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Intra-individual stability of longitudinal urinary steroid profiles in Swedish athletes

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Abstract

The steroid module of the athlete biological passport (ABP) aims to detect doping with endogenous steroids by longitudinally monitoring epitestosterone (E), testosterone (T), and four metabolically related steroids and their ratios. There are large variations in the urinary levels of the androgen metabolites due to genetic polymorphisms, drug use, menstrual cycle, and other factors. In this study, we aimed to increase our understanding of the natural, within-individual variations of the established ABP markers in males and females over time, looking at samples collected both in and out-of-competition (IC/OOC). Urinary steroid profiles from 323 Swedish athletes, with at least five samples per athlete, were extracted from ADAMS together with information on type of sport, IC/OOC, and time of day. Data were analyzed using coefficient of variation (CV%) to examine within-subject variability and linear mixed effects models to estimate within-subject change in the metabolites over time. The metabolites and ratios expressed higher individual CV% in females (23–56) than in males (18–39). Samples taken OOC showed larger intra-individual variations than samples collected IC for most of the ABP metabolites in both sexes. The median concentrations were higher IC for some metabolites, particularly testosterone being 52% higher among females. Time of day influenced the intra-individual variation of the urinary steroid profile with decreases in androgen metabolites over time, if measured in evening versus daytime. These findings can aid in the testing strategies and interpretation of the steroidal module of ABP.

KEYWORDS

athlete biological passport, doping, testosterone, urinary steroid profile

1 | INTRODUCTION

The steroidal module of the athlete biological passport (ABP) aims to detect doping with endogenous steroids, for example, testosterone, by longitudinally monitoring the concentrations and ratio of testosterone (T), epitestosterone (E), and T/E, as well as four other steroid concentrations and ratios, that is, 5α-androstane-3α,17β-diol/5β-androstane-3α, 17β-diol (5αAdiol/5βAdiol), androsterone/T (A/T), A/etiocholanolone (A/Etio), and 5αAdiol/E (1). Studies show that the steroid ABP module increases the chance to detect testosterone administration as compared with population-based cut-off T/E values in men[1,2] and women.[3,4] This is also demonstrated by an increased T

detection rate according to yearly testing figures published by the World Anti-Doping Agency (WADA). Nevertheless, more knowledge is still warranted for better interpretation of natural variation of the ABP biomarkers.

In addition to doping, other factors may influence the urinary steroid profile. For example, the inter-individual variation of T/E ratio is largely dependent on copy number variation of the UGT2B17 gene.[5] Microbial degradation, administration of alcohol, and various permitted drugs, for example, antifungals and aromatase inhibitors, may influence the urinary steroid profile.[6] Subsequently, markers for these agents are analyzed together with the steroid profile to improve the interpretation of the passport result. In women, hormonal contraceptives and pregnancy also affect the urinary steroid profile, particularly the excretion rate of E.[7–9] The concentrations of the urinary steroid metabolites are highly dependent on the menstrual cycle; consequently, higher variations of the androgen metabolites and ABP ratios are found in female athletes.[10] Moreover, inherently lower metabolite concentrations in female urine are more prone to methodological variation; that is, at lower concentrations, a higher level of uncertainty is accepted according to the guidelines,[11] which may result in a higher coefficient of variation (CV).

In a large register study including 11,000 urinary steroid profiles, the impact of confounders on the *inter-individual* variation of ABP steroid ratios was studied. It was noted that 16% of the variation could be explained by the following confounders: age, type of sport, in-competition (IC) or out-of-competition (OOC), time of day, and time of year.[10] In addition, a recent population study of 10,000 and 12,000 samples collected IC and OOC, respectively, showed that samples taken IC displayed higher T/E, A/etio, and 5αAdiol/5βAdiol IC than OOC.[12] However, of greater importance is to study the *within-subject* variations of the ABP biomarkers to understand how the testing time point (IC/OOC, circadian time) could impact the adaptive steroid module of the ABP. Therefore, we conducted longitudinal analyses of repeated measures of urinary profile test results from Swedish athletes that have been subject to doping control five times or more between 2014 and 2020. The specific aims were to study the intra-individual variations of ABP biomarkers in relation to time of day and to examine if testing performed in IC or OOC in male and female athletes was associated with a change in the biomarkers.

