

1 **Requirement for Specific Gravity and Creatinine Adjustments for Urinary**  
2 **Steroids and Luteinizing Hormone Concentrations in Adolescents**

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4 **Running Head: Specific Gravity and Creatinine Adjustments for Urinary**  
5 **Hormones**

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47 **Abstract**

48 **Objectives:**

49 Urinary hormone concentrations are often adjusted to correct for hydration status. We  
50 aimed to determine whether first morning void urine hormones in growing  
51 adolescents require adjustments and, if so, whether urinary creatinine or specific  
52 gravity (SG) are better adjustments.

53

54 **Design and Methods:**

55 The study population was adolescents aged 10.1 to 14.3 years initially who provided  
56 fasting morning blood samples at 0 and 12 months (n=343) and first morning urine  
57 every three months (n=644). Unadjusted, creatinine and SG-adjusted hormonal  
58 concentrations were compared by Deming regression and Bland-Altman analysis and  
59 grouped according to self-rated Tanner stage or chronological age. F-ratios for self-  
60 rated Tanner stages and age groups were used to compare unadjusted and adjusted  
61 hormonal changes in growing young adolescents. Correlations of paired serum and  
62 urinary hormonal concentration of unadjusted and creatinine and SG adjusted were  
63 also compared.

64

65 **Results:**

66 Fasting first morning void hormone concentrations correlated well and were unbiased  
67 between unadjusted or adjusted by either creatinine or SG. Urine creatinine  
68 concentration increases with Tanner stages, age and male gender whereas, urine SG  
69 was not influenced by Tanner stage, age or gender. Adjustment by creatinine or SG of  
70 urinary luteinizing hormone, estradiol, testosterone, dihydrotestosterone and

71 dehydroepiandrosterone concentrations did not improve correlation with paired serum  
72 concentrations.

73

74 **Conclusions:**

75 Urine steroid and LH concentrations in first morning void samples of adolescents are

76 not significantly influenced by hydration status and may not require adjustments;

77 however, if desired, both creatinine and SG adjustments are equally suitable.

78 **Introduction**

79 Measurements of urinary gonadotropins and steroids in children and adolescents  
80 emerged as methods to estimate pubertal development and gonadal function early in  
81 the immunoassay era.<sup>1-3</sup> Urine sampling provides an integrated measurement  
82 especially for hormones secreted in pulsatile manner such as luteinizing hormone  
83 (LH)<sup>4</sup> or diurnally like sex steroids in early puberty, and is more acceptable to  
84 children and adolescents than venipuncture. The relatively high hormone  
85 concentrations in urine compared with blood or saliva, together with the ability to  
86 concentrate urine, is advantageous for assays with low sensitivity or analytes at low  
87 concentrations. However, an inherent problem of using urine is the wide and  
88 unregulated variation reflecting the individual's fluid status.

89  
90 Urine dilution or concentration creates corresponding changes in urine solute  
91 concentrations so that adjustment of urine concentration may be required to avoid  
92 misinterpreting hormone excretion due to variation in hydration.<sup>5</sup> Osmolality, specific  
93 gravity (SG) and creatinine measurements are used to adjust for hydration.<sup>6</sup> Although  
94 measurement of osmolality by freezing point depression is considered the reference  
95 method<sup>7</sup>, it is laborious, time consuming and expensive so is usually replaced by SG  
96 and creatinine measurements particularly for large scale, field studies. Urinary SG is  
97 measured using a refractometer to compare light refraction of a urine sample against  
98 pure water standard or by reagent strips which measure the ionic strength of urine by  
99 color changes. Urine SG of sample is normalized to a population reference value.

100 While SG measurement has been largely superseded by urine creatinine adjustment in  
101 clinical laboratories, SG adjustment for urine dilution remains standard in anti-doping  
102 laboratories and is used in some toxicology studies.<sup>8</sup> Creatinine adjustment is based

103 on the assumption that (a) this end-product formed endogenously from muscle  
104 creatine is released into the bloodstream and excreted in urine at a constant rate  
105 depending only on total muscle mass<sup>9</sup> and (b) endogenous hormones and creatinine  
106 undergo renal excretion at the same rate.<sup>6</sup> Yet, CR excretion rate may be influenced  
107 by the growing muscle mass during puberty leading to potential systemic errors in  
108 using creatinine adjustments.<sup>10</sup>

109

110 Some<sup>11, 12</sup> but not other<sup>13-17</sup> studies suggest creatinine or SG adjustment for  
111 measurement of urinary substances although such adjustments may be either  
112 unnecessary or even introduce additional measurement errors. Furthermore, none  
113 have focused on situations where creatinine is changing systematically due to somatic  
114 growth. Thus the present study aimed to determine whether the first morning void  
115 hormonal assessments carried out in growing young adolescents at various stages of  
116 pubertal progression require adjustments and, if so, to determine whether creatinine or  
117 SG adjustment was better.

