confidence intervals for each fixed effect predictor were calculated by parametric bootstrapping. Collinearity was assessed using the function "vif" of the package car and revealed no higher values than 2.4, indicating collinearity was not an issue. Post hoc tests were performed using the function 'glht' of the package 'multcomp' to investigate significant differences between factor levels. The package 'emmeans' was used to investigate significant interaction terms. P values were adjusted for multiple comparisons using the Tukey method. Oe heutput tables for full and reduced models including model stability estimates and confidence intervals for each predictor can be found in the SI.

To investigate the correlations between urinary and salivary hormone concentrations, correlations between absolute values were calculated at baseline (pre-run), post-run, and post-rest. In addition, concentration changes were calculated by subtracting the basal values from the post-run (delta1) and post-rest values (delta2) and used for correlational analyses. Data were first tested for normal distribution using the Shapiro-Wilk test. In case of not normally distributed data, Spearman's rank correlation coefficients were calculated. All correlations were run for each group (G1, G2) separately due to the differences in group composition and running scheme outlined above. For G1, the saliva samples collected after 3 ×10 min running (S4) were used as the post-run samples. To avoid inflating type I error rates due to performing multiple correlations on the same data, the significance thresholds (alpha levels) were adjusted according to Holm's sequential Bonferroni procedure (Abdi, 2010).

3. Results

Descriptive results of all measures can be found in the SI (Tables S-1 and S-2).

3.1. Effect of running on pulse rate

Pulse rate measures increased significantly in both groups from basal to post-run (estimate = 0.60, se = 0.03, P < 0.01, Table SI-4) and stayed significantly elevated compared to baseline also after 30 min of resting (estimate = 0.14, se = 0.03, P < 0.01). Participants of G1 (running 3 ×10 min) had overall significantly higher pulse rates than participants of G2 (running 1 ×60 min) (main effect of group, estimate = −0.22, se = 0.07, P < 0.01, Table SI-4).

3.2. Effect of running on OXT concentrations in urine and saliva

The interaction between timepoint and group did not reveal significance (ChiSq = 3.78, P = 0.15, Table SI-5). Instead, OXT concentrations in urine increased significantly from basal to post-run (levels of factor timepoint, estimate = 1.15, se = 0.28, P < 0.01, Fig. 2A), basal to post-rest (levels of factor timepoint: estimate = 0.39, se = 0.15, P = 0.02, Fig. 2A), and decreased from post-run to post-rest (levels of factor timepoint: estimate = −0.76, se = 0.23, P < 0.01, Fig. 2A) in both groups. We also identified a marginal effect of group insofar as G2 runners tended to have higher OXT concentrations than G1 runners in urine (factor group: estimate = 0.51, se = 0.25, P = 0.05, Table SI-6).

