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EXPERIMENTAL STUDY

Does the 2nd and 4th digit ratio reflect prenatal androgen exposure?

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ABSTRACT
OBJECTIVES: The aim of our study was to describe the effect of prenatal testosterone exposure on 2D:4D in both sexes, and to determine whether this effect is mediated via the androgen receptor. In addition, the sex differences in lengths of 2D, 4D, and 2D:4D ratio were analyzed.
BACKGROUND: Clinical studies suggest a negative correlation between prenatal testosterone exposure and ratio of the lengths of the second and fourth digits (2D:4D). However, less is known about the underlying molecular mechanisms.
METHODS: Pregnant rats were treated with olive oil, testosterone, flutamide or testosterone with flutamide daily from the fourteenth day of pregnancy until delivery. The finger lengths of adult offspring were measured using both, digital scanning of the paws and μCT analysis of the phalanges.
RESULTS: None of the aforementioned methods revealed any effect of testosterone on 2D:4D. μCT measurements showed that prenatal hyperandrogenism in both sexes leads to shorter 2D compared to controls. Moreover, the testosterone treatment in males resulted in the shortening of 4D when compared to controls.
CONCLUSION: Prenatal hyperandrogenism leads to shorter lengths of 2D and 4D; however, it does not affect 2D:4D ratio. Whether other steroid hormones and/or testosterone metabolites affect the 2D:4D ratio requires further investigation *(Tab. 5, Fig. 3, Ref. 32)*. Text in PDF *www.elis.sk.*
KEY WORDS: finger length, morphometry, prenatal development, testosterone.

Introduction

Testosterone belongs to the steroid hormones, which play an important role in the physiology of both sexes. The effect of prenatal testosterone is important in the sexual differentiation of the fetus (1). One of the sexual dimorphisms, which is probably related to intrauterine development, is the ratio of the lengths of second and fourth digits (2D:4D) (2,3). The 2D:4D is associated with the concentration of prenatal testosterone (4–6). However, the causal relationship is not clear. It has previously been reported that the inactivation of the androgen receptor leads to a shorter 4D and a higher 2D:4D, while the inactivation of the estrogen receptor has the opposite effect (6). In females, the 2D:4D is higher than in males (6). The 2D:4D is also higher in the right hand compared to the left hand (2, 7). Previous experimental studies have reported prolongation of 4D after prenatal testosterone treatment (2, 6, 8, 9), which led to a lower 2D:4D in rats (9). However, neither the effect of prenatal application of flutamide (an androgen receptor blocker), nor the effect of dihydrotestosterone on 2D:4D was confirmed in mice (2, 6, 8, 9).

In children with autism spectrum disorder, which is associated with a prenatal exposure to high concentrations of testosterone, a lower 2D:4D has been observed (10). An association between 2D:4D and gastric cancer (11), knee and hip osteoarthritis (12), and prostate cancer (13) has also been reported. Additionally, a higher 2D:4D (low prenatal testosterone concentration) has been linked to multiple sclerosis (14) coronary heart disease (15, 16), and breast cancer (16–18). Therefore, both maternal testosterone and 2D:4D in the offspring may be a predictive factor for these diseases.

Despite several clinical and experimental studies, the mechanism of action of prenatal testosterone exposure on finger length remains unclear. Therefore, in the present study, flutamide was used to study the molecular mechanism of endogenous testosterone action. Moreover, the combination of testosterone and flutamide was used to monitor the effect of exogenous testosterone. The aim

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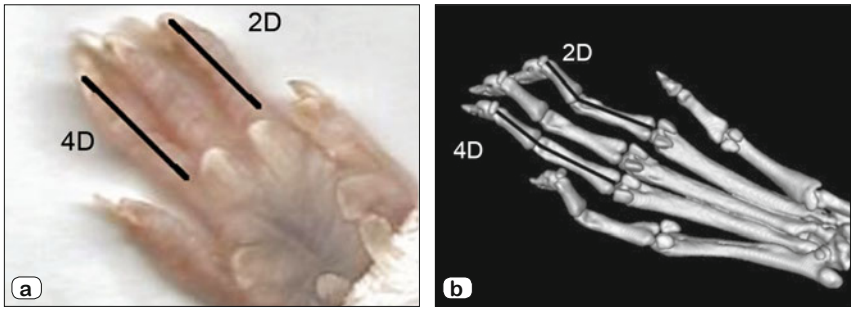

Fig. 1. Representative images of digital scan (a) and µCT (b) methods used for the measurement of 2D and 4D.

of the present study was to describe the effect of prenatal testosterone on 2D:4D in both sexes and to prove whether this effect is mediated *via* the androgen receptor.

