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Maternal corticosterone but not testosterone level is associated with the ratio of second-to-fourth digit length (2D:4D) in field vole offspring (*Microtus agrestis*)

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ABSTRACT

The steroid environment encountered by a foetus can strongly affect its post-natal physiology and behaviour. It has been proposed that steroid concentrations experienced *in utero* could be estimated from adults by measuring their second-to-fourth digit length ratio (2D:4D). However, there is still little direct evidence that intra-uterine steroid levels affect individual 2D:4D. We examined whether maternal pre-pregnancy testosterone and corticosterone levels (as estimates of intra-uterine testosterone and corticosterone exposure) affected the 2D:4D of pups in non-domesticated field voles (*Microtus agrestis*), measured by X-rays at the age of weaning (21 days). Furthermore, for the first time in a non-human species, we studied whether testosterone and corticosterone levels correlated with 2D:4D in adult females. We found that the maternal pre-pregnancy level of testosterone was not associated with offspring 2D:4D in either the left or the right paw. Instead, maternal pre-pregnancy corticosterone level was positively correlated with offspring 2D:4D in the right paw, but unrelated to 2D:4D in the left paw. In addition, the 2D:4D of adult females was not associated with either their circulating testosterone or corticosterone levels. Our results suggest that in field voles maternally administered testosterone is not a major determinant of offspring 2D:4D, whereas maternal stress appears to account for some of the variation in the 2D:4D of their offspring.

1. Introduction

The ratio of second-to-fourth digit length (hereafter 2D:4D) has been highlighted as a useful adult-age marker of prenatal exposure to steroids [25,26,31] that are likely to have strong effects on vertebrate physiology, behaviour and reproduction [33]. In many species, the steroid environment encountered by developing foetuses is very difficult to measure accurately, due to ethical and practical reasons [32]. Therefore, the notion that 2D:4D is a reliable marker of prenatal exposure to steroids rests mainly on indirect evidence, for example regarding sex differences in adult 2D:4D (humans; [26,44], birds; [4,37,41,45], non-human mammals; [5,21,27], reptiles; [8,42]), and the temporal stability of 2D:4D with age [30,47].

However, not all studies have managed to establish a sex difference in 2D:4D [2,11,24] or found that 2D:4D remains unaltered from birth [1,5,6,22,28,29]. Currently the strongest evidence for a link between prenatal steroid concentrations and 2D:4D is provided by recent experimental studies in mammals [46] and birds [45]. For example, Talarovičová et al. [46] found that experimentally elevated levels of maternal testosterone in rats resulted in decreased 2D:4D ratios of offspring. However, these results were based on a rather small sample size, as only three pregnant females were manipulated with testosterone and the subsequent digit ratio measures were based on only eight pups born. More studies, especially on mammals, are therefore highly called for.

Although there is evidence for a leakage of testosterone from male foetuses to neighbouring foetuses (i.e., effects of intra-uterine positioning (IUP), [43]), it is likely that steroid levels in maternal circulation approximately reflect those experienced by the developing embryos, as testosterone levels should be rather stable across maternal tissues [32]. This assumption is supported by reports that have found that systematic administration of testosterone to pregnant female rats in laboratory conditions results in behavioural masculinization of female offspring [17]. Systematic administration of testosterone to pregnant sheep has also been found to result in behavioural and physical masculinization in lambs [39]. Pregnant rats have also shown considerable differences in blood androgen levels, which may, in part, be a possible source of the variation in masculinization of offspring between litters [12]. Transfer of androgens from maternal blood to foetal circulation was provided as an explanation for these findings. It could thus be predicted that maternal testosterone level might also influence the 2D:4D of pups [9]. However, to our knowledge, an association between maternal serum testosterone and offspring 2D:4D has not yet been studied.

