Brief Communication: Latent Toxoplasmosis and Salivary Testosterone Concentration—Important Confounding Factors in Second to Fourth Digit Ratio Studies

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ABSTRACT A sexually dimorphic characteristic, the second to fourth digit ratio (2D:4D ratio), has been shown to reflect the prenatal concentration of sex steroid hormones and to correlate with many personality, physiological, and life history traits. The correlations are usually stronger for the right than the left hand. Most studies have shown that the 2D:4D ratio does not vary with age or postnatal concentration of sex steroid hormones. Recently, a strong association between left hand 2D:4D ratio and infection with a common human parasite *Toxoplasma* has been reported. We hypothesized that the confounding effect of *Toxoplasma* infection on left hand 2D:4D ratio could be responsible for the stronger association between different traits and right hand rather than left hand 2D:4D ratio. This confounding effect of toxoplasmosis could also be responsible for the difficulty in finding an association between 2D:4D ratio and age or postnatal steroid hormone concentration. To test this hypothesis, we analyzed the association between sex and age and 2D:4D ratio in a population of 194 female and 106 male students with and without controlling for the confounding variables of *Toxoplasma* infection and testosterone concentration. Our results showed that the relationship between age and sex and 2D:4D ratio increased sharply when *Toxoplasma* infection and testosterone concentration were controlled. These results suggest that left hand 2D:4D ratio is more susceptible to postnatal influences and that the confounding factors of *Toxoplasma* infection, testosterone concentration and possibly also age, should be controlled in future 2D:4D ratio studies. Because of a stronger 2D:4D dimorphism in *Toxoplasma*-infected than *Toxoplasma*-free subjects, we predict that 2D:4D ratio dimorphism as well as right hand/left hand 2D:4D ratio dimorphism will be higher in countries with a high prevalence of *Toxoplasma* infection than in those with a low prevalence. Am J Phys Anthropol 137:479–484, 2008. © 2008 Wiley-Liss, Inc.

Males and females in many species (Brown et al., 2002a; Roney et al., 2004; Rubolini et al., 2006) including humans (Manning, 2002) differ in second to fourth digit ratio (2D:4D ratio). A low 2D:4D ratio in humans is thought to be associated with a high prenatal testosterone to estrogen ratio (Brown et al., 2002b; Okten et al., 2002; Lutchmaya et al., 2004). The 2D:4D ratio is formed in early embryogenesis probably under the influence of Hox genes products (Manning et al., 1998) and seems to be fairly stable after the age of 2 years (Manning, 2002). The correlation between postnatal concentrations of sex steroid hormones and 2D:4D ratio is minimal or absent (Neave et al., 2003).

The 2D:4D ratio covaries with sperm quality (Wood et al., 2003), fecundity (Manning et al., 2000a; Saino et al., 2006), sexual orientation (Hall and Love, 2003; Lippa, 2003), autism (Manning et al., 2001), numerical competence (Fink et al., 2006a), and several personality traits including aggression, intelligence, agreeableness, cooperativeness, and sensation-seeking (Fink et al., 2004; Bailey and Hurd, 2005; Luxen and Buunk, 2005; Fink et al., 2006b; Millet and Dewitte, 2006). With some exceptions, the association between 2D:4D ratio and various traits tends to be stronger for the right than the left hand. Moreover, right hand compared to left hand 2D:4D ratio expresses higher heritability (i.e., the ratio between genetically determined variability and total variability of the trait) (Paul et al., 2006).

Recently, an association between left hand 2D:4D ratio and infection with a common protozoan parasite *Toxoplasma gondii* (worldwide prevalence varies between 20 and 70% in different countries, depending on climate, hygiene standards, and eating habits) has been observed (Flegr et al., 2005). The probability of infection with this intracellular parasite is expected to correlate negatively with the activity of the cellular arm of the immune sys-

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tem, which is known to be inhibited by a high concentration of steroid hormones (Roberts et al., 2001; Schuster and Schaub, 2001). However, *Toxoplasma* is also known to induce behavioral and neurophysiological changes in infected human or animal hosts. Increased dopamine and testosterone levels are suspected to play an important role in the observed changes; for a review, see Flegr (2007). Nevertheless, the statistical association between a low 2D:4D ratio and *Toxoplasma* infection does not tell us whether subjects with a low 2D:4D ratio have a higher probability of *Toxoplasma* infection or whether *Toxoplasma* modifies the concentration of steroid hormones (and the 2D:4D ratio) in infected hosts.