118

## 119 **Materials and Methods**

120

### 121 **Samples**

122 Adolescents aged 10.1 to 14.3 years initially were recruited from local secondary  
123 schools in the state of New South Wales (NSW), Australia. Ethical approval was  
124 obtained from the Human Research Ethics Committee, University of Sydney (HREC  
125 13094). Fasting morning blood samples were collected at 0 and 12 months (n=343)  
126 and first morning urine collected three monthly after 12 hr fasting at home by the  
127 adolescents between 7.00 am and 8.30 am (n=644). Post-menarcheal girls provided

128 samples in the mid-follicular phase (day 7-10) with the assumption of 28-32 day  
129 cycle. Serum and urine samples were stored at -80 C until analysis. The adolescents  
130 also provided a self-rating of puberty using line drawings based on the Tanner stages  
131 at 0 and 12 months.

132

### 133 **Assays**

134 Urine SG was measured by immersing a reagent strip (ChoiceLine 10, Roche  
135 Diagnostics) in freshly voided urine samples. Dipstick color changes were compared  
136 visually with the color chart to estimate the SG. Urine samples were subsequently  
137 stored at -80 C and subjected to three freeze-thaw cycles for luteinizing hormone  
138 (LH), creatinine and steroid analysis. The urine samples were first thawed and  
139 assayed for LH measurements with the Immulite 1000 LH (Siemens) as described  
140 previously.<sup>18</sup> The within-assay coefficients of variation were <10%. The samples  
141 underwent second freeze-thaw cycle for the creatinine measurements. Urine  
142 creatinine concentrations were determined by the colorimetric alkaline-picrate (Jaffé)  
143 method (CREJ2, Roche Diagnostics, Cat. No. 04810716 190) on a Cobas C501  
144 analyzer (Roche Diagnostics GmbH, Indianapolis, IN). Calibrators (Roche  
145 Diagnostics, Cat. No. 10759350 190) were used for this automated system to generate  
146 a linear curve ranging between 375 and 55000  $\mu\text{mol/L}$  and the limit of detection of  
147 375  $\mu\text{mol/L}$ . The final urine thaw was for urine steroid measurement. Urinary and  
148 serum estradiol ( $\text{E}_2$ ), testosterone (T), dihydrotestosterone (DHT) and  
149 dehydroepiandrosterone (DHEA) were measured by liquid chromatography tandem  
150 mass spectrometry (LC-MS/MS) as modified from a previously described method for  
151 serum<sup>19</sup> and adapted for urine specimens following deconjugation, with details  
152 described in the Supplementary Materials.

153

154 The LH and steroid concentrations were adjusted to standard SG of 1.020 according  
155 to the formula  $[\text{hormone concentration}_{\text{sample}} \times (1.020-1)/(\text{SG}_{\text{sample}}-1)]^{20}$  and to  
156 standard CR measurement of the present study adolescent population (12.40 mmol/L  
157 [n=644; F 331]) using the formula  $[\text{hormone concentration}_{\text{sample}} \times$   
158  $(\text{Creatinine}_{\text{population}}/\text{Creatinine}_{\text{sample}})]$  where  $\text{Creatinine}_{\text{population}}$  was defined as the mean  
159 of the urinary creatinine of the whole sample.

160

### 161 **Data Analysis**

162 The steroid and LH concentrations unadjusted and adjusted for SG or creatinine were  
163 compared by Deming (orthogonal) regression and deviance (Bland-Altman) analysis  
164 using MedCalc software. Based on using the same analyte with different adjustments,  
165 the variance ratio in the Deming regression was assumed to be unity. Non-  
166 independence according to variations in the number of samples provided by each  
167 individual was ignored in this analysis. Descriptive statistics including mean, standard  
168 deviation (SD) and standard error of mean (SEM) were performed by SPSS version  
169 21. The F- ratios of age and Tanner stage group comparison for each hormone were  
170 calculated by one-way ANOVA separately for each gender.