In addition to maternal steroids, maternal stress also affects the development of the embryos [16,50]. For example, high levels of

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maternal corticosterone during pregnancy have been found to negatively affect the masculinization of male foetuses in rats [49], and to masculinize female behaviour in guinea pigs [52] and prairie voles [40]. Developmental stress may also be manifested as fluctuating or directional asymmetry [36]. Therefore, maternal corticosterone may also be predicted to affect both absolute 2D:4D and its degree of lateral symmetry in offspring. Previous studies have not, however, measured whether maternal stress during pregnancy has an effect on offspring 2D:4D.

In humans, it has also been discussed that 2D:4D may reflect adult steroid levels as well [25,28,29,32]. However, the issue remains yet controversial, as a recent meta-analysis failed to find clear evidence for such associations [19]. To our knowledge, this question has not yet been directly addressed in non-human species. A recent study, however, found indirect evidence for an association between circulating corticosterone level and 2D:4D in adult mice, by showing that a line selected for high running, which was also associated with a 2-fold increase in circulating corticosterone level, had higher 2D:4D in the right paw, as compared to a control line [51]. Interestingly, this indicates that elevated corticosterone levels may affect also the degree of lateral symmetry in digit ratios.

We studied whether pre-pregnancy maternal testosterone and corticosterone levels correlated with offspring 2D:4D in the left and the right paws in non-domesticated field voles (*Microtus agrestis*). More specifically, we predict that both high maternal testosterone and corticosterone levels result in a lower, more masculine, 2D:4D in pups, and that these effects differ between the sexes and between the left and the right paws. In addition, we examine whether 2D:4D correlates with pre-pregnancy testosterone and corticosterone levels in adult females.

2. Materials and methods

2.1. General methods and study species

The study was conducted in autumn 2005 on first-generation laboratory-born field voles, whose parents were trapped from the wild in south-western Finland during the spring of 2005, and on their offspring from their first litter. Before pairing, the voles were housed in same-sex pairs in standard mouse cages (Tecniplast, Italy), maintained on a 16 h light/8 h dark light-cycle at approximately 20 °C, and fed with Rat/Mouse Breeding Diet pellets (Altromin GmbH, Germany), oats and potatoes *ad libitum*. Dry peat and hay were used as bedding.

Maternal circulating corticosterone level was measured from faecal samples. The rationale for measuring corticosterone from faecal samples instead of from blood was to avoid confounding baseline corticosterone levels with the effect of a short-term rise in corticosterone levels due to handling and sampling [14]. Samples were obtained just prior to pairing by placing females into bedding-free cages, lined with tissue to absorb urine. After this, approximately 30 faecal pellets were collected, inserted into a microtube, their total wet mass measured to the nearest 0.001 g and thereafter immersed in 1 ml of 95% ethanol. Samples were immediately stored until further analyses at −20 °C.

Blood samples for testosterone were collected from mothers prior to breeding from the submandibular area (cheek pouch) using 5 mm Goldenrod Animal Lancets (Medipoint Inc., USA). Approximately 200 µl blood was drawn into microtubes and from there into heparinized 75 µl capillary tubes (Hirschmann Laborgeräte, Germany). Capillary tubes were centrifuged for 5 min at 10 000 rpm (Hettich Mikro 12-24, Germany), after which the plasma was separated from the centrifuged tubes and stored in microtubes at −20 °C until analysis.

Because maternal levels of circulating testosterone likely vary according to the reproductive state of animals [7,34], the oestrous cycle phase of females was recorded according to vaginal cytology [20]. To diminish the confounding effect of circadian rhythm on levels of circulating corticosterone in females [7,34], animals were always sampled in the morning, between 8:00 and 10:00 o'clock.

We formed 28 random breeding pairs of adult voles with no formation of sibling pairs (see [15] for details). The voles were paired for 14 days to ensure fertilisation, after which the males were removed. All pups born ($n = 129$) were sexed visually under a microscope immediately after their birth on the basis of the anogenital distance. This procedure took usually less than a minute per litter to conduct, during which the pups were kept warm and they showed no signs of distress. All litters (mean litter size ± SD = 4.2 ± 1.8) were monitored daily and dead pups ($n = 18$, 14% of all pups born) recovered for gender verification by necropsy. No experimental procedures that could have caused pup mortality were practised. At the age of weaning, (21 days; [53]), all pups were euthanized by CO_2 gas, after which the paws were removed from the individuals for 2D:4D measurements. After this, the sex of all pups was verified by necropsy. Pups that died prior to weaning age (most pup mortality occurred within a few days from birth) were excluded from the study. The study was approved by the Lab-Animal Care & Use Committee of the University of Turku (license number 1534/05).