2 | MATERIAL AND METHOD

A data set of all steroid passports from Swedish athletes from 2014 until 2020, who have at least five samples in their ABP, were included. Even though all samples have been reported as negative, the doping/not doping status of endogenous anabolic steroids remains unknown. The urinary steroid profiles, including all metabolites (i.e., T, E, A, Etio, 5αAdiol, and 5βAdiol) and ratios (i.e., T/E, 5αAdiol/5βAdiol, A/T, A/Etio, and 5αAdiol/E), were extracted from the WADA's Anti-Doping Administration and Management System (ADAMS) to Microsoft Excel,

and the identity of the athletes removed and replaced with a code to know which profiles belong to the same athlete. The urinary steroid profiles were analyzed using gas chromatography coupled with tandem mass spectrometry (GC–MS/MS) following the current versions of the Technical Documents for Endogenous Anabolic Androgenic Steroids.[13,14] All steroid concentrations were corrected for specific gravity. Concentrations below the limit of quantification (LOQ) are according to WADA requirements 100 ng/mL for A and Etio, 1 ng/mL for T and E, and 10 ng/mL for the diols.[11] Additional information extracted from ADAMS included type of sport, IC or OOC testing, and time of day of sampling. The time of day when the samples were collected were sub-grouped into morning (05:00–06:59), daytime (07:00–18:59), and evening (19:00–01:32).

To further anonymize the data set, the sports disciplines were divided into sport classifications according to the recommendations from an exercise physiologist. The sport categories were power/strength sports, VO$_2$ max endurance sports, muscular endurance sports, ball and team sports, fight sports, aiming sports, and gymnastics sports. The full list of what sports belong to what category can be found in the supplemental material of the study by Mullen et al.[10]

The study has been approved by the Ethics Review Board, Stockholm (DNR 2017/516-31/4).

3 | STATISTICAL ANALYSES

The CV% and median values of the metabolites and ABP ratios in each athlete were calculated. The majority of the within-subject data were normally distributed, whereas the distribution of individual CV% and medians showed non-normal distributions, so that the CV% and median values were compared between males and females with Mann–Whitney. When comparing each athletes' levels/ratios between IC and OOC, Wilcoxon paired tests were done, including at least two analyses in each group. Multiple comparison testing was needed as the analyses were performed in separate models. The analyses were done using GraphPad Prism, version 7. Results were considered significant for $p < 0.05$ (two-sided tests).

To further investigate the change in each metabolite/ratio over time at the intra-individual level, while adjusting for confounders, separate linear mixed effects models for each exposure on each outcome were employed in R (version 4.1.3, Austria, Vienna) using package lme4.[15] The models included random slopes and intercepts at the subject level, with an unstructured variance–covariance matrix pattern, using restricted maximum likelihood in order to estimate beta-coefficients, and taking missings into account. Time of day and IC/OOC were mutually adjusted for, with type of sport included as a confounder. When studying the association between IC/OOC on metabolite change over time, IC/OOC, time of day, and type of sport were included as fixed effects and IC/OOC and subject as random effects. In the models, testing time of day on change in the metabolites, time of day, IC/OOC, and type of sport were included as fixed effects and time of day and subject as random effects. All models were stratified by sex.

4 | RESULTS AND DISCUSSION

4.1 | Study population

In Table 1, the demographic information of the study population can be found. A sample of 323 athletes (157 males and 166 females) were included in this study with a total of 4218 steroid profiles. Even though all samples have been reported as negative, and all the steroid passports have been reviewed by an Athlete Passport Management Unit (APMU), the true doping/not doping status remains unknown. However, the risk of undetected doping with endogenous anabolic steroids to such an extent that it will impact the results in this large data set is considered very low.

The ethnicity of the athletes is not known, but the majority of the athletes in the study population are of Caucasian descent.

Most athletes were engaged in VO₂ endurance sports (47%), followed by muscular endurance sports (17.7%), and power/strength sports (19.5%) (Table 1). There were more samples from men in the VO₂ max endurance sports and ball/team sports categories, whereas

the other categories had more samples from women. The median numbers of samples analyzed per individual were lowest among athletes engaged in ball sports, between 5 and 9 samples/athlete, whereas highest numbers of samples per athlete were noted in the VO₂ endurance category (median 13 in both males and females) (Table 1).

In total, 893 samples were collected IC and 3315 samples OOC (Table 1). With the exception of the ball and team sports, a higher number of samples were collected OOC than IC in all sports categories. This is in line with the sample collection approach in other ABP studies.[12,16] The distribution of samples between sports and individuals was based on Anti-Doping Sweden's (ADSE) risk assessment and the number of athletes in the Registered Testing Pool (RTP) who reported their whereabouts on a daily basis. Athletes that belong to a high-risk sport and/or belong to the RTP are tested more often. The international-level athletes that participate in international competitions are also tested more often IC by the international federation. Only samples with ADSE as Testing Authority are included in this study, hence the discrepancy in the number of samples between IC and OOC tests in some steroid profiles.

Most of the analyses were done in the Stockholm Doping Control Laboratory (95.3%) followed by Oslo, Norway; Cologne, Germany; and Salt Lake City, USA. Approximately 1% was analyzed by each of these doping control laboratories, and 2% analyzed by 15 other doping control laboratories.

TABLE 1 Demographic table showing the number of male (n = 157) and female (n = 166) athletes in each sport category and the number of steroid profiles measured IC and OOC for each sport category (italics).