Similarly, in saliva, there was no interaction effect of group and

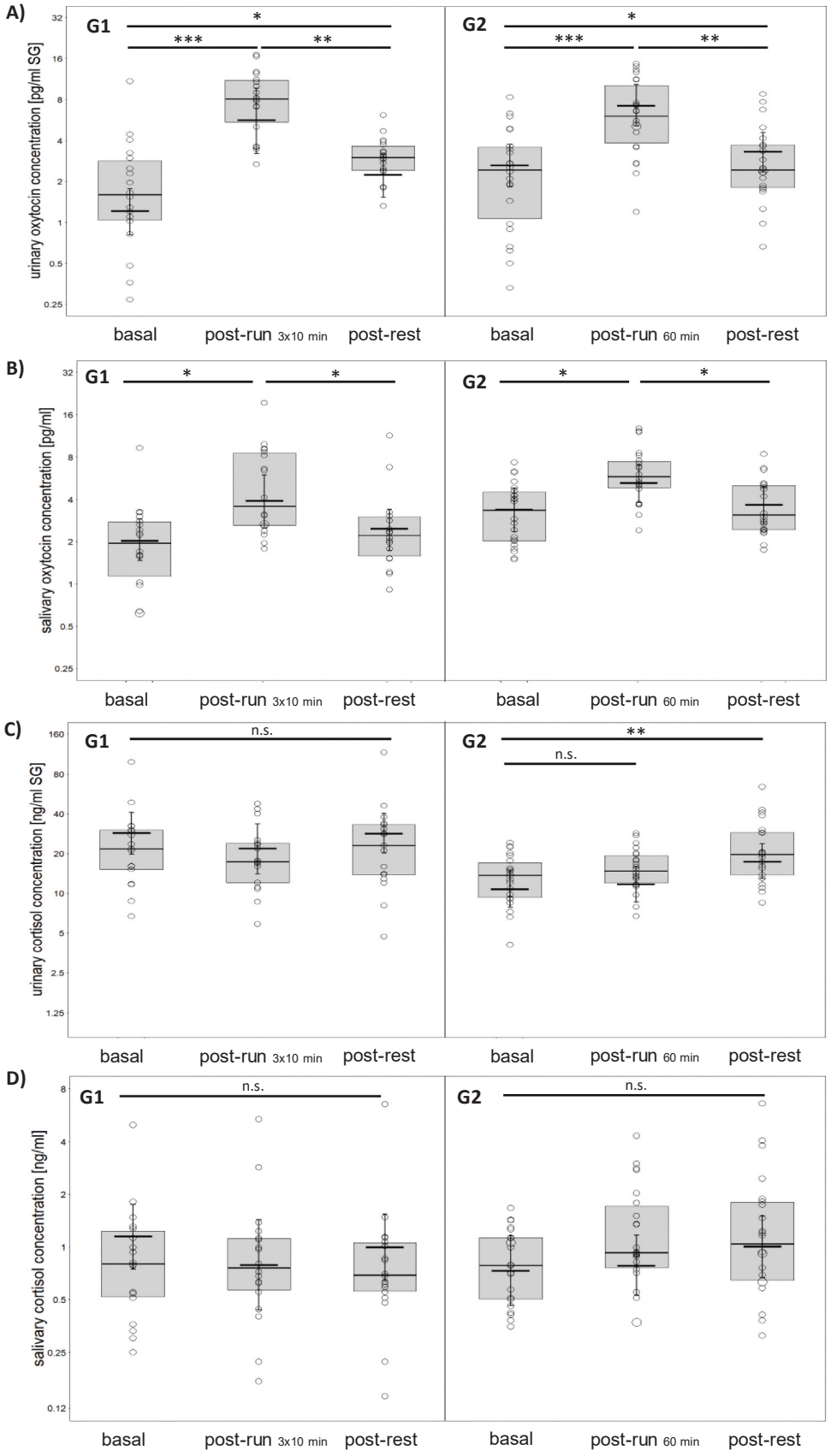

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Fig. 2. A-D. Effect of running on urinary oxytocin (A), salivary oxytocin (B), urinary cortisol (C) and salivary cortisol (D) concentrations in samples of healthy male and female volunteers under basal conditions (timepoint 'basal'), immediately after finishing a 3 ×10 min (G1, left panels) or a 60 min (G2, right panels) running period (timepoint 'post-run'), and after 30 min of resting following the run (i.e., 60 min post stressor onset for G1, and 90 min post stressor onset for G2) (timepoint 'post-rest'). Urinary hormone concentrations were corrected for specific gravity (SG). Shown are the datapoints (circles), medians and quartiles (rectangular boxes), as well as the fitted models with upper and lower confidence intervals (black lines). *** P < 0.001, ** P < 0.01, * P < 0.05, n.s. = not significant (P values adjusted for multiple comparisons).

timepoint (ChiSq = 2.06, P = 0.36, Table SI-7). Rather, OXT concentrations increased from basal to post-run (levels of factor timepoint: estimate = 0.47, se = 0.17, P = 0.02, Fig. 2B) and decreased from post-run to post-rest (levels of factor timepoint: estimate = −0.35, se = 0.15, P = 0.04, Fig. 2B) in both groups. Accordingly, both exercise intensities (running for 3x10 min or running for 60 min) resulted in a substantial rise in OXT concentrations in both urine and saliva.