An association between toxoplasmosis and increased testosterone concentration was postulated on the basis of both indirect and direct evidence. Infected males are taller, have a lower left hand 2D:4D ratio (Flegr et al., 2005), and are perceived as more dominant and masculine (Hodkova et al., 2007). Infected females have a (nonsignificantly) lower left hand 2D:4D ratio and are more likely to give birth to a boy than a girl (Kaňková et al., 2007a); the latter is also true for laboratory infected mice (Kaňková et al., 2007b). Recently, a case control study showed that *Toxoplasma*-infected males had a (nonsignificantly) higher ($F_{1,81}$ = 3.5, P = 0.06) and, surprisingly, *Toxoplasma*-infected females had a lower ($F_{1,149}$ = 15.6, P < 0.001) concentration of testosterone than *Toxoplasma*-free controls (Flegr et al., 2008).

Here, we tested our hypotheses that the confounding effect of *Toxoplasma* infection could be responsible for the stronger association between right than left hand 2D:4D ratio and various traits, as well as for the difficulty in finding any association between 2D:4D ratio and age or postnatal steroid hormone concentration. To test these hypotheses, we analyzed the effects of sex, age, *Toxoplasma* infection, and salivary testosterone on 2D:4D ratio in a population of 194 female and 106 male students.

MATERIALS AND METHODS

Subjects

Undergraduate biology students of the Faculty of Sciences, Charles University, Prague, were approached during regular biology lectures and were invited to participate in the study on a voluntary basis. One hundred and six (106) male students and one hundred and ninety-four (194) female students were enrolled in the study and signed an informed consent form. All participants provided 2 ml of blood for serological testing and three saliva samples of about 200 μl (collected at approximately 9:00, 11:30, and 13:00). Meanwhile, all participants performed an identical set of psychological and behavioral tests (Lindová et al., 2006). All sera and saliva samples were stored in a freezer at −20°C until assayed. The recruitment of the study subjects and data handling practices complied with the Czech regulations in force.

Anthropometry

The anthropometric parameters, namely the length of the second and fourth fingers (on the ventral surface of the hand from the basal crease of the digit to the tip), were measured on the right and left hands using a sliding caliper with a resolution of 0.1 mm. The same person, a trained anthropologist (VP), did all measurements. Neither the participant nor the anthropologists were aware of the *Toxoplasmosis* status of the participant.

Immunological tests for toxoplasmosis

All serological tests were carried out in the National Reference Laboratory for Toxoplasmosis, National Institute of Public Health, Prague. Specific anti-*Toxoplasma* IgG in all subjects and IgM in high IgG-subjects were determined by ELISA (IgG: SEVAC, Prague, IgM: TestLine, Brno) optimized for early detection of acute toxoplasmosis (Pokorny et al., 1989), and by the complement fixation test (CFT) (SEVAC, Prague), which is more reliable in old *T. gondii* infections as decrease of CFT titers is more regular (Warren and Sabin, 1942). *Toxoplasma* antibody titers in the sera were measured at dilutions between 1:4 and 1:1,024. Participants who tested IgM negative by ELISA (positivity index < 0.9) and who had CFT titers higher than 1:4 were considered latent toxoplasmosis positive.