Sports discipline	Males	Females
VO₂ max endurance sports	n = 85	n = 67
Median samples/athlete (range)	13 (6–31)	13 (6–35)
IC	*260*	*227*
OOC	*928*	*756*
Muscular endurance sports	n = 20	n = 37
Median samples/athlete (range)	11 (6–24)	12.5 (6–22)
IC	*53*	*80*
OOC	*200*	*431*
Power/strength sports	n = 28	n = 35
Median samples/athlete (range)	9.5 (7–29)	10 (7–22)
IC	*73*	*103*
OOC	*244*	*328*
Fight sports	n = 14	n = 23
Median samples/athlete (range)	8.5 (6–15)	11 (6–23)
IC	*22*	*41*
OOC	*103*	*240*
Gymnastics	n = 3	n = 4
Median samples/athlete (range)		8.5 (8–13)
IC	*3*	*6*
OOC	*30*	*32*
Ball/team sports	n = 7	n = 0
Median samples/athlete (range)	7 (5–9)	
IC	*25*	*0*
OOC	*23*	*0*

Abbreviations: IC, in-competition; OOC, out-of-competition.

4.2 | Intra-individual variation in ABP metabolites and ratios in males and females

The intra-subject variations of the ABP metabolites and ratios for each athlete with at least five samples were calculated and compared between males (n = 155) and females (n = 166). Eleven females displayed T concentrations below the LOQ. These females are not included in the analyses of T and A/T, whereas the T/E ratios determined by the laboratories from corrected peak heights/areas were used, as described in the WADA technical document.[11]

The CV% was significantly higher in women for T, E, A, and 5βAdiol (Table 2). The largest differences in intra-individual variation were seen for E in females. The large CV% may to some degree reflect the impact the menstrual cycle exerts on the steroid metabolites, in particular on E.[17] Urinary E concentrations are strongly associated with both estrogen and progesterone levels peaking during the ovulation and the luteal phase.[4,18] Another confounding factor not accounted for here is the use of hormonal contraceptives, which is known to affect the excretion rate of E.[8] Consequently, the ratios with E (T/E and 5αAdiol/E) showed the highest fluctuation in women and were in line with previous intra-individual CV% values.[10]

The individual median concentrations of all the metabolites, except Etio, were higher in males than in females and similar to reference ranges noted in other athlete population studies.[10,19,20] Individual median ratios showed significant differences between males and females: females have higher A/T and 5αAdiol/E and lower T/E,

TABLE 2 Individual CV% and median SG corrected steroid concentrations (ng/mL), as well as interquartile range of male ($n = 157$) and female ($n = 166$) athletes tested ≥5 times between 2014 and 2020.

		Males	Females	
T (M157/F155)	CV%	36.82 (29.44–44.85)	40.89 (33.81–54.11)	****
	ng/mL	27.51 (19.57–37.86)	4.20 (3.11–6.02)	****
E (M157/F166)	CV%	38.28 (31.60–45.94)	56.52 (43.85–72.08)	****
	ng/mL	19.76 (13.46–29.82)	5.34 (3.03–7.44)	****
A (M157/F166)	CV%	32.43 (26.93–39.59)	34.68 (28.07–43.08)	*
	ng/mL	2458 (1892–3,202)	1448 (1028–1950)	****
5αAdiol (M157/M166)	CV%	35.85 (29.61–42.71)	34.25 (29.26–43.41)	n.s.
	ng/mL	43.56 (29.67–60.48)	15.72 (11.81–19.77)	****
5βAdiol (M157/F166)	CV%	39.15 (31.25–48.35)	42.64 (34.53–53.10)	**
	ng/mL	115.7 (68.1–184.1)	51.17 (31.97–91.39)	****
Etio (M157/M166)	CV%	33.30 (26.01–40.29)	34.65 (28.33–41.44)	0.0560
	ng/mL	1909 (1549–2452)	1850 (1390–2341)	n.s.
T/E (M157/F166)	CV%	18.05 (15.35–22.27)	40.39 (33.84–51.23)	****
	Ratio	1.44 (0.85–2.00)	0.90 (0.60–1.36)	****
A/T (M157/155)	CV%	26.40 (22.63–32.66)	28.82 (22.66–34.57)	n.s.
	Ratio	84.42 (65.86–128.30)	329.7 (90.3–9,353)	****
A/Etio (M157/F166)	CV%	22.39 (18.58–27.39)	23.48 (20.32–29.26)	0.0502
	Ratio	1.27 (0.97–1.67)	0.81 (0.61–1.08)	****
5αAdiol/5βAdiol (M154/160)	CV%	25.94 (19.42–33.23)	35.12 (26.85–48.01)	****
	Ratio	0.41 (0.29–0.59)	0.30 (0.18–0.48)	****
5αAdiol/E (M157/F166)	CV%	28.54 (21.35–37.00)	46.95 (37.73–58.17)	****
	Ratio	2.17 (1.45–3.20)	3.56 (2.19–4.99)	****

Abbreviation: n.s., not significant.
*$p < 0.05$. **$p < 0.01$. ****$p < 0.0001$.