Materials and Methods

Animals and housing conditions

In the present study, pregnant Lewis rats (10–13 weeks old, Anlab, Prague, Czech Republic) were used. All rats were kept under controlled laboratory conditions (temperature 25 ± 2 °C, humidity 55 ± 10 %, 12:12 light-dark cycle) and had *ad libitum* access to food and water. Rats were housed in polycarbonate cages (36 x 20 x 19 cm). All procedures were conducted according to Slovak legislation and were approved by the ethical committee of the Institute of Molecular Biomedicine, Comenius University, Bratislava.

Prenatal treatment

Pregnant female Lewis rats (n = 19) were randomly divided into four groups: control (C), testosterone (T), flutamide (F), and testosterone with flutamide (TF). The control dams received the vehicle (olive oil, Galvex, spol s.r.o., Banská Bystrica, Slovak Republic). The T group was treated with testosterone (2 mg/kg body weight) (19), the F group with flutamide (5 mg/kg body weight, an androgen receptor blocker) (20), and the TF group with testosterone and flutamide (2 mg/kg body weight and 5 mg/kg body weight), all of which were dissolved in olive oil prior to administration. Daily intramuscular injections of the substances (Sigma Aldrich, St. Louis, MO, USA) were applied during the third week of pregnancy until delivery (21, 22). After delivery, the offspring were weaned on postnatal day 21.

Determination of anogenital distance in the adult offspring

The anogenital distance of the offspring was measured using vernier calipers with an experimenter blind to treatment conditions. Measurements were taken three times and the arithmetic mean was used for statistical analysis.

Digit length measurements

The 2D:4D was determined using two different methods. The first method was based on a digital scan of paws, and the length of digits was determined using the ImageJ program. The measurements were conducted by three observers blind to the sex and treatment conditions of the animals. The length of 2D and 4D was determined from the mid-point of the basal crease to the tip of the digits, excluding the toenails (Fig. 1a) (23). The average length of these three measurements was used for statistical analysis. The second method was based on an analysis of the phalanges using µCT (IVIS® SpectrumCT, PerkinElmer, Waltham, Massachusetts). The 2D:4D was determined using the distance between the proximal and middle phalanges (6) of the 2D and 4D (Fig. 1 b). In the second method, the lengths of the phalanges were measured using the Living Image program specifically designed for IVIS® SpectrumCT. As with the first method, the measurements were conducted by an observer blind to the sex and treatment conditions of the animals.

Blood collection and hormonal assay

At the end of pregnancy (gestational day 21), blood was taken from the tail vein of dams and the testosterone concentration was assessed using a commercially available ELISA kit (DRG Diagnostic, Marburg, Germany) according to the instructions of the manufacturer.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.01 (GraphPad Software, Inc., CA, USA). To analyze the concentration of circulating testosterone in dams, the anogenital distance, or finger characteristics (lengths of the fingers, 2D:4D) of the adult offspring, one-way and two-way ANOVA (independent factors: testosterone and flutamide treatment) with Bonferroni-corrected post-hoc t-test were used. Two-way ANOVA with Bonferroni-corrected post-hoc t-test was also used to analyze sex differences (independent factors: treatment and sex) and laterality (independent factors: laterality and treatment). P values lower than 0.05 were considered statistically significant. Data are presented as the mean plus standard deviation (SD).

Results

Circulating testosterone concentration in pregnant rats

There were significant differences in the circulating testosterone concentrations of pregnant dams measured on gestational day 21 ($F_{3,15} = 21.7$, $p < 0.001$) (Tab. 1). The pregnant dams that

Tab. 1. Circulating testosterone concentration in pregnant dams on gestational day 21.

Groups of animals	Females			
	C	T	F	TF
Testosterone (nmol/l)±SD	1.36±0.55	37.3±14.6***	2.93±1.33	42.2±10.8^{+++}

C – control group, T – group treated with testosterone, F – group treated with flutamide, TF – group treated with testosterone and flutamide; ***p < 0.001 vs C, $^{+++}$p < 0.001 vs F. Data are presented as means ± SD

Tab. 2. The length of 2D and 4D on both the left and right forepaw.

Digital scan											
Paw		Females					Males				
		C (n=15)	T (n=14)	F (n=9)	TF(n=12)	One-way ANOVA	C (n=16)	T (n=14)	F (n=9)	TF (n=9)	One-way ANOVA
Left	2D	5.91±0.66	5.77±0.51	6.08±0.33	6.10±0.44	p=0.38	6.50±0.61	6.16±0.60	6.53±0.22	6.19±0.39	p=0.18
	4D	4.03±0.48	3.74±0.46	3.86±0.34	4.03±0.42	p=0.24	4.21±0.66	4.28±0.45	4.16±0.27	4.49±0.40	p=0.49
	2D:4D	1.48±0.17	1.56±0.11	1.58±0.17	1.54±0.20	p=0.41	1.58±0.24	1.45±0.20	1.59±0.13	1.39±0.15	+p=0.07
Right	2D	6.07±0.44	5.81±0.52	6.42±0.36	6.08±0.46	p<0.05	6.60±0.85	6.38±0.57	6.44±0.17	6.69±0.37	p=0.61
	4D	3.90±0.65	3.83±0.53	4.02±0.44	4.20±0.50	p=0.36	4.18±0.71	4.29±0.44	4.08±0.26	4.27±0.40	p=0.79
	2D:4D	1.59±0.24	1.54±0.15	1.61±0.22	1.46±0.19	p=0.30	1.59±0.19	1.50±0.19	1.58±0.11	1.57±0.11	p=0.46