3.3. Effect of age, pulse rate, gender and cycle phase, and running experience on oxytocin concentrations in urine and saliva

Age was negatively associated with urinary OXT concentrations (estimate = −0.52, se = 0.11, P < 0.01, Table SI-6, Fig. SI-1), i.e., older participants had lower urinary OXT concentrations compared to younger ones, but this effect was not present in saliva (estimate = −0.06, se = 0.13, P = 0.67, Table SI-8). In saliva, but not in urine (urine: estimate = 0.07, se = 0.12, P = 0.58, Table SI-6), a higher pulse rate tended to be linked to higher OXT concentrations (saliva: estimate = 0.15, se = 0.08, P = 0.06, Table SI-8). Gender and cycle phase had no statistically significant effect on OXT concentrations in urine (estimate = 0.55, se = 0.26, P = 0.16, Table SI-6) and saliva (estimate = −0.14, se = 0.31, P = 0.09, Table SI-8), and neither did running experience (i.e., the number of times participants reported to run for at least 30 min per month) with urinary (estimate = −0.34, se = 0.19, P = 0.27, Table SI-6) or salivary OXT concentrations (estimate = −0.27, se = 0.23, P = 0.54, Table SI-8).

3.4. Effect of running on cortisol concentrations in urine and saliva

In urine, CORT concentrations differed in response to running between the two groups (interaction group and timepoint: ChiSq = 9.63, P < 0.01, Table SI-9). While participants of G1 showed no significant changes from basal to post-run and post-rest CORT concentrations (ratio = 1.31, se = 0.32, P = 0.87 and ratio = 1.01, se = 0.13, P = 1, respectively, Fig. 2C), participants of G2 had a significant increase in urinary CORT concentrations from basal to post rest (ratio = 0.62, se = 0.07, P < 0.01, Fig. 2C), but not from basal to post run (ratio = 0.92, se = 0.18, P = 1, Fig. 2C). G2 participants also had lower CORT concentrations than G1 at baseline (ratio = 2.66, se = 0.86, P = 0.04, Fig. 2C).

In saliva, no significant changes were found in either group from basal to post run and post rest (non-significant full-null model comparison: ChiSq = 7.99, df = 5, P = 0.16, Fig. 2D).

3.5. Effect of age, pulse rate, gender and cycle phase, and running experience on cortisol concentrations in urine and saliva

Age did not significantly affect urinary (estimate = 0.02, se = 0.12, P = 0.86, Table SI-9) or salivary (estimate = 0.05, se = 0.16, P = 0.77, Table SI-10) CORT concentrations, neither did pulse rate (for urine: estimate = 0.05, se = 0.09, P = 0.58, Table SI-7; for saliva: estimate = 0.17, se = 0.12, P = 0.16, Table SI-10) or gender and cycle phase (for urine: estimate = −0.23, se = 0.29, P = 0.32, Table SI-9; for saliva: estimate = −0.01, se = 0.38, P = 0.77, Table SI-10). In urine, running less than 5 times per month tended to be associated with lower CORT concentrations than running between 5 and 10 times and more than 10 times per month (estimate = −0.55, se = 0.22, P = 0.06, Table SI-9, Fig. SI-2).

3.6. Correlations of oxytocin and cortisol concentrations in urine and saliva

Absolute OXT concentrations did not correlate significantly in urine and saliva at any timepoint in either group (Table 1). In contrast, the running-induced rise in OXT concentrations in urine and saliva from basal to post-run (delta1) showed a significant positive correlation in both groups (Spearman's rank correlation coefficient, rho = 0.77 and 0.81 respectively for G1 and G2, both P = 0.000, Table 2, Fig. 3A).