TABLE 3 Percent changes in-competition (IC) compared with out-of-competition (OOC) for median SG corrected steroid concentrations (ng/mL) and ratios of male ($n = 115$) and female ($n = 103$) athletes tested at least twice both IC and OOC.

	Males	Females
IC % change for median concentrations and ratios		
T	−2% n.s.	52% ****
E	−19% ****	16% **
A	16% ****	23% ****
Etio	1% n.s.	−4% n.s.
5αAdiol	−4% n.s.	9% n.s.
5βAdiol	−32% ****	−23% ****
T/E	8% ****	34% ****
A/T	19% ****	−26% ****
A/Etio	25% ****	20% ****
5αAdiol/5βAdiol	7% ***	−10% *
5αAdiol/E	36% ****	42% ****

Abbreviation: n.s., not significant.
*$p < 0.05$. **$p < 0.01$. ***$p < 0.0001$.

A/Etio, and 5αAdiol/5βAdiol ratios compared with males. This is in line with a previous study where the T/E ratios were higher among males than females expressing UGT2B17.[21] The UGT2B17 del/del genotype frequency herein is not known. Sixteen males (10.2%) and 16 females (9.6%) were phenotypically identified as del/del based on T/E < 0.4 in males and T < LOQ in females. The frequency of athletes phenotypically identified as UGT2B17 del/del was in line with previously reported allele frequencies in Caucasians.[22]

4.3 | Intra-individual variation in ABP metabolites and ratios IC and OOC

The IC and OOC samples were analyzed in subjects with at least two values (males $n = 115$, females $n = 103$) in both the IC and OOC group. Table 3 shows how the metabolite concentrations and the ABP ratios increase or decrease in samples collected OOC compared with IC. In males, all the ABP ratios and A appeared significantly higher IC, whereas E and 5βAdiol were lower IC. No difference was seen in male T levels. In contrast, the largest difference in females was seen in T levels, being 52% higher IC compared with OOC. Figures 1 and 2 illustrate the differences between samples collected IC and OOC in one randomly selected male and female, respectively.

It could be speculated that the samples collected IC are more homogenous compared with the OOC samples. The majority of IC samples are non-fasting, daytime samples collected after strenuous activity, and OOC samples are collected at different times of day, after
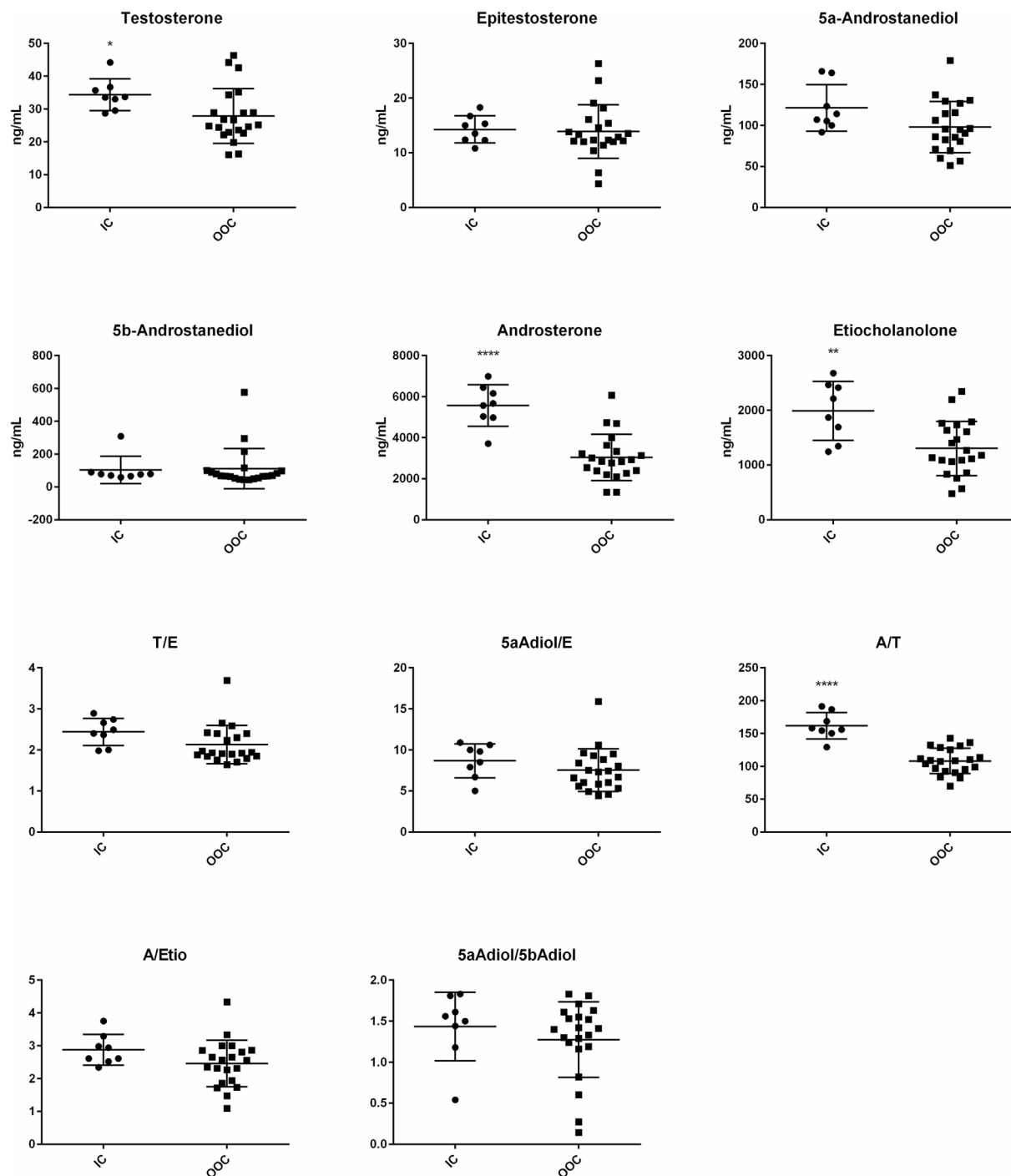
FIGURE 1   Differences in concentrations and ratios between samples collected in-competition (IC) and out-of-competition (OOC) in a male athlete randomly selected from the cohort. The asterisks show significant differences between IC and OOC using Mann–Whitney test. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