171

### 172 **Results**

173 In first morning urine void samples (n=644), the mean (SD, range) creatinine  
174 concentration was 12.4 (4.5, 1.4 - 31.5) mmol/L with an overall gender difference  
175 being higher in males (P<0.05). The SG was 1.020 (0.0054, 1.005 - 1.030) without  
176 significant gender difference (P=0.054). Urine creatinine concentrations were  
177 progressively increased according to chronological age and to Tanner stage (Figure 1)

178 for both genders. Pooling genders, there were significant differences in urine  
179 creatinine concentrations by age and Tanner stage ( $P < 0.05$ , two-way ANOVA) but  
180 not for urine SG according to age ( $P = 0.29$ ) or Tanner stages ( $P = 0.22$ ) (data not  
181 shown).

182

183 Urinary LH,  $E_2$ , T, DHT and DHEA concentrations, adjusted for either SG or  
184 creatinine, are compared according to Deming regression line and the deviance plots  
185 are shown in Figure 2. For each urinary hormone concentration, there was a good  
186 correlation between the SG and creatinine adjusted concentrations ( $R^2$ : 0.69 - 0.85)  
187 free from proportional bias between adjustment methods.

188

189 Similarly, Deming regression and Bland-Altman comparison between the unadjusted  
190 and adjusted hormone concentrations with either SG or creatinine (Table 1) also  
191 demonstrate lack of bias whether adjusted or not by either creatinine or SG.

192

193 The mean, SD and F-ratios of the unadjusted, SG adjusted and creatinine adjusted  
194 hormones according to Tanner stages and age groups in females and males are shown  
195 in Supplemental data, Tables 3-4 and Supplemental data, Table 5-6, respectively. The  
196 results show consistent estimates and progression according to age and Tanner stage  
197 of unadjusted, SG adjusted and creatinine adjusted urine LH and steroid  
198 concentrations. The mean, SD and F-ratios of serum hormones according to Tanner  
199 stage and age groups are shown in Supplemental data, Table 7 and 8.

200

201 The correlation coefficient of paired urinary and serum hormone concentrations is  
202 given in Table 2. The unadjusted and adjusted (creatinine and SG) urinary LH,  $E_2$ , T,

203 DHT and DHEA concentration showed similar correlation against serum. The  
204 samples were also grouped into three creatinine and SG percentile ranges (up to 25th  
205 percentile, between 25th to 75th percentile and above 75th percentile) and regression  
206 analysis was performed between unadjusted/adjusted urine hormone concentrations  
207 against serum hormone concentrations (data not shown). There were no  
208 improvements in the correlation coefficient values within the groups. Dividing the  
209 same percentiles according to gender also did not improve the correlations between  
210 the urine unadjusted/adjusted hormones against serum hormones concentrations (data  
211 not shown).

212

### 213 **Discussion**

214 Urinary measurement of reproductive hormones is a convenient means to evaluate  
215 pubertal status and gonadal function for field population studies. In clinical settings,  
216 adjustment based on the assumption of stable urine creatinine excretion is commonly  
217 used to adjust for variations in hydration although other techniques such as regression  
218 normalization or log transformation are proposed.<sup>10, 21</sup> As an end metabolite of muscle  
219 creatine, urine creatinine is determined by total muscle mass in addition to other  
220 factors such as age, gender, diet (meat consumption), physical activity, and body mass  
221 index some of which exert their effects via changes in muscle mass.<sup>6, 17, 22, 23</sup> Hence  
222 one aim of the present study was to determine for the first time whether creatinine  
223 adjustment was valid or required for longitudinal studies of growing adolescents.

224

225 Our findings confirm that the first morning urine creatinine concentration increases  
226 with age and Tanner stages and was higher among males. However, adjustment for  
227 urine creatinine was no better or worse than adjustment for SG or even no adjustment.

228 This may reflect the fact that we studied first morning void urine samples which  
229 control hydration, whereas similar interpretation may not apply to urine sampled at  
230 random when hydration state may vary more. Our findings are consistent with  
231 previous studies showing prominent intra- and inter-individual variability in  
232 creatinine excretion of second morning and 24h urine samples in adults due to  
233 variable fluid intake.<sup>17</sup> Significantly higher creatinine levels in morning versus  
234 afternoon,<sup>24</sup> in evening spot samples<sup>6</sup> and creatinine loss due to multiple freeze-thaw  
235 cycles have also been reported<sup>25, 26</sup> all of which introduce systematic errors in use of  
236 urine creatinine for dilution adjustments. Thus, although studies have suggested  
237 alternative adjustment based on SG in adult humans and primates,<sup>27, 28</sup> none have  
238 focused on the need for SG adjustments in first morning voids of growing  
239 adolescents.