Absolute CORT concentrations in urine and saliva samples correlated significantly at baseline in G1 (Spearman's rank correlation coefficient, rho = 0.66, P = 0.005, Table 1). Post-rest they correlated significantly in G2 (rho = 0.75, P = 0.000 for G2, Table 1) whereas in G1 the correlation was not significant after adjusting the alpha level for multiple testing (rho = 0.59, P = 0.014, adj. alpha = 0.005, Table 1). The running-induced rise in CORT in urine and saliva from basal to post-run (delta1) did not reach significance after adjustment of the alpha level in G1 (rho = 0.57, P = 0.017, adj. alpha = 0.013, Table 2), but the changes in CORT from basal to post-rest (delta2) showed strong, positive correlations in both groups (rho = 0.72 and 0.79, respectively, in G1 and G2, both P < 0.01) (Table 2, Fig. 3B).

4. Discussion

The present study confirms that physical exercise is a robust stimulus for OXT secretion into the periphery as reflected by elevated concentrations in both saliva and urine in healthy human participants. Importantly, although absolute OXT concentrations in urine and saliva were not found to be correlated, the exercise-induced rise in OXT levels in both body fluids showed correlations in both groups of runners, i.e., independent of running experience and running patterns. Previous work using blood sampling demonstrated the activation of the oxytocinergic system in response to physical exercise (Landgraf et al., 1982, Hew-Butler et al., 2008). The current study now confirms that both urine and saliva are adequate substitutes for blood sampling to monitor peripheral OXT concentrations, both during basal activity of the OXT system and in response to relevant stimuli. Saliva and urine sampling provides crucial advantages over blood collection: it is non-invasive, does not require medical personnel and both can be collected stress-free by the study participants themselves, even allowing home collection.

Here, we show that OXT in saliva was elevated in relation to baseline after just 10 min of moderate running and further increased after 20, 30, and 60 min of running. This is in line with previous findings (de Jong

Table 1
Correlation coefficients of absolute oxytocin and cortisol concentrations in urine and saliva at different timepoints separated by group.

		Basal	Post run	Post rest
G1	**OXT: urine – saliva**	Rho=0.32, p=0.195	Rho=−0.14, p=0.584	Rho=−0.33, p=0.182
	CORT: urine – saliva	***Rho=0.66, p=0.005***	Rho=0.27, p=0.289	Rho=0.59, p=0.014*
G2	**OXT: urine – saliva**	Rho=0.08, p=0.709	Rho=0.31, p=0.165	Rho=−0.16, p=0.469
	CORT: urine – saliva	Rho=0.33, p=0.134	Rho=0.34, p=0.123	***Rho=0.75, p=0.000***

* Not significant after Holm-Bonferroni correction for multiple comparisons (adjusted alpha level = 0.005).

Table 2
Correlation coefficients of relative changes (delta) in oxytocin and cortisol concentrations in urine and saliva separated by group.

		Basal – Post run (delta1)	Basal – Post rest (delta2)
G1	**OXT: urine – saliva**	***Rho=0.77, p=0.000***	Rho=−0.24, p=0.327
	CORT: urine – saliva	Rho=0.57, p=0.017*	***Rho=0.72, p=0.001***
G2	**OXT: urine - saliva**	***Rho=0.81, p=0.000***	Rho=0.19, p=0.389
	CORT: urine - saliva	Rho=0.38, p=0.085	***Rho=0.79, p=0.000***

* Not significant after Holm-Bonferroni correction for multiple comparisons (adjusted alpha level = 0.013).