The lengths of digits were measured using digital scans. Data are expressed as means±SD. C (Females: n=15; Males: n=16), T (Females: n=14; Males: n=14), F (Females: n=9; Males: n=9) and TF (Females: n=12; Males: n=9).

Tab. 3. The length of 2D and 4D on both the left and right forepaw.

Analysis of phalanges by μCT measurements											
Paw		Females					Males				
		C (n=15)	T (n=14)	F (n=8)	TF(n=11)	One-way ANOVA	C (n=16)	T (n=14)	F (n=9)	TF (n=8)	One-way ANOVA
Left	2D	6.67±0.21	6.43±0.22	6.43±0.12	6.65±0.19	p < 0.01	7.24±0.22	6.94±0.27	7.03±0.19	6.84±0.26	p < 0.001
	4D	7.54±0.16	7.29±0.35	7.34±0.24	7.39±0.30	p = 0.09	8.07±0.25	7.67±0.28	7.72±0.22	7.66±0.24	p < 0.001
	2D:4D	0.88±0.04	0.88±0.04	0.88±0.04	0.90±0.04	p = 0.52	0.90±0.03	0.90±0.03	0.91±0.02	0.89±0.02	p = 0.46
Right	2D	6.74±0.15	6.46±0.12	6.50±0.11	6.66±0.17	p < 0.001	7.23±0.19	6.96±0.20	7.02±0.16	6.83±0.30	p < 0.001
	4D	7.56±0.19	7.31±0.24	7.34±0.23	7.45±0.36	p = 0.06	8.04±0.20	7.71±0.28	7.70±0.21	7.60±0.28	p < 0.001
	2D:4D	0.89±0.03	0.88±0.02	0.88±0.04	0.90±0.04	p = 0.38	0.90±0.03	0.90±0.03	0.91±0.02	0.90±0.02	p = 0.64

The lengths of digits were measured using μCT images. Data are expressed as means ± SD. C (Females: n = 15; Males: n = 16), T (Females: n = 14; Males: n = 14), F (Females: n = 8; Males: n = 9) and TF (Females: n = 11; Males: n = 8)

Tab. 4. The laterality in lengths of 2D, 4D, and 2D:4D on both the left and right forepaw.

Digital scan											
		Females					Males				
		Left	Right	Two-way ANOVA			Left	Right	Two-way ANOVA		
				Laterality	Treatment	Interaction			Laterality	Treatment	Interaction
2D	C	5.91±0.18	6.07±0.18	p = 0.20	p < 0.05	p = 0.62	6.50±0.20	6.60±0.20	p = 0.13	p = 0.29	p = 0.46
	T	5.77±0.19	5.81±0.19				6.16±0.21	6.38±0.21			
	F	6.07±0.23	6.42±0.23				6.53±0.27	6.44±0.27			
	TF	6.1±0.20	6.08±0.20				6.19±0.27	6.69±0.27			
4D	C	4.03±0.18	3.90±0.18	p = 0.45	p = 0.14	p = 0.63	4.21±0.18	4.18±0.18	p = 0.45	p = 0.42	p = 0.90
	T	3.74±0.19	3.84±0.19				4.28±0.19	4.29±0.19			
	F	3.86±0.23	4.02±0.23				4.16±0.24	4.08±0.24			
	TF	4.03±0.20	4.20±0.20				4.49±0.24	4.27±0.24			
2D:4D	C	1.48±0.07	1.59±0.07	p = 0.74	p = 0.38	p = 0.29	1.58±0.06	1.59±0.06	p = 0.12	p = 0.05	p = 0.38
	T	1.56±0.07	1.54±0.07				1.45±0.07	1.50±0.07			
	F	1.58±0.09	1.61±0.09				1.59±0.09	1.58±0.09			
	TF	1.54±0.07	1.46±0.07				1.39±0.09	1.57±0.09			

The lengths of digits were measured using digital scans. Data are expressed as means ± SD. C (Females: n=15; Males: n=16), T (Females: n=14; Males: n=14), F (Females: n=9; Males: n=9) and TF (Females: n=12; Males: n=9).