240

241 SG is readily measured by reagent strip for field studies without needing a laboratory.  
242 Previous studies demonstrate good agreement between SG measurements by reagent  
243 strip versus refractometer<sup>29, 30</sup> or osmolality.<sup>30-32</sup> SG measurement by reagent strip is  
244 widely used in clinical applications.<sup>32, 33</sup> Although refractometer urine SG may be  
245 influenced by disease states leading to high serum protein or glycosuria,<sup>7, 34</sup> reagent  
246 strip SG is not affected by glucose, only minimally by urea and albumin, but may be  
247 affected by the rare instances of alkaline urine.<sup>31</sup> Urine SG reading may also be  
248 influenced by diet, environment and the renal reabsorption capacity.<sup>35</sup> Among  
249 adolescents, we find that urine SG measured with reagent strips is systematically not  
250 influenced by age or gender consistent with previous reports.<sup>22, 36</sup>

251

252 Limits of acceptable creatinine and SG measurements vary between studies.  
253 Generally, urine is considered too dilute when the SG and creatinine levels are lower  
254 than 1.010 and 0.5 g/l (4.4 mmol/L), respectively, and too concentrated where SG and  
255 creatinine levels higher than 1.030 (or 1.035) and 3 g/l (26.5 mmol/L), respectively.<sup>17,</sup>  
256 <sup>35</sup> However, due to the standardized method of collection and hydration (first morning  
257 void), the present study did not discard any samples as too dilute or too concentrated.  
258  
259 The present study demonstrated that the fasting first morning void urine hormone  
260 concentrations adjusted by creatinine correlated well with those adjusted by SG in this  
261 adolescent population. This is consistent with previous reports that used randomly  
262 collected or timed urine collection from children and adults showing good  
263 correlations when creatinine and SG adjustments were compared directly<sup>5, 12, 21, 23, 35,</sup>  
264 <sup>37</sup> or with adjustment according to both<sup>5, 27, 28</sup> including a reduced variation using  
265 these adjustments in some studies.<sup>10, 12</sup> However, the present study shows that neither  
266 of the adjustment methods for first morning void urine sample of adolescents were  
267 significantly improved compared to unadjusted hormone concentrations. These  
268 observations are consistent with previous reports for creatinine adjustment of urine  
269 steroid measurements in adult women.<sup>38, 39</sup>  
270  
271 In studies where the urinary hormone concentrations were correlated with paired  
272 circulating serum concentrations, the urinary unadjusted concentration or  
273 concentration expressed by volume of urine correlates better than the adjustment  
274 based on analyte to creatinine ratios,<sup>11, 13, 40</sup> although some studies have shown  
275 improved correlation with creatinine adjustments.<sup>41, 42</sup> The present study demonstrated  
276 that the urinary hormone concentrations adjusted with creatinine and SG did not

277 improve the correlation with paired serum concentrations. These samples were also  
278 grouped into three creatinine and SG percentile ranges (25th, 25-75th and 75th) to  
279 replicate non-fasting conditions with wider variation in hydration status. However, no  
280 improvement was observed in terms of correlation between the unadjusted or adjusted  
281 urine hormone and paired serum concentrations. These findings further support that  
282 the adjustments may not be necessary for first morning void urine samples.

283

284 In conclusion, the present study shows that adjustment of urinary steroid and LH  
285 concentration for hydration state may not be required for first morning void  
286 specimens of even growing adolescents. If adjustments are required, then either  
287 creatinine or SG are equally suitable and provide comparable results. Reagent strip  
288 SG measurements are simple and sufficiently reliable, economical and time-saving for  
289 large numbers of urine sampling in long-term field studies.

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406

407 **Tables**

408 Table 1: Comparison of unadjusted against SG and CR adjusted urinary hormone  
 409 measurements. The slope, intercept and 95% confidence interval (CI) were  
 410 determined by Deming regression. The mean and 95% CI ( $\pm 1.96$  SD) were derived  
 411 from the Bland-Altman plots.