a full night's sleep or after a full day of movement, after a heavy meal or after fasting for several hours. It has been shown that active male and female athletes excrete lower levels of the urinary androgens in comparison with sedentary controls,[18,23] and the training volume among athletes showed negative correlations with the urinary excretion rate of the ABP metabolites.[18] It may be speculated that athletes excrete a higher percentage of their androgens in sweat. It has been

shown that sulfate conjugated androgens, in particular, are highly abundant in axillary sweat.[24]

Moreover, a significant association between whether the samples were collected IC or OOC and the change in the metabolite over time could be seen when using a linear mixed model, adjusting for time of day and type of sport (Table 4). In male athletes, compared with OOC, there was a statistically significant increase in 5α-diol/5β-diol, A, T/E,
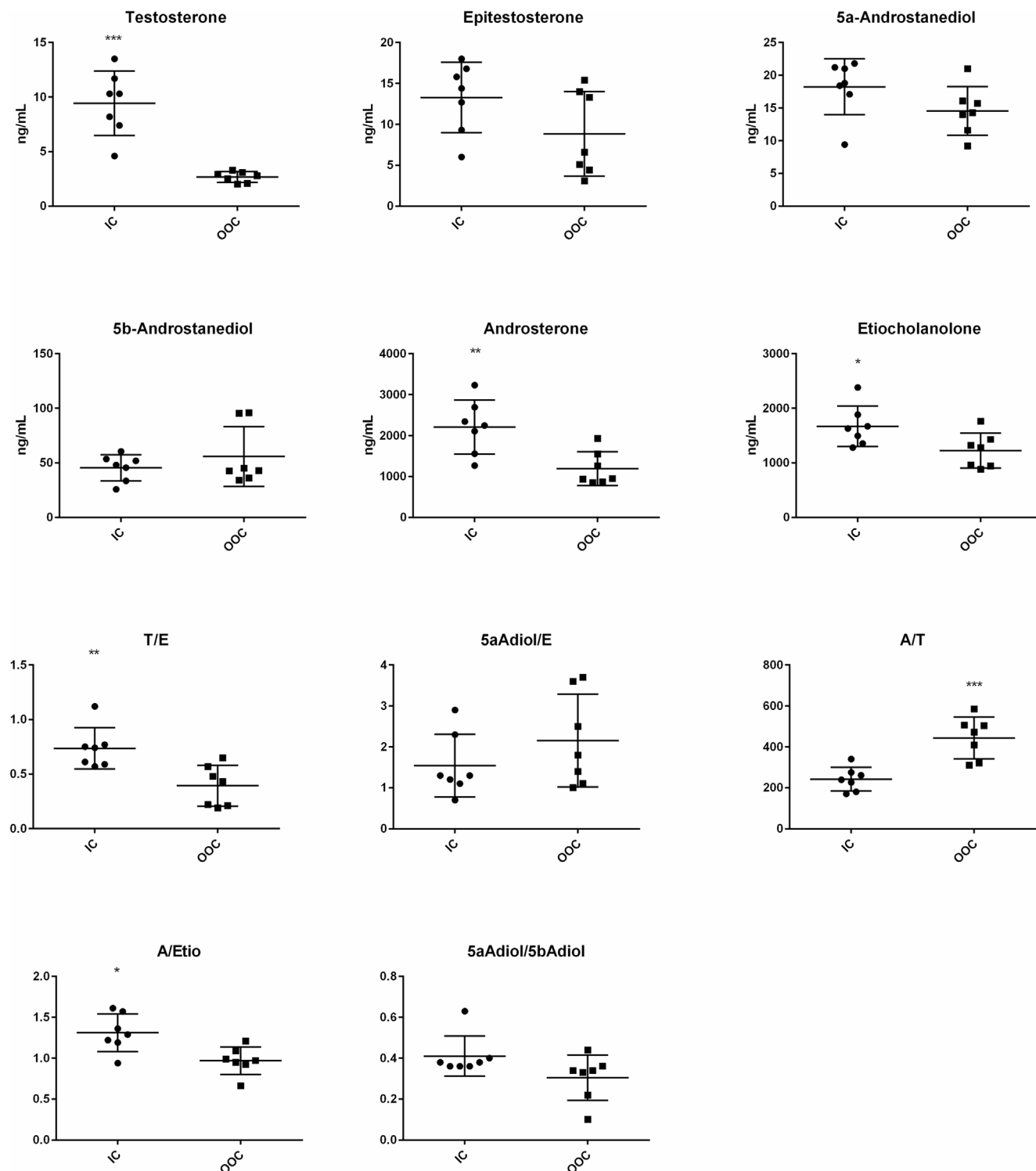
FIGURE 2 Differences in concentrations and ratios between samples collected in-competition (IC) and out-of-competition (OOC) in a female athlete randomly selected from the cohort. The asterisks show significant differences between IC and OOC using Mann–Whitney test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