Radioimmunoassay test for testosterone

All testosterone assays were performed at the Institute of Endocrinology, Prague. Saliva samples and controls or blanks (bidistilled water), 1.0 ml each, were spiked with [^{3}H]testosterone (Radiochemical Centre, Amersham, UK, 1200 dpm/sample), and extracted in duplicate with diethyl ether (4 ml) in stoppered glass tubes. The aqueous phase was frozen in solid carbon dioxide, the organic phase was decanted and the ether was fully evaporated. The extracts were dissolved in ethanol (500 μl); 100 μl of the solution was removed to quantify loss during extraction, and the rest was evaporated again and taken for radioimmunoassay (RIA). A standard curve consisting of 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 nmol/l testosterone was prepared in duplicate. Antiserum (rabbit anti-testosterone-3-CMO: BSA, working dilution 1:100,000) and the tracer ([^{125}I] iodohistaminyl testosterone derivative, 15,000 cpm), 100 μl each, were added; the volume was adjusted to 300 μl with the working buffer (20 mmol sodium phosphate saline containing sodium azide and BSA, 0.1% each) and the tubes were equilibrated at room temperature for 1 h or overnight at 4°C. After incubation, 1 ml of dextran-coated charcoal suspension (0.025 and 0.25 g/100 ml, respectively) was added to each tube to separate the free fraction, and the radioactivity of ^{125}I was measured in the supernatant using a 12-channel gamma counter (Berthold, FRG). Results were calculated from the standard curve using a log-logit transformation, corrected for recovery and expressed as nmol of testosterone per liter of sample. The samples were assayed in triplicates and the concentration of testosterone was calculated as the arithmetic mean in these triplicates. Because of the low volume of some saliva samples, testosterone concentration data were obtained for only 135 females and 73 males.

Statistics

The relationship between 2D:4D ratio and the independent factors sex, age, testosterone, and *Toxoplasma* infection was tested with General Linear Models (GLM), with simple models with a single confounding factor, i.e. testosterone, and with nonparametric Kendall partial rank correlation (Sheskin, 2003). Because the statistical significance of the corresponding parametric and nonparametric tests was qualitatively identical, we present here only the results of the GLM analysis in which control for two confounding factors (testosterone concentration and toxoplasmosis) is possible. There was virtually no difference between results of statistical tests performed with or without $\log(1 + x)$ transformation of the testosterone concentration data.

RESULTS

The average age of 194 females was 21.05 years, range 18–28; average concentration of testosterone (data available for 135 females) was 0.23 nmol/l, range 0.04–0.8; average right hand 2D:4D ratio was 0.99, range 0.92–1.07; and average left hand 2D:4D ratio was 0.99, range 0.91–1.10. The average age of 106 males was 20.94 years, range 18–27; average concentration of testosterone (data available for 73 males) was 0.41 nmol/l, range 0.08–1.09; average right hand 2D:4D ratio was 0.97, range 0.91–1.05; and average left hand 2D:4D ratio was 0.98, range 0.91–1.07. The difference between females and males was not significant for age ($t_{298} = 0.47$, $P = 0.64$) or left hand 2D:4D ratio ($t_{298} = 1.70$, $P = 0.09$). Males had significantly higher levels of testosterone ($t_{206} = 7.46$, $P < 0.001$) and a lower right hand 2D:4D ratio ($t_{299} = 3.44$, $P < 0.001$) than females. Thirty-one (15.9%) females and 25 (23.6%) males were *Toxoplasma*-infected. Infected males had higher and infected females had lower testosterone levels than *Toxoplasma*-free males and females, respectively (Toxo × Sex interaction: $F_{1,204} = 7.52$, $P = 0.007$, Fig. 1). Differences between *Toxoplasma*-infected and *Toxoplasma*-free subjects were not significant for the right hand 2D:4D ratio; however, when tested for each sex separately, the *Toxoplasma*-infected males had a lower left hand 2D:4D ratio than *Toxoplasma*-free males ($t_{71} = 2.73$, $P = 0.008$). The difference was not significant for females ($t_{133} = 0.59$, $P = 0.56$).

Multivariate GLM analysis with the dependent variable 2D:4D ratio and independent variables sex, testosterone concentration, and age showed a significant association between sex and right hand 2D:4D ratio ($F_{1,203} = 7.22$, $P = 0.008$) but not left hand 2D:4D ratio ($F_{1,203} = 2.55$, $P = 0.11$). In the model that also included the factor *Toxoplasma* infection, we detected an association between *Toxoplasma* infection and left hand 2D:4D ratio ($F_{1,201} = 7.14$, $P = 0.008$). Furthermore, the strength of the association between sex and both right and left hand 2D:4D ratios increased ($F_{1,201} = 8.79$, $P = 0.003$ and $F_{1,201} = 5.40$, $P = 0.021$, respectively).