Tab. 5. The laterality in lengths of 2D and 4D and 2D:4D on both left and right forepaw.

Analysis of phalanges by µCT measurements												
		Females					Males					
				Two-way ANOVA					Two-way ANOVA			
		Left	Right	Laterality	Treatment	Interaction	Left	Right	Laterality	Treatment	Interaction
2D	C	6.67±0.06	6.74±0.06	p = 0.20	p < 0.001	p = 0.89	7.24±0.08	7.23±0.08	p = 0.97	p < 0.001	p = 0.98
	T	6.43±0.06	6.46±0.06				6.94±0.08	6.96±0.08			
	F	6.43±0.09	6.50±0.09				7.03±0.11	7.02±0.11			
	TF	6.65±0.07	6.65±0.07				6.84±0.11	6.83±0.11			
4D	C	7.55±0.1	7.56±0.1	p = 0.71	p < 0.01	p = 0.99	8.07±0.09	8.04±0.09	p = 0.73	p < 0.001	p = 0.90
	T	7.29±0.1	7.31±0.1				7.67±0.09	7.71±0.09			
	F	7.34±0.13	7.34±0.13				7.72±0.12	7.70±0.12			
	TF	7.39±0.11	7.45±0.11				7.66±0.12	7.60±0.12			
2D:4D	C	0.88±0.01	0.89±0.01	p = 0.74	p = 0.18	p = 0.97	0.90±0.01	0.90±0.01	p = 0.74	p = 0.25	p = 0.98
	T	0.88±0.01	0.88±0.01				0.90±0.01	0.90±0.01			
	F	0.88±0.01	0.88±0.01				0.91±0.01	0.91±0.01			
	TF	0.90±0.01	0.90±0.01				0.89±0.01	0.90±0.01			

The lengths of digits were measured using µCT images. C (Females: n=15; Males: n=16), T (Females: n=14; Males: n=14), F (Females: n=8; Males: n=9) and TF (Females: n=11; Males: n=8).

received testosterone (T, TF groups) had higher plasma testosterone concentrations compared to the C group (T: $t_{15} = 5.93$, $p < 0.001$; TF: $t_{15} = 6.37$, $p < 0.001$). Two-way ANOVA revealed the main effect of testosterone ($F_{1,15} = 61.9$, $p < 0.001$), but not flutamide (n.s.), on circulating testosterone concentration. The interaction between these two factors was not significant.

The anogenital distance in adult offspring

Significant differences in anogenital distance were found among both females ($F_{3,47} = 16.6$, $p < 0.001$) and males ($F_{3,45} = 15.3$, $p < 0.001$). Among females, the T group had a longer anogenital distance when compared to the C group (by 19 %, $t_{47} = 6.23$, $p < 0.001$) and the TF group (by 14 %, $t_{47} = 4.68$, $p < 0.001$). Two-way ANOVA showed a significant effect of both testosterone ($F_{1,47} = 26.0$, $p < 0.001$) and flutamide ($F_{1,47} = 10.9$, $p < 0.01$) treatment on anogenital distance. The testosterone x flutamide interaction was statistically significant ($F_{1,47} = 9.06$, $p < 0.01$).

Among males, a shorter anogenital distance was found in the T (by 6 %) and F (by 8 %) groups compared to the C group (T vs C: $t_{45} = 3.21$, $p < 0.05$; F vs C: $t_{45} = 3.75$, $p < 0.01$). T males had longer anogenital distances compared to males in the TF group (by 9 %, $t_{45} = 3.71$, $p < 0.01$). Two-way ANOVA showed the main effect of both testosterone ($F_{1,45} = 17.0$, $p < 0.001$) and flutamide ($F_{1,45} = 27.8$, $p < 0.001$). The interaction between these two factors was not significant.

Analysis of 2D length, 4D length, and 2D:4D using digital scan and µCT measurements

Based on digital scans, neither females nor males showed significant differences in 2D length, 4D length, or 2D:4D (Tab. 2). Therefore, we performed an analysis of the phalanges based on measurements of µCT images. The µCT analysis revealed significant differences in the 2D lengths of the left and right forepaws (females: (left: $F_{3,44} = 5.54$, $p < 0.01$, right: $F_{3,44} = 11.6$, $p < 0.001$); males: (left: $F_{3,43} = 6.52$, $p < 0.001$, right: $F_{3,43} = 7.5$, $p < 0.001$) (Tab. 3).

Among females, prenatal T and F resulted in a shorter left and right 2D compared to the C group (left: (T: by 4 %; F: by 4 %); right: (T: by 4 %; F: by 4 %)); (left: (C vs T: $t_{44} = 3.28$, $p < 0.01$, C vs F: $t_{44} = 2.82$, $p < 0.05$), right: (C vs T: $t_{44} = 5.39$, $p < 0.001$, C vs F: $t_{44} = 3.88$, $p < 0.01$)). In addition, the T group had a shorter 2D than the TF group (left: by 3 %; $t_{44} = 2.75$, $p < 0.05$; right: by 3 %; $t_{44} = 3.47$, $p < 0.01$).