5α-diol/E, A/T, and A/Etio over time but decrease in 5βAdiol, E, and Etio when collected IC. These results were similar for women, except that E and Etio were not significant, and 5αAdiol/E and A/T decreased over time. T increased over time for women IC compared with OOC, but no statistically significant change was seen for testosterone in men.

Mental stress has been suggested to explain higher T (and T/E ratios) in samples collected IC.[12] However, stress is a complex condition that is often accompanied with increased cortisol production and a reduction in T, at least in endurance events.[25,26] The urinary steroids in relation to stress have been poorly studied. In a study including

TABLE 4 Results from linear mixed effects models showing intra-individual changes in metabolites over time in relation to whether collected in-competition (IC) compared with out-of-competition (OOC; reference) and stratified by sex.

Metabolite	Males		Females	
	β-coefficient (95% CI)	p value	β-coefficient (95% CI)	p value
5α-diol/5β-diol	**0.10 (0.07,0.14)**	**<0.001**	**0.11 (0.08,0.13)**	**<0.001**
5α-androstanediol	−0.09 (−4.64,4.42)	0.97	1.34 (−0.08,2.77)	0.07
5β-androstanediol	**−34.69 (−44.81,−24.66)**	**<0.001**	**−14.90 (−20.21,−9.63)**	**<0.001**
Androsterone (A)	**486.60 (305.50,668.30)**	**<0.001**	**261.30 (155.40,368.20)**	**<0.001**
Epitestosterone (E)	**−4.02 (−5.81,−2.20)**	**<0.001**	0.37 (−0.25,1.00)	0.25
Etiocholanolone (Etio)	**−145.00 (−264.50,−25.37)**	**0.02**	−57.70 (−180.90,64.96)	0.36
Testosterone (T)	−1.15 (−3.31,1.01)	0.30	**2.39 (1.82,2.96)**	**<0.001**
T/E	**0.15 (0.09,0.21)**	**<0.001**	**0.29 (0.22,0.37)**	**<0.001**
5α-diol/E	**0.50 (0.17,0.82)**	**<0.001**	**−0.35 (−0.66,−0.05)**	**0.02**
A/T	**59.17 (35.65,82.88)**	**<0.001**	**−68.81 (−119.90,−22.20)**	**<0.001**
A/Etio	**0.33 (0.27,0.38)**	**<0.001**	**0.34 (0.03,0.66)**	**0.03**

Note: All models were run separately for each metabolite and adjusted for time of day and type of sport. Significant results are shown in bold.
Abbreviation: CI, confidence interval.

TABLE 5 Results from linear mixed effects models showing intra-individual changes in metabolites over time in relation to time of day, with daytime being the reference and stratified by sex.