	UA vs SG					UA vs CR				
	Deming Regression			Bland-Altman		Deming Regression			Bland-Altman	
	Slope (95% CI)	Intercept (95% CI)	r* (95% CI)	Mean	95% CI	Slope (95% CI)	Intercept (95% CI)	r* (95% CI)	Mean	95% CI
LH (IU/L)	0.94 (0.84 to 1.03)	0.03 (-0.67 to 0.73)	0.91 (0.90 to 0.92)	0.6	8.3, -7.2	1.03 (0.94 to 1.11)	-0.39 (-0.98 to 0.19)	0.89 (0.88 to 0.91)	0.1	8.1, -7.8
E2 (ng/mL)	0.92 (0.75 to 1.09)	0.02 (-0.19 to 0.22)	0.92 (0.91 to 0.94)	0.1	2.0, -1.8	1.08 (0.93 to 1.22)	-0.09 (-0.24 to 0.07)	0.92 (0.91 to 0.93)	0.0	1.8, -1.8
T (ng/mL)	0.98 (0.89 to 1.06)	-0.17 (-0.75 to 0.42)	0.95 (0.94 to 0.96)	0.4	11.5, -10.7	1.20 (1.08 to 1.31)	-1.23 (-1.99 to -0.47)	0.94 (0.93 to 0.95)	-0.7	11.7, -13.2
DHT (ng/mL)	1.12 (0.74 to 1.50)	-0.63 (-1.94 to 0.68)	0.95 (0.94 to 0.95)	0.2	3.5, -3.2	1.53 (0.71 to 2.35)	-1.75 (-4.44 to 0.94)	0.90 (0.89 to 0.92)	-0.1	4.8, -5.0
DHEA (ng/mL)	0.90 (0.81 to 0.99)	0.65 (-0.92 to 2.22)	0.87 (0.85 to 0.89)	1.5	17.9, -14.9	1.07 (0.94 to 1.20)	-1.58 (-3.67 to 0.50)	0.82 (0.79 to 0.84)	0.2	17.6, -17.2

412 The slope, intercept and 95% confidence interval were determined by Deming regression. The mean and 95% CI ( $\pm 1.96$  SD)  
 413 were derived from the Bland-Altman plots.

414 UA: unadjusted; SG: specific gravity adjusted; CR: creatinine adjusted; R2: correlation of determination; CI: confidence interval;  
 415 E2: serum estradiol; T: testosterone; DHT: dihydrotestosterone; DHEA: dehydroepiandrosterone; LH: luteinizing hormone. For  
 416 the Deming regression, the variance ratio was assumed to be unity.

417  
 418 \*All the P values were <0.0001.

419 Table 2: Pearson's correlation coefficient and confidence intervals (in parentheses) of  
 420 paired urinary and serum LH, E2,T, DHT and DHEA (n=343).

Urine versus serum	LH	E <sub>2</sub>	T	DHT	DHEA
UA	0.56 (0.48 to 0.63)	0.73 (0.67 to 0.77)	0.79 (0.74 to 0.82)	0.44 (0.35 to 0.52)	0.63 (0.56 to 0.69)
SG	0.57 (0.50 to 0.64)	0.72 (0.66 to 0.77)	0.79 (0.74 to 0.83)	0.42 (0.33 to 0.50)	0.60 (0.52 to 0.66)
CR	0.56 (0.48 to 0.63)	0.79 (0.74 to 0.82)	0.80 (0.76 to 0.84)	0.35 (0.25 to 0.44)	0.66 (0.59 to 0.71)