et al., 2015), and extends them to include the measurement of OXT in urine. The OXT increase in saliva peaked after three 10-min intervals of running (G1) and remained elevated compared to baseline levels even after 30 min of post-run resting, i.e., 60 min after the start of the exercise. In urine, OXT levels peaked post running and remained elevated compared to baseline even after 30 min of post-run resting. The increase in urinary OXT concentrations was robust, as 36 out of 40 (90 %) participants showed a substantial rise from basal to post-run (average percent rise: 645 % or 6.45-fold increase). In saliva, 27 out of 40 (67.5 %) participants showed a rise from basal to post-run (average percent rise: 148 % or 1.48-fold increase). As such, the results concur with previous findings (de Jong et al., 2015): Salivary OXT concentrations showed an approximately 2.5-fold rise from basal to post-stimulus (running, sexual self-stimulation and psychosocial stress) levels and a relative increase of 20 % or more was detected in 53 out of 61 samples (86.8 %). The much larger increase in urinary than salivary OXT concentrations most likely reflects the accumulation of OXT in the urinary bladder and emphasizes the need to standardize stimulus-urination intervals in study designs based on urine sampling (see Verspeek et al., 2021, for similar findings on CORT concentrations). The fact that an OXT increase was detected in 97.5 % of participants' urine samples, but only 67.5 % of participants' saliva samples, may relate to the possible pulsatile release and short half-time of OXT: if the OXT signal is