Metabolite	Time of day Ref. daytime	Males		Females	
		β-coefficient (95% CI)	p value	β-coefficient (95% CI)	p value
5α-diol/5β-diol	Morning	0.02 (−0.04,0.09)	0.51	−0.01 (−0.04,0.01)	0.20
	Evening	0.03 (0.00,0.07)	0.06	0.02 (−0.01,0.04)	0.14
5α-androstanediol	Morning	1.84 (−2.02,5.70)	0.35	−0.01 (−1.18,1.15)	0.99
	Evening	−4.41 (−12.14,3.34)	0.26	**−2.46 (−3.99,−0.93)**	**<0.001**
5β-androstanediol	Morning	3.72 (−8.97,16.51)	0.57	1.93 (−3.84,7.67)	0.51
	Evening	**−28.44 (−38.09,−18.92)**	**<0.001**	**−14.02 (−19.31,−8.71)**	**<0.001**
Androsterone (A)	Morning	**−203.40 (−351.70,−52.31)**	**0.01**	**−107.30 (−209.00,−5.62)**	**0.04**
	Evening	**−559.40 (−693.60,−427.70)**	**<0.001**	**−355.50 (−455.30,−256.10)**	**<0.001**
Epitestosterone (E)	Morning	0.78 (−0.79,2.35)	0.33	−0.50 (−1.19,0.19)	0.16
	Evening	**−9.31 (−10.81,−7.80)**	**<0.001**	**−1.91 (−2.56,−1.26)**	**<0.001**
Etiocholanolone (Etio)	Morning	**−118.80 (−234.90,−2.53)**	**0.05**	−109.80 (−225.60,5.67)	0.06
	Evening	**−565.20 (−662.70,−469.30)**	**<0.001**	**−585.50 (−696.50,−474.40)**	**<0.001**
Testosterone (T)	Morning	0.55 (−1.64,2.74)	0.62	**−0.76 (−1.13,−0.40)**	**<0.001**
	Evening	**−9.55 (−11.57,−7.55)**	**<0.001**	**−1.35 (−1.77,−0.91)**	**<0.001**
T/E	Morning	−0.002 (−0.06,0.05)	0.94	0.01 (−0.06,0.08)	0.79
	Evening	**0.16 (0.11,0.21)**	**<0.001**	**0.14 (0.06,0.22)**	**<0.001**
5αAdiol/E	Morning	0.06 (−0.17,0.28)	0.64	**0.45 (0.14,0.76)**	**<0.001**
	Evening	**1.21 (0.53,1.88)**	**<0.001**	**0.60 (0.21,1.00)**	**<0.001**
A/T	Morning	−10.87 (−25.31,3.56)	0.14	**196.30 (60.60,332.00)**	**0.01**
	Evening	**37.01 (15.97,58.04)**	**<0.001**	8.78 (−16.35,33.91)	0.49
A/Etio	Morning	−0.03 (−0.08,0.02)	0.26	0.01 (−0.25,0.26)	0.95
	Evening	**0.06 (0.01,0.11)**	**0.02**	−0.06 (−0.33,0.20)	0.64

Note: All models were run separately for each metabolite and adjusted for in- and out-of-competition and type of sport. Significcant results are shown in bold.
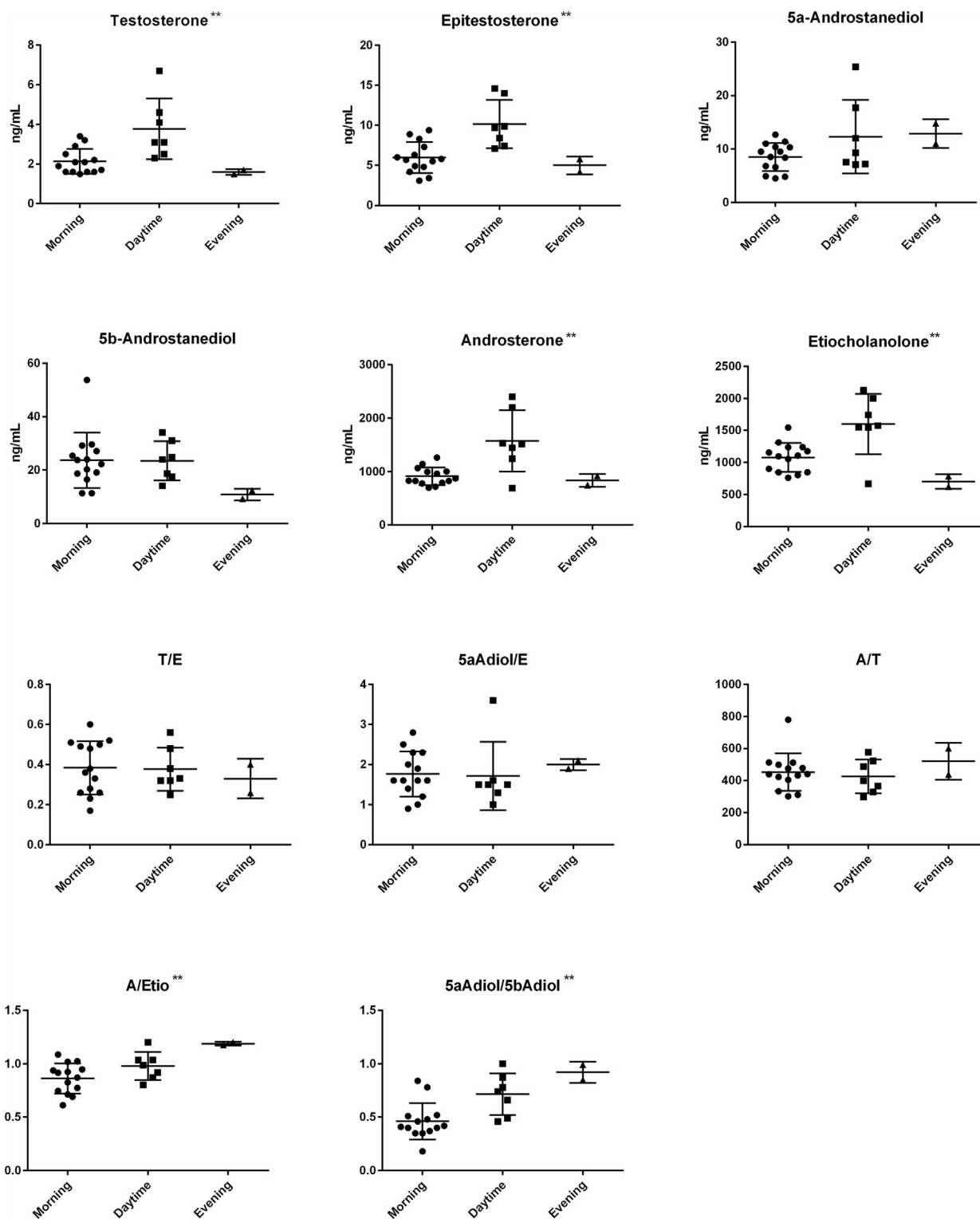Abbreviation: CI, confidence interval.