Because of the existence of the sex–*Toxoplasma* interaction, we performed further analyses separately for males and females. For males, the simple GLM model revealed no significant relationship between age and right or left hand 2D:4D ratio ($F_{1,104} = 2.00$, $P = 0.16$ and $F_{1,104} = 1.28$, $P = 0.26$, respectively). In the model that also included the covariate testosterone concentration, the relationship between age and the right hand ratio became significant ($F_{1,70} = 4.48$, $P = 0.038$), while that between age and left hand 2D:4D ratio remained nonsignificant ($F_{1,70} = 1.72$, $P = 0.193$). No correlation was observed between testosterone concentration and 2D:4D ratio (right hand: $F_{1,71} = 0.70$, $P = 0.407$, left hand $F_{1,71} = 0.17$, $P = 0.686$). The most complex model containing the independent factors age, testosterone concentration, and *Toxoplasma* infection showed a significant relationship between age and both right and left hand 2D:4D ratios (Table 1). The association between testosterone concentration and 2D:4D ratio remained

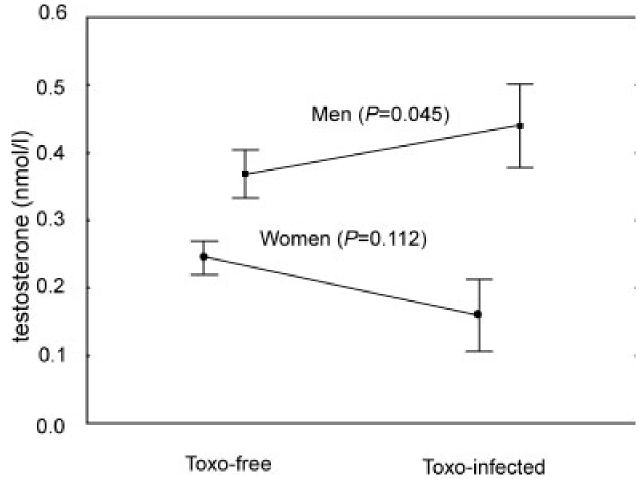

Fig. 1. Testosterone levels in saliva of *Toxoplasma*-free and *Toxoplasma*-infected males and females. Vertical bars denote 0.95 confidence intervals.

nonsignificant; however, the relationship between age and 2D:4D ratio dropped below the formal level of statistical significance. In the simpler models, where the testosterone concentration was not controlled, the effect of age was not significant (right hand: $F_{1,102} = 3.30$, $P = 0.07$, left hand: $F_{1,102} = 3.00$, $P = 0.09$).

For females, no significant relationship between 2D:4D ratio and age or testosterone concentration was observed (Table 1).

DISCUSSION

Our study has shown that the observed association between sex and 2D:4D ratio increased sharply when *Toxoplasma* infection and testosterone concentration were controlled. The results also show a negative relationship between age and 2D:4D ratio in males aged 18–27. This relationship was stronger for the right than the left hand. It reached the formal level of statistical significance for the right hand when the confounding variables, i.e., concentration of testosterone in saliva and *Toxoplasma* infection, were controlled. The relationship was in the opposite direction (positive) for females, but did not reach a formal level of statistical significance. In agreement with previous observations, no relationship between 2D:4D ratio and testosterone concentration was observed.