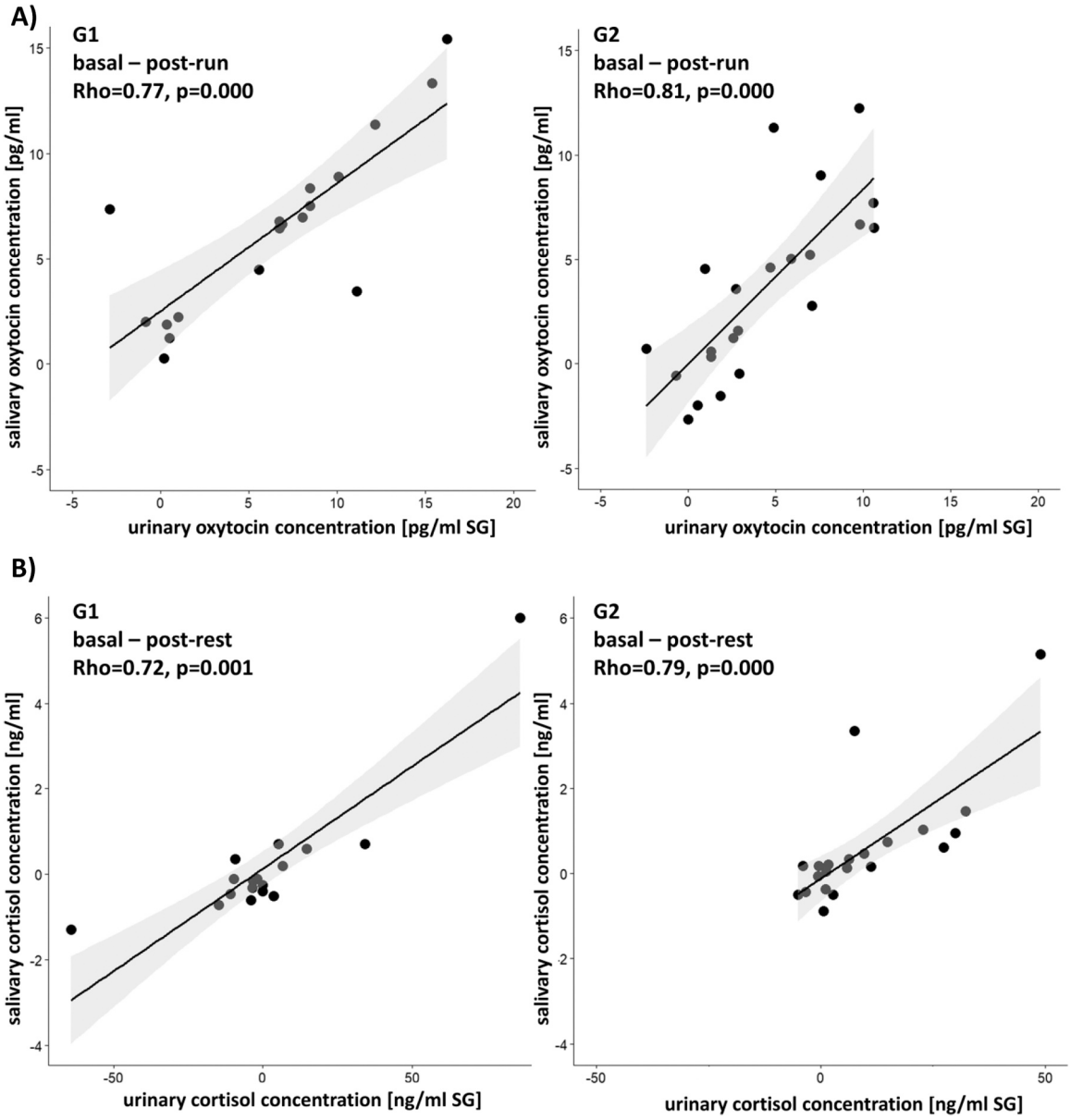

Fig. 3. A-B. Correlation plots of A) oxytocin concentration changes (delta1) in urine and saliva from basal to post-run timepoints for G1 (left) and G2 (right), and B) cortisol concentration changes (delta2) in urine and saliva from basal to post-rest timepoints for G1 (left) and G2 (right). Shown are the datapoints (black dots), the regression line (black line), and the 95 % confidence interval (in grey).

relatively transient in saliva, it may be missed in the moment of sampling saliva. However, whether OXT is secreted in a pulsatile fashion (as found in response to the suckling stimulus in women, Ueda et al., 1994, and more recently also in male participants at rest, Baskaran et al., 2017) during physical exercise is not known. Consequently, urine offers a stronger, more stable and more robust OXT signal than saliva, when monitoring of the precise temporal dynamics of secretory processes does not play a dominant role in the research design.

Urinary CORT concentrations were significantly elevated compared to baseline in the long-running group (G2), but, surprisingly, not right after 60 min of continuous running (post-run), but only after the 30-min resting period (post-rest, i.e., 90 min after the onset of exercising). In the short-running group (G1) no significant rise in CORT concentrations was detected in either urine or saliva, neither post-run nor post-rest. Both groups taken together, out of 39 runners (urine samples of one participant were excluded due to exceeding the detection range of the assay), 20 (51 %) showed an increase (average percent rise: 35 % or 0.35-fold) in urinary CORT from basal to post-run, and 22 (56 %) showed a rise (average percent rise: 136 % or 1.36-fold) in urinary CORT from basal to post-rest. Thus, it seems that CORT responses to physical exercise appear rather delayed in human urine samples. This seemingly contrasts with rats and mice, which reliably respond with significantly increased plasma corticosterone concentrations at 5 min post exposure to the elevated plus-maze (Neumann et al., 1999) and immediately after exposure to a brief 3-min swim stress (Feng et al., 2021), although peak plasma corticosterone concentrations were measured at 15 min (vehicle-treated control animals, Neumann et al., 1999) and 30 min post stressor (Feng et al., 2021), and it is currently unknown, when the peak in urinary corticosterone would appear in these animals. Previous results of non-human primates, cats, and dogs administering radio-labelled cortisol showed that the clearance window into urine may indeed be rather long, i.e., several hours (Bahr et al., 2000; Schatz and Palme, 2001). In humans, it has been demonstrated that intravenous administration of hydrocortisone results in a rapid peak of cortisol in plasma (within half an hour), saliva (within 1 hour) and urine (within 1–2 hours), whereas oral administration of the same drug resulted in delayed peaks (plasma: 1.7 hours, saliva: 1–2 hours, urine: 1–3 hours) (Jung et al., 2014). The present study shows that urinary CORT levels in healthy human participants started to increase significantly from baseline after 1.5 hours (90 min) of stressor onset.