FIGURE 3 Differences in concentrations and ratios between samples collected out-of-competition (OOC) in the morning, daytime, or evening in a female athlete randomly selected from the cohort. The in-competition (IC) samples were removed from this illustration, because the IC samples are almost exclusively collected during daytime and would dilute the circadian effect. The concentrations and ratios shown here are those that showed a significant difference in the mixed model. The asterisks show significant differences between the three groups using Kruskal–Wallis.

Swedish athletes, there was no association between serum cortisol levels and urinary steroid metabolites or ABP ratios.[27] It is possible that additional factors explain the differences noted, that is, lower

excretion rate of urinary metabolites OOC, particularly the large difference in T levels in female athletes. IC samples are more often collected from "winning" athletes about 30 min to 5 h after competing.

Studies have shown that winning athletes have elevated levels of T directly after competing.[28,29] It is plausible that elevated levels of dopamine and adrenaline as a result of the euphoric sensation of winning may lead to increased T production IC. The sex difference can to some extent be explained by the fact that males have naturally higher testosterone levels than females, and a small increase in production from the adrenal gland due to increased dopamine may not have a large effect on the total T, whereas a small increase in females will lead to a larger relative difference.

4.4 | Intra-individual variations of ABP metabolites and ratios in relation to time of day

In order to study the within variations of the ABP markers over time, linear mixed effects models were applied. Most of the samples were collected daytime ($n = 2069$), followed by morning ($n = 1127$) and evening ($n = 1025$). There was a decrease in all the metabolites that were considered statistically significant over time, if measured in the evening compared with daytime measurements for both men and women, adjusting for time of day and type of sport. For the metabolite ratios, there was a significant increase over time in the evening versus daytime (Table 5). Therefore, time of day can have an intra-individual effect on several metabolites and ratios found in the ABP, which agrees with population data.[10]

The reason for dividing the time between morning, daytime, and evening is an attempt to make the grouping as homogenous as possible. For the morning samples, we aimed to include samples collected immediately after waking up, before breakfast, and before training. Most of the evening samples are non-fasting and after training. However, the daytime samples are more heterogenous, that is, regarding both fasting and training status. It is well-known that circulatory T displays circadian rhythmicity with peak concentrations in the morning in males[30,31] and similar diurnal variation but, to lesser degree, has been reported in females.[32] During the day, circulatory T levels can also be influenced by food intake, declining shortly after a meal[33] and after exercise.[34] Urine samples contain the metabolites from circulatory steroids from the last few hours up to a full night prior, and the circadian rhythmicity may not be as clearly displayed in urine compared with serum samples. However, the mixed model still clearly showed strong correlations between time of collection and metabolite concentrations. Figure 3 shows the differences between the concentrations and ratios in samples collected OOC in a randomly selected athlete from the cohort. Although the effect from the circadian rhythm on the steroid concentrations may not be of major importance when interpreting the ABP profiles, awareness of these time effects is still relevant.

5 | CONCLUSION

In this longitudinal study of 323 athletes, it was observed that female athletes showed larger fluctuations (CV%) for all ABP metabolites and ratios. In female athletes, the median concentration of T was 52% higher IC than OOC, whereas no difference in urinary T levels among male athletes was detected between IC and OOC samples. Moreover, time of day was also shown to significantly affect some of the concentrations and ratios of the ABP, depending on what time of day the collection was made. A significant number of biological passports return Atypical Passport Findings (ATPFs) due to ratios being outside of the athlete's individual thresholds. These findings may be of substantial help when evaluating whether steroid passports are suspicious or if the variation can be due to natural causes, such as time of day and IC and OOC collection.

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