Among males, prenatal treatment led to a shorter 2D (T treatment) and 4D (T and F treatment) when compared to the C group (2D: (left: (T by 4 %; C vs T: $t_{43} = 3.47$, $p < 0.01$), right: (T by 4 %; C vs T: $t_{43} = 3.38$, $p < 0.01$)), 4D: (left: (T by 5 %; C vs T: $t_{43} = 4.29$, $p < 0.001$, F by 4 %; C vs F: $t_{43} = 3.28$, $p < 0.01$), right: (T by 4 %; C vs T: $t_{43} = 3.65$, $p < 0.01$, F by 4 %; C vs F: $t_{43} = 3.35$, $p < 0.01$))). There were no significant differences in female 4D measurements among groups or in 2D:4D of both sexes among groups (Tab. 3).

Laterality in 2D length, 4D length, and 2D:4D

Using µCT, two-way ANOVA showed a significant effect of treatment on lengths of 2D and 4D in both sexes. Bonferroni post-

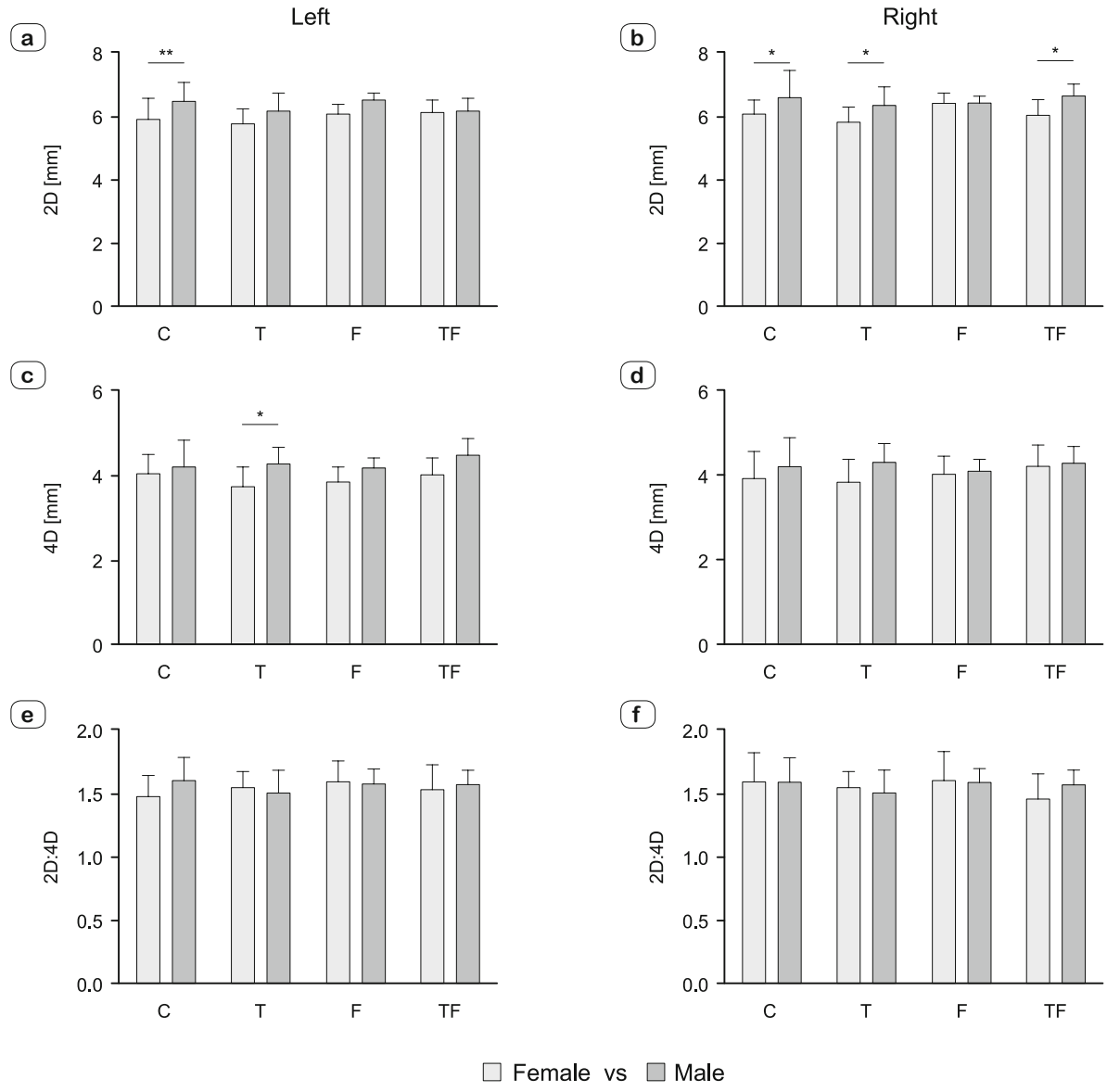

Fig. 2. Sex differences in 2D length (a, b), 4D length (c, d), and 2D:4D (e, f). Females: C: (n=15), T: (n=14), F: (n=9), TF: (n=12); Males: C: (n=16), T: (n=14), F: (n=9), TF: (n=9). The lengths of digits were determined using digital scan. Data are expressed as means + SD. *p<0.05, **p<0.01, ***p<0.001.

hoc test revealed no differences among treatment groups. Similarly, the lengths of the left and right forepaws were not different. In both measurements, the lengths of 2D and 4D, the treatment x laterality interaction was not significant (Tab. 5).

In the case of the digital scans, the main effect of treatment was observed only with the lengths of 2D in females. Bonferroni post-hoc test did not show any differences among the groups. No significant differences were found among the groups in the lengths of 4D for both sexes (Tab. 4). Using both methods, neither effect of treatment nor laterality were observed on 2D:4D in females as well as in males (Tabs 4 and 5).

Sex differences – digital scan

Sex differences in 2D length, 4D length, and 2D:4D were determined using two-way ANOVA (independent factors: sex and treatment). The main effect of sex was observed in the lengths of left and right 2D and left 4D (2D: left: ($F_{1,90} = 12.6$, $p < 0.001$); right: ($F_{1,90} = 15.1$, $p < 0.001$)); 4D: left ($F_{1,90} = 14.5$, $p < 0.001$)). This effect was not found on the right 4D (n.s.). Females had a shorter 2D than males in: the C group, on the left forepaw by 9 % and the right forepaw by 8 %; the T and TF groups, on the right forepaw by 9 % (left: (C: $t_{90} = 3.14$, $p < 0.01$) (Fig. 2a); right: (C: $t_{90} = 2.76$, $p < 0.05$; T: $t_{90} = 2.78$, $p < 0.05$; TF: $t_{90} = 2.6$, $p < 0.05$) (Fig. 2b). T group females had a shorter left 4D compared to T