Salivary CORT concentrations were not significantly affected by our paradigm, although 21 runners (53 %; 15 from G2) had an increase in CORT levels from basal to post-run (average percent rise: 157 % or 1.57-fold) and 22 runners (56 %; 16 from G2) had an increase from basal to post-rest (average percent rise: 185 % or 1.85-fold). These results both from urinary and salivary CORT demonstrate that the duration, and likely the intensity, of exercise affects adrenal CORT secretory responses. A previous study on marathon runners using saliva samples to monitor adrenal activity found that although CORT concentrations increased during the run, they peaked only 30 min after completion of the run and returned to baseline within 4 hours (Cook et al., 1987). Interestingly, the authors also reported a sudden surge of salivary CORT levels after 21 miles (approx. 34 km), which they relate to the phenomenon known by runners as “hitting the wall”. This usually refers to the point, when the body has depleted its stored glycogen and depends largely on an individual’s training background, whether glycogen stores are replenished during exercising (by consuming carbohydrates), as well as the intensity of the physical activity. In the present study, participants of G2 averaged about 9–10 km, whereas the round course for G1 was approx. 1.5 km, which the runners covered 3 times with a 3-min gap in between. These rather short distances likely explain the lack of robust CORT increases particularly in G1.

The present results confirm that the temporal dynamics of OXT and CORT concentrations in urine and saliva following a relevant stimulus differ. Whereas OXT concentrations increased rapidly at the first possible time point assessed, i.e., 10 min and 30 min after the start of running in both saliva and urine, respectively (G1), CORT concentrations increased much slower and were only significantly elevated in urine after the 30-min resting period, i.e., 90 min after the participants had started to run (G2). A previous study in bonobos showed that CORT concentrations peaked only after approx. 2.5 hours following a stressor in urine and saliva (Verspeek et al., 2021). In dogs, a radiolabelling study by Schatz and Palme (2001) demonstrated that urinary CORT peaked approx. 3 (plus or minus 1 hour) hours after a stressor, and in humans, salivary OXT peaked considerably faster than salivary CORT in response to a psychosocial stressor (Trier Social Stress Test, TSST, de Jong et al., 2015). This contrasts with the assumption that CORT as a lipophilic steroid hormone may pass quicker through capillaries into saliva or urine than the hydrophilic neuropeptide OXT (Gröschl, 2008). On the other hand, CORT is highly protein-bound in plasma with a half-time of approx. 90 min (Walker and Seckl, 2001) and heavily metabolised (Wang et al., 2018), which could delay its appearance in peripheral substrates such as saliva and urine. In expectation of an earlier peak timepoint, however, we did not collect any more samples after the resting period and, therefore, cannot determine, when exactly CORT concentrations would have reached their peak in this study. Such information is vital for future study designs, especially when both measures of OXT and CORT are required and should be tested for each intended study species and stimulus prior to data collection.

Another aim of the current study was to investigate correlations between hormonal concentrations in urine and saliva. Interestingly, we found that the rise in OXT concentrations (delta1) was significantly correlated in urine and saliva. In other words, participants with a strong increase from basal to post-run OXT concentrations in saliva also had a strong increase in urinary OXT levels. In contrast, the absolute OXT concentrations in urine and saliva were not correlated at any timepoint. As such, our results align with previous studies that found significant correlations between ‘percent change from baseline’ levels of urinary and plasma OXT levels in healthy adults post-MDMA administration and ASD patients post-intranasal OXT administration (Francis et al., 2016), but no correlations between baseline OXT levels (Francis et al., 2016, Feldman et al., 2011). These discrepancies may stem from the different timescales captured by plasma/saliva vs. urine, as already outlined above. Hence, the OXT signal in plasma and saliva may be too variable with a high temporal dynamics compared to urinary OXT levels, leading to non-correlated results when comparing single timepoint measurements (Francis et al., 2016).