2D:4D ratio is thought to indicate prenatal concentrations of sex steroid hormones. The results of cross-sectional studies have suggested that this ratio remains constant during the postnatal development of males and females. For instance, Manning et al. (1998) found no evidence of a change in 2D:4D ratio with age either among 400 males and 400 females at the age of 2–25 years or in a sample of 69 males, mean age of 34 years, and 62 females, mean age of 32 years. No relationship between age and 2D:4D ratio was observed in 798 children aged 5–14 years drawn from Caucasian populations (Berberos, $n = 90$, and Asian Uygurs, $n = 438$), Orientals (Chinese Han, $n = 118$), and an Afro-Caribbean population (Jamaicans, $n = 152$) (Manning et al., 2004). Opposite results have been obtained in longitudinal studies. A study on a sample of 54 male and 54 female Jamaican children aged 7–13 (first measurement) and 11–17

TABLE 1. Effects of age, sex, testosterone concentration, and toxoplasmosis on 2D:4D ratio estimated with GLM tests

	Right hand 2D:4D ratio				Left hand 2D:4D ratio			
	β	*F*	*P*	η^2	β	*F*	*P*	η^2
Females (n = 135, right hand R^2 = 0.014, left hand R^2 = 0.007)								
Age	0.040	0.21	0.649	0.002	0.055	0.39	0.533	0.003
Testosterone	−0.056	0.40	0.526	0.003	0.037	0.18	0.687	0.001
Toxoplasmosis	−0.089	1.02	0.315	0.008	0.047	0.28	0.587	0.002
Males (n = 73, right hand R^2 = 0.096, left hand R^2 = 0.149)								
Age	−0.283	5.76	**0.019**	0.077	−0.239	4.38	**0.040**	0.060
Testosterone	−0.056	0.22	0.639	0.003	0.041	0.13	0.720	0.002
Toxoplasmosis	0.169	1.95	0.167	0.027	0.371	9.95	**0.002**	0.126
Females and males (n = 208, right hand R^2 = 0.078, left hand R^2 = 0.060)								
Age	−0.070	1.05	0.306	0.005	−0.058	0.70	0.405	0.003
Testosterone	−0.066	0.72	0.396	0.004	0.037	0.23	0.634	0.001
Toxoplasmosis	0.007	0.01	0.920	0.001	0.187	7.14	**0.008**	0.034
Sex	0.289	8.78	**0.003**	0.042	0.229	5.40	**0.021**	0.026
Sex-toxoplasmosis	−0.122	1.90	0.170	0.090	−0.170	3.64	0.058	0.018

Significant results are printed in bold.
The headers of three parts of the table show number of cases and full model R^2.
The values of partial η^2 indicate effect size for particular factors.

(second measurement) found the 2D:4D and 2D:3D ratio to increase with age at similar rates in all age classes while 3D:5D and 4D:5D ratios decreased during the same period of time (Trivers et al., 2006). Similar results were obtained in a study on 130 Jamaican children aged 5–11 years (Manning et al., 2000b) and for preschool children in Scotland aged 2–5 years (Williams et al., 2003). Another longitudinal study included 111 children from the Ohio area. Radiographs of children's left hands were taken at the age of 1, 5, 9, 13, and 17 years. The 2D:4D ratio increased at a constant rate between the ages of 1 and 13 years in females and 1 and 17 years in males (McIntyre et al., 2005). Between 13 and 17 years, the 2D:4D ratio remained constant for females but increased for males. However, the shape of a similar growth function for the 3D:4D ratio suggested a downward trend after the age of 17 for both males and females. Based on our data, negative results of cross-sectional studies can possibly be explained by a masking effect of the confounding factors such as postnatal concentration of testosterone and *Toxoplasma* infection. When this effect was controlled, the relationship between age and the 2D:4D ratio could be detected even in our relatively age-homogeneous population.

Most published studies have found no relationship between postnatal testosterone concentration and the 2D:4D ratio (Manning, 2002; Neave et al., 2003). Manning et al. (1998) reported a negative association between testosterone concentration and right hand 2D:4D ratio (P = 0.03) and a nonsignificant negative association between testosterone concentration and left hand 2D:4D ratio (P = 0.08) for 58 males. The relationship between 2D:4D ratio and testosterone concentration lost statistical significance when controls were made for weight, height, and age. The problem with the measurement of postnatal levels of steroid hormones, and testosterone in particular, is that they fluctuate widely with season, time of day (Dabbs, 1990a, b), and in response to various external stimuli (Bernhardt et al., 1998). This makes it difficult to estimate long-term concentrations of testosterone based on one-shot measurements (Shirtcliff et al., 2002). The temporal fluctuations of hormonal levels, together with the effects of confounding variables such as toxoplasmosis, could be the reason for the absence of a relationship between 2D:4D ratio and postnatal testosterone concentration in the published studies.