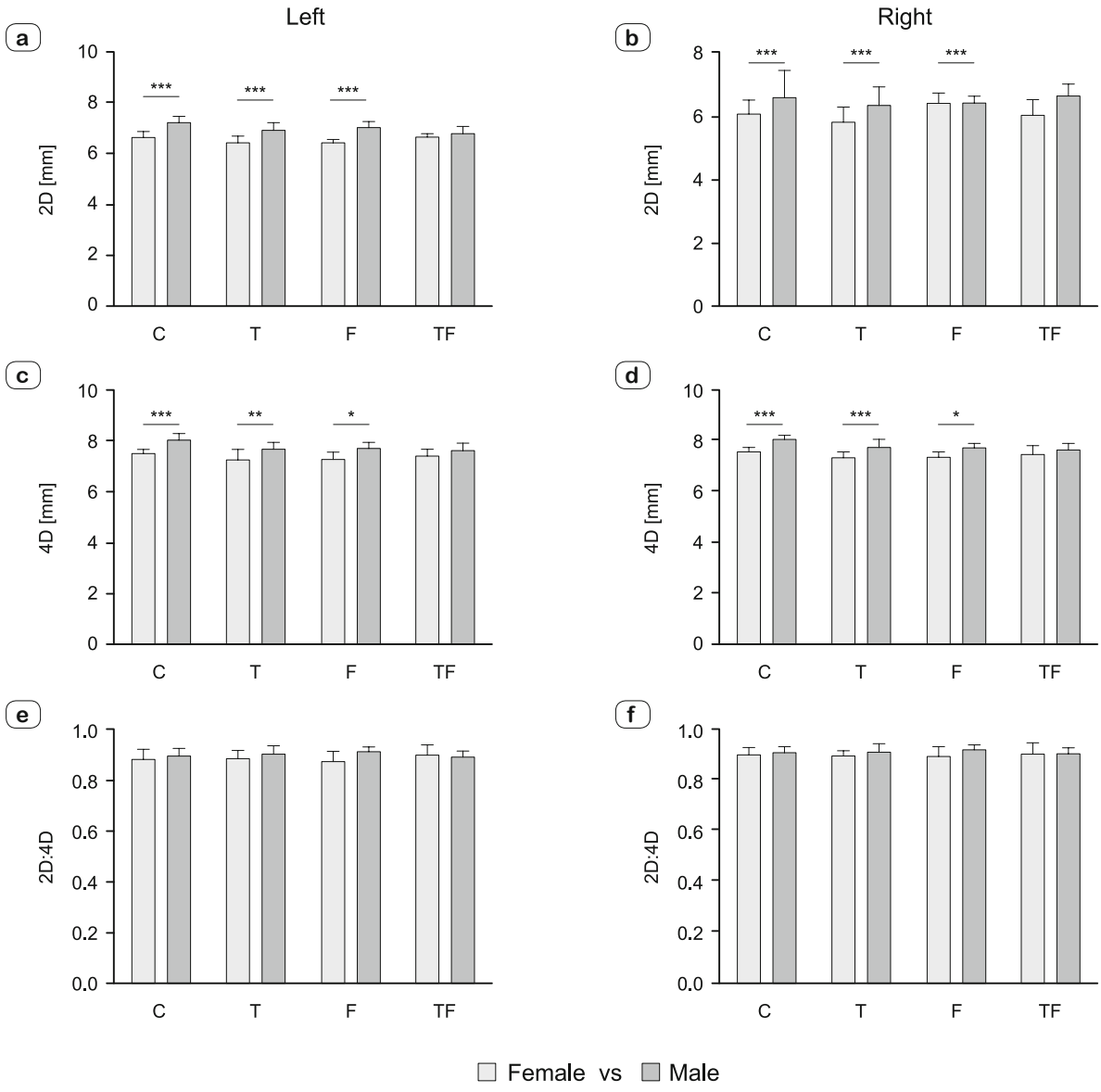

Fig. 3. Sex differences in 2D length (a, b), 4D length (c, d), and 2D:4D (e, f). Females: C: (n=15), T: (n=14), F: (n=8), TF: (n=11); Males: C: (n=16), T: (n=14), F: (n=9), TF: (n=8). The lengths of digits were determined using analysis of phalanges based on µCT images. Data are expressed as means + SD. *p<0.05, **p<0.01, ***p<0.001.

group males by 13 % (t_{90} = 3.06, p < 0.05) (Fig. 2c). The effect of treatment was not revealed in the lengths of 2D and 4D (2D: (left, right: n.s.); 4D: (left, right: n.s.)). The interaction between sex and treatment was not significant (2D: (left, right: n.s.); 4D: (left, right: n.s.)). Analysis of 2D:4D revealed neither an effect of sex nor an effect of treatment (sex: (left, right: n.s.; treatment (left, right: n.s.) (Figs 2e, 2f). The treatment x sex interaction was not significant (left, right: n.s.).

Sex differences – analysis of phalanges by µCT measurements

The analysis of the phalanges based on µCT measurements also showed differences between females and males in the lengths of 2D and 4D (2D: left: ($F_{1,87}$ = 103, p < 0.001), right: ($F_{1,87}$ = 122, p < 0.001); 4D: left: ($F_{1,87}$ = 48.5, p < 0.001); right ($F_{1,87}$ = 43.3, p < 0.001)). Males had a longer average 2D than females in the C (left: by 8 %; right: by 7 %), T (left: by 7 %; right by 7 %), and F groups (left: by 9 %; right: by 6 %) but not in