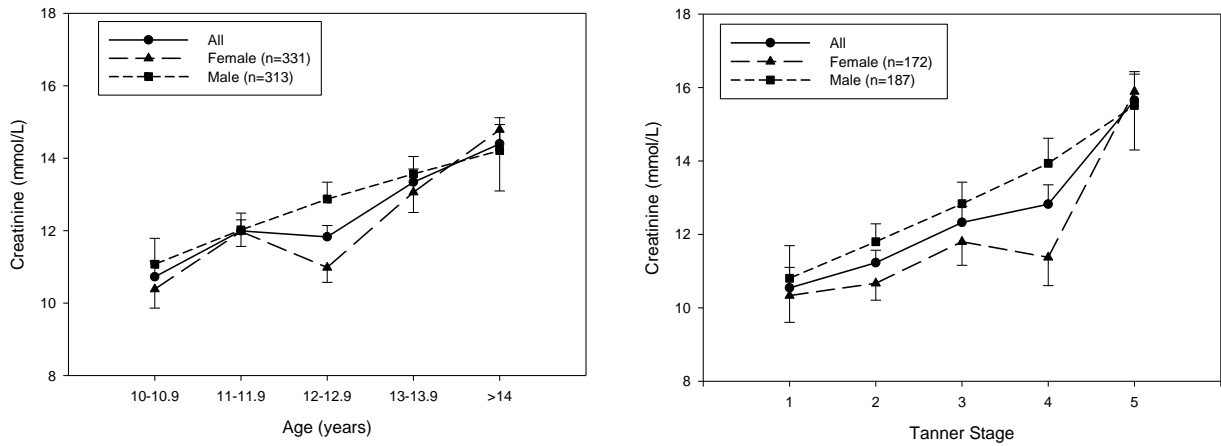
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UA: unadjusted; SG: specific gravity adjusted; CR: creatinine adjusted; E<sub>2</sub>: serum estradiol; T: testosterone; DHT:

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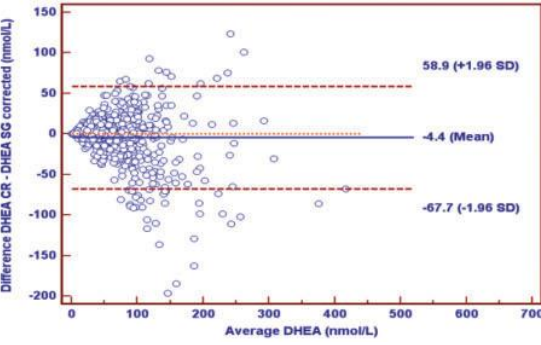
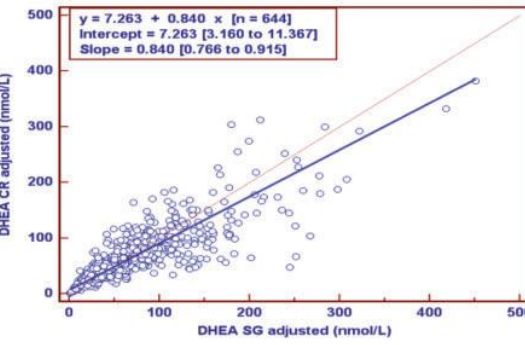
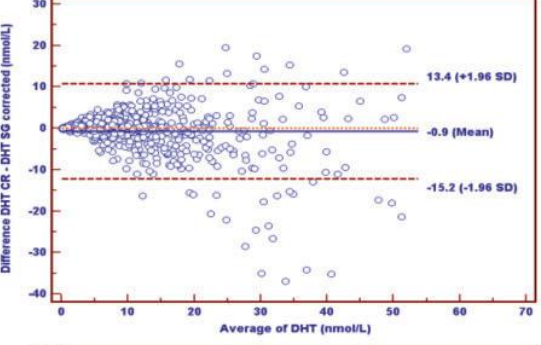
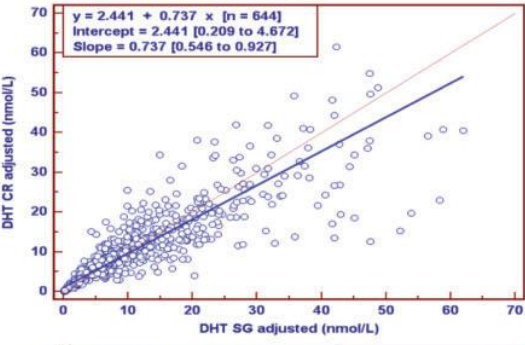
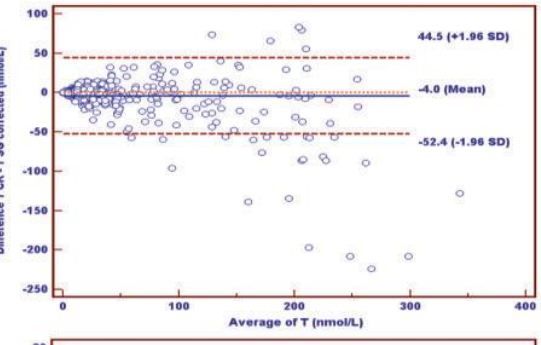
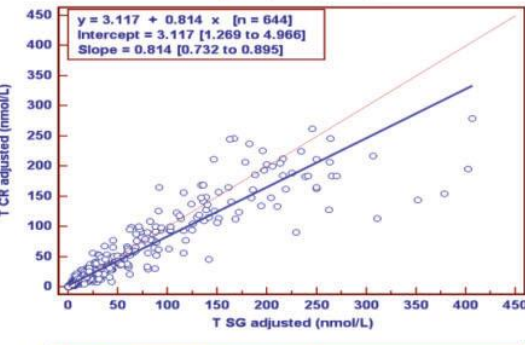
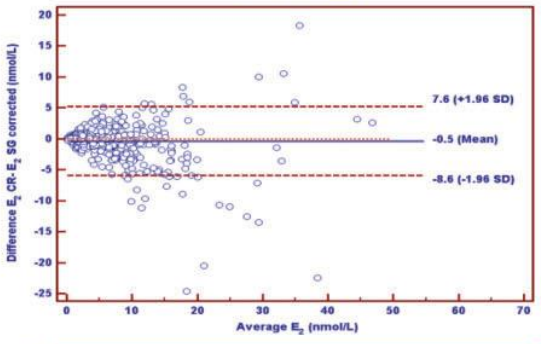
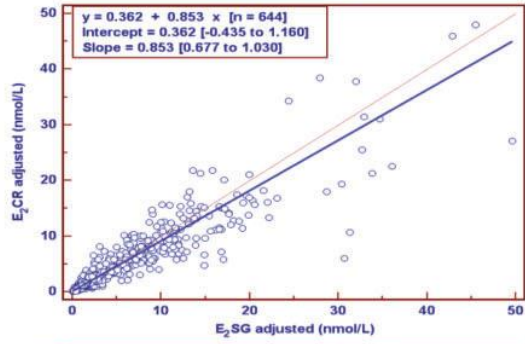
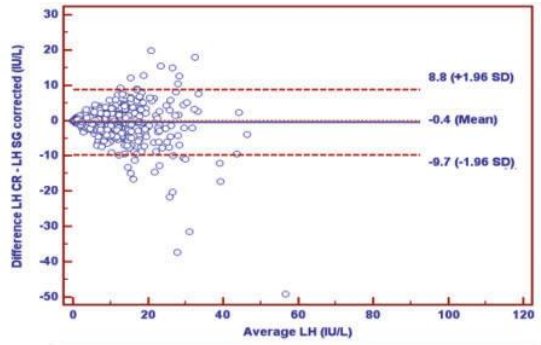
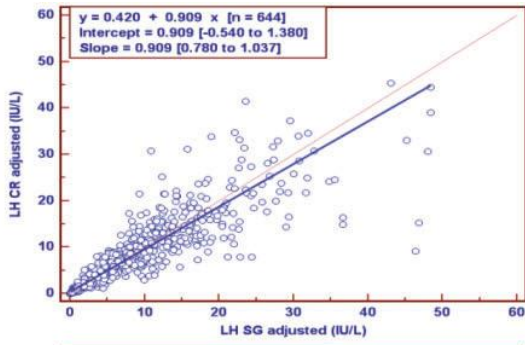
dihydrotestosterone; DHEA: dehydroepiandrosterone; LH: luteinizing hormone.

424 **Figures**



425

426 Figure 1: Plot of urinary creatinine measurements of adolescents groups according to  
427 age (left panel) and Tanner stage (right panel). Data represents the creatinine mean  
428 and SEM. For the age plot, samples were from 3-monthly intervals (n=644) whereas  
429 for the Tanner stage plot, samples were from 0 and 12 months (n=359).



431 Figure 2: Comparison of urinary LH, E<sub>2</sub>, T, DHT and DHEA concentrations adjusted  
432 by SG and creatinine. Comparison were made according to Deming regression  
433 analysis (left panels) and Bland-Altman plots (right panels). For the Deming plots, the  
434 slope is shown as a solid line and line of identity in fine dotted line. Insets are the  
435 regression formula and 95% confidence limits on the intercept and slope. The Bland-  
436 Altman plots represent the differences between creatinine and SG adjusted hormone  
437 concentrations against the averages of the hormone concentrations adjusted with the  
438 two correction method. The solid line and the dashed lines represent the observed  
439 average and the 95% limit of confidence ( $\pm 1.96$  SD), respectively.