The confounding factor *Toxoplasma* infection has a stronger association with left than right hand 2D:4D ratio. This could explain the stronger relationship between different physiological and psychological traits and 2D:4D ratio for the right hand that is usually found (Manning et al., 2000a). For example, a higher age stability of right hand 2D:4D ratio was observed by Trivers et al. (2006). In their study of the effect of age on 2D:4D ratio, they observed that Cohen's d (the measure for effect size) was 0.27 and 0.51 for right and left hand, respectively. The rate of change of 2D:4D ratio was 0.0025 per year for the right hand, and 0.0036 per year for the left hand; this difference between right and left hand was highly significant (P = 0.006). The higher susceptibility of the left hand 2D:4D ratio to environmental factors such as *Toxoplasma* infection is also supported by the results of a twin study, which showed higher heritability of right hand than left hand 2D:4D ratio (Paul et al., 2006). Toxoplasmosis is negatively associated with both right hand and left hand 2D:4D ratio in males, but not in females. Because of a stronger 2D:4D dimorphism in *Toxoplasma*-infected than *Toxoplasma*-free subjects, we predict that 2D:4D ratio dimorphism as well as right hand/left hand 2D:4D ratio dimorphism will be higher in countries with a high prevalence of *Toxoplasma* infection than in those with a low prevalence. Neither of these predictions has yet been tested.

The proximal mechanism for the association between lower 2D:4D ratio and toxoplasmosis is not clear. *Toxoplasma* infection is associated with a significantly lower left hand 2D:4D ratio in males and a nonsignificantly lower left hand 2D:4D ratio in females (Flegr et al., 2005). *Toxoplasma* infection was also shown to be associated with higher testosterone levels in males and lower testosterone levels in females (Flegr et al., 2007). Most studies report a positive correlation between testosterone and estradiol concentrations (Kirchengast, 1993; Handa et al., 1997; Ankarberg and Norjavaara, 1999; Troisi et al., 2003). Thus, the females with low testosterone are expected also to have low levels of estradiol. Theoretically, the positive correlation between estrogen levels and 2D:4D ratio in females can explain the observed lower 2D:4D ratio in *Toxoplasma*-infected females (Manning et al., 1998). It must be pointed out, however, that the 2D:4D ratio in females is generally expected to be influenced by the testosterone/estrogen (T/E) ratio rather than estrogen levels alone (Manning, 2002). Therefore, no clear prediction about 2D:4D ratio in females with low testosterone can be made before the qualitative relationships between T/E and 2D:4D ratios and testosterone and estrogen levels are resolved.

As noted in previous studies (Flegr et al., 2005; Hodková et al., 2007), a case control study cannot confirm whether *Toxoplasma* infection induces changes in the 2D:4D ratio, or whether low 2D:4D ratio subjects (i.e., subjects with higher testosterone concentrations) have a higher probability of acquiring *Toxoplasma* infection. High concentrations of testosterone are known to have immunosuppressive effects (Roberts et al., 2001; Schuster and Schaub, 2001), which could result in a higher probability of acquiring *Toxoplasma* infection. It must be stressed, however, that the lower concentration of testosterone in *Toxoplasma*-infected females makes the immunosuppression based explanation of the association between *Toxoplasma* infection and low 2D:4D ratio less likely.

Our study has shown that confounding factors such as age, postnatal testosterone concentration, and *Toxoplasma* infection can correlate with 2D:4D ratio. The relationship between 2D:4D ratio and *Toxoplasma* infection is particularly strong for the left hand. This suggests that right hand 2D:4D ratio should be preferentially used to quantify prenatal testosterone levels, themselves an indicator of fertility, behavior, and health. Whenever possible, the confounding factors of age, testosterone concentration, and in particular *Toxoplasma* infection should be controlled.

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