the TF group (C: (t_{87} = 7.31, p < 0.001; right: t_{87} = 7.54, p < 0.001); T: (left: t_{87} = 6.17, p < 0.001; right: t_{87} = 7.49, p < 0.001); F: (left: t_{87} = 5.76, p < 0.001; right: t_{87} = 6, p < 0.001); TF: (left, right: n.s.). Regarding the lengths of left and right 4D, a significant effect of sex (left: ($F_{1,87}$ = 48.5, p < 0.001); right ($F_{1,87}$ = 43.3, p < 0.001)) and treatment (left: ($F_{3,87}$ = 9.19, p < 0.001); right: ($F_{3,87}$ = 8.85, p < 0.001)) was found. The sex x treatment interaction was not significant (left:

($F_{3,87} = 0.94$, $P = 0.42$) (Fig. 3c); right: ($F_{3.87} = 1.67$, $P = 0.18$) (Fig. 3d). Males displayed a longer 4D compared to females in the C (left: by 6 %; right: by 6 %), T (left: by 5 %; right: by 5 %), and F groups (left: by 5 %; right: by 5 %) but not in the TF group (C: (left: $t_{87} = 5.53$, $p < 0.001$; right: $t_{87} = 5.31$, $p < 0.001$); T: (left: $t_{87} = 3.81$, $p < 0.01$; right: $t_{87} = 4.31$, $p < 0.001$); F: (left: $t_{87} = 3.01$, $p < 0.05$; right: $t_{87} = 2.98$, $p < 0.05$); TF: (left, right: n.s.);. The effect of sex on 2D:4D in both left ($F_{1,87} = 4.94$, $p < 0.05$, Fig. 3e) and right ($F_{1,89} = 4.41$, $p < 0.05$) (Fig. 3f) forepaws was observed. The prenatal treatment did not affect the 2D:4D (left, right: n.s.); (Figs 3e, 3f)). The sex x treatment interaction was not significant (left, right: n.s.) (Figs 3e, 3f).