For CORT, the rise from basal to post-rest concentrations (delta2) was significantly correlated in urine and saliva, as were absolute CORT concentrations in urine and saliva at baseline (only in G1, hence this result should be interpreted with caution) and post-rest (in G2). This difference between OXT and CORT with respect to the correlation of urine and saliva concentrations at specific timepoints is noteworthy and has implications for future studies. Hence, we cannot draw inferences about urinary OXT concentrations from OXT levels measured in saliva, and *vice versa*. As both urinary and salivary OXT originates from plasma, either differences in the dynamics of transport of OXT molecules into saliva and into urine, or local metabolism may explain this finding. Yet, the strong positive correlations between the delta rise in urinary and salivary OXT concentrations demonstrate that following a biologically relevant stimulus of OXT release (in this case, physical exercise) measuring OXT in either urine or saliva provides meaningful results. Thus, the choice of substrate should be made based on practical considerations relating to study design.

Interestingly, of the control variables included in the statistical analyses, we found that age had a significant effect on urinary OXT concentrations, with older participants having lower OXT levels in urine than younger ones (Fig. SI-1). This aligns with previous work in rodents, which has demonstrated an age-related decline in circulating (plasma) OXT levels (Elabd et al., 2014). Another notable finding was the lack of a gender and cycle phase effect: Based on previous meta-analytical work (Engel et al., 2019) we would have expected to find higher OXT

concentrations in women during their follicular than their luteal phase. In fact, for saliva, we found this to be the case, but the effect did not reach significance, possibly due to a large proportion of menopausal women in our study population.

Finally, based on the present findings, we would like to address practical implications for experimental research attempting to measure peripheral oxytocinergic and glucocorticoid responses. An advantage of saliva sampling is the higher temporal resolution, because samples can be collected more frequently, whereas urine samples need to be spaced out temporally to allow for sufficient sample volume. This may introduce additional noise, as it is rarely possible to fully control the environment of participants over a longer time course. On the other hand, in urine samples the running-induced rise in OXT was found to be more robust, as reflected by a higher average percent increase, and by more participants showing such rise, than in saliva samples. In contrast to urine, saliva samples reflect points in time (similarly to plasma samples) rather than accumulative measures. Thus, the temporal dynamics of OXT secretion may, therefore, be better captured by sampling from saliva, whereas the accumulative secretory response to a given stimulus is better reflected in urine samples. On the other hand, urinary hormone concentrations are sensitive to changes in the body's hydration status as a direct function of the renal system counteracting dehydration by retaining water via urine concentration. Thus, urinary hormone concentrations always require a correction for the osmolarity of the sample. This can be achieved by either measuring creatinine and expressing the hormone as a ratio of hormone to creatinine, or specific gravity (SG) of the sample. Using Miller et al.'s (2004) formula to arrive at the so-called SG correction factor, an individual with highly concentrated urine and, thus, higher urinary SG than the population average would end up with "artificially" lower hormone concentrations after applying the correction. To counteract this issue, one may decide to exclude participants with high SG values from statistical analyses (e.g., samples with more than two standard deviations from the average SG). Salivary hormone concentrations in contrast do not usually require a correction for concentration (but see Lindsay and Costello (2017) for suggestions how to incorporate salivary flow rate into salivary metabolite measures), but might be sensitive to other confounding variables, such as certain foods or drinks, and even the swabs used to collect the samples (MacLean et al., 2018). Moreover, it needs to be mentioned that urine samples need an extraction procedure prior to radioimmunological analysis, whereas saliva samples do not (see above; de Jong et al., 2015).

5. Conclusions

To conclude, urine appeared to be more robust than saliva to assess OXT and CORT concentrations following a physical stressor in healthy human adults. Yet, saliva samples may be more useful, when higher temporal resolution or multiple samples are required. As urinary hormone concentrations may be confounded by differences in osmolarity, it is crucial to correct for creatinine concentration or SG of the samples, and to exclude extreme outliers. In the current study, we demonstrate strong positive correlations between the increase of OXT and CORT concentrations in urine and saliva samples from basal to post exercise and confirm the longer time-lag from stimulus to rising CORT compared to OXT levels in the periphery.

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CRediT authorship contribution statement

Catherine Crockford: Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Gwendolyn Wirobski:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Inga D. Neumann:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Tobias Deschner:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of Competing Interest

None.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.psyneuen.2024.107144.

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