Discussion

In the present study, the effect of prenatal testosterone on 2D length, 4D length, and 2D:4D in relation to sex was examined. Anogenital distance measurements confirmed a prenatal exposure to a hyperandrogenic environment. Prenatal T masculinized the anogenital distance in females and feminized it in males. In females, the analysis of phalanges by μCT measurements indicated that prenatal T, as well as F, leads to a shorter 2D. Regarding the digit lengths of male rats, prenatal hyperandrogenism resulted in a shorter 2D and 4D, while F only caused a shortening of 4D. Neither digital scans nor μCT measurements indicated the effect of prenatal T on the 2D:4D. μCT measurements revealed shorter lengths of 2D and 4D in females compared to males (C, T, and F groups), while the digital scan showed only subtle sex differences. These results indicate a potential lack of sensitivity in using digital scans for digit length measurements.

Anogenital distance, defined as the distance between the genitalia and the anal opening, is an external morphological marker of prenatal androgen exposure in rodents (24, 25). In our study, T females had longer anogenital distances compared to C females. In T males, a shorter anogenital distance was observed compared to C males indicating that testosterone might be metabolized to estradiol. However, previous studies reported no alterations in anogenital distance of males after prenatal testosterone treatment (26–28). F females displayed shorter anogenital distances, an effect consistent with previously published data (29). In addition, TF males also had shorter anogenital distance compared to T males.

The effect of prenatal testosterone on the lengths of the digits was formerly studied in both mice and rats (6, 8, 9). These studies have reported a positive correlation between testosterone concentration and the length of 4D (2, 6, 8, 9) resulting in a lower 2D:4D (9). In contrast, our results did not show any prolongation of 4D after prenatal testosterone treatment. There are only a few studies reporting no effect of prenatal T on the length of 2D (6, 8). In our study, both treatments resulted in the shortening of 2D, an effect also reported by Talarovičová et al (9). Huber et al (8) showed no effect of either T or F on 2D:4D. Similarly, in our study, there were no differences in the 2D:4D among treatments. These results are in contrast with previously published studies (6, 9) showing a relationship between the concentration of testosterone and a lower 2D:4D.

Digit lengths showed sexual dimorphism (females < males), a finding that has been demonstrated by other studies (9, 30). Similar to the findings of Talarovičová et al (9), males in all groups had a longer 2D and 4D in both the left and right forepaws compared to females. No sex differences were observed in the 2D:4D, a finding that has also been demonstrated by previous studies (9, 23, 31). Our results, however, are contradictory to the results of Brown et al (32), where the 2D:4D was higher in females compared to males. The authors found a higher 2D:4D in the right forepaws of females compared to that of males.

According to our results, when comparing the left and right forepaws, no differences were found in the 2D lengths, 4D lengths, and 2D:4D in both sexes. As far as animal studies are concerned, our study is the first investigating laterality in 2D length, 4D length, and 2D:4D. In humans, there are studies reporting a higher 2D:4D in the right hand compared to the left (2, 7). Contradictory findings may arise from differences in methodology used in the aforementioned studies, including different strains of animals (6, 8, 9), regimen of testosterone exposure (9), dosing and type of testosterone (6, 8, 9), and different methods of treatment administration (6, 8). Moreover, the use of different techniques (6, 8, 9) and software (8) may have affected the outcome.

The current study has limitations that should be pointed out. In the present study, the testosterone administration was restricted to the last week of gestation. The use of different doses of testosterone during different periods of gestation may be more informative. Despite some limitations, to the best of our knowledge, this is the first study analyzing the effect of prenatal hyperandrogenism on 2D:4D in relation to sex differences in laboratory rats using two different methodologies. The results of the anogenital distance measurements suggest that the dose of testosterone was adequate. Despite these findings, in both sexes, prenatal testosterone, exogenous or endogenous, had no effect on the 2D:4D. The results of our study can be explained by Manno et al (2008), who reported that the intrauterine formation of 2D and 4D is a complex process (31). This process can also be related to different testosterone metabolites and other steroids hormones, not only to testosterone.

In conclusion, the results of our study demonstrate that the 2D:4D does not reflect prenatal androgen exposure. The effect of prenatal testosterone was observed only in the decreased lengths of 2D and 4D compared to controls. Further studies focusing on the influence of estrogen and other testosterone metabolites in different periods of gestation on 2D:4D are necessary.

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