2.2. Maternal faecal corticosterone assay

Faecal samples were collected into calibrated tubes for the measurement of wet mass. After adding 1 ml of 95% ethanol, the samples were frozen in −20 °C until suspended with a RW 16 homogenizer (IKA®-Werke GmbH & Co. KG, Staufen, Germany). Ethanol was evaporated before lyophilization (Freeze dryer Beta 2-16, Christ, USA) and the dry mass of the sample was recorded. Pulverized samples were then suspended in 1 ml of 100% ethanol with ultrasonication (MSE ultrasonic disintegrator). Corticosterone was extracted with boiling at 75 °C for 20 min. After centrifugation at 12 000 × *g* for 5 min, the supernatants were collected and the faecal material was re-suspended in ethanol. The extraction phase was repeated and the supernatants of each sample were pooled. Ethanol was evaporated and the extract was reconstituted with ultrasonication (MSE ultrasonic disintegrator) in 200 µl of phosphate buffered saline (pH 7.4) containing 1% of bovine serum albumin (Sigma Aldrich, Steinheim, Germany). After reconstitution, the samples were centrifuged at 12 000 × *g* for 5 min and the supernatants collected and stored in −20 °C until analysis. Faecal corticosterone content was analysed using a commercial double antibody 125I radioimmunoassay kit (MP BioMedicals, Orangeburg, New York, USA) according to the procedure described by the manufacturer. The assay sensitivity was 7.7 ng/ml when the limit of detection was defined as the value which is two standard deviations below the mean of the zero standard measurement values. The intra-assay coefficient of variation (CV) was below 10.2%, and the inter-assay CV below 7.2%. The results are expressed as the concentration of corticosterone (ng) per dry mass of faeces (mg) analysed per individual.

2.3. Maternal serum testosterone assay

Maternal plasma testosterone levels were measured with a direct radioimmunoassay (Dia Sorin, Stillwater, MN, USA) using antibody-coated tubes and iodine-labelled T tracer according to the manufacturer's instructions. The analytical sensitivity of the assay was 20 pg/ml. The intra-assay variations were less than 8.06% and the inter-assay variations were below 7.58%.

2.4. Digit ratio measurement

We measured the 2D:4D of weaned pups and their mothers from X-ray scans. In this species, the measurement of 2D:4D from X-rays is superior to measurements from photographs, due to a much higher measurement accuracy ($r = 0.88$ vs. $r = 0.50$, see [22]). The front paws

were removed and glued on to separate labelled hard plastic sheets and X-rayed at the Turku University Hospital with a high-definition dentistry X-ray machine. The X-ray scans were transferred to digital media during this procedure. The second and fourth digit lengths were measured with Image-J software (http://rsb.info.nih.gov/ij/) one phalanx at a time, with three phalanges per digit. The phalanges were then summed to calculate the length of the digits. The digit measurements were calibrated with a precisely measured lead ball (diameter 4.742 mm), which was placed in every X-ray scan. See Lilley et al. [22] for more measurement details.

2.5. Statistical analyses

Prior to the analysis, the raw data was screened for outliers using Grubbs' outlier test [3]. All members of one litter (three offspring) were excluded from the analysis as outliers due to the considerably high corticosterone level of their mother (Grubbs test: $Z=4.6$, Critical $Z=2.89$). The maternal corticosterone level for the removed litter was 5.26 ng/mg, while the mean of the whole data was 0.02 ± 1.14 ng/mg. A total of 25 adult females and 46 male and 60 female pups were included in the analyses. The potential dependence of female testosterone and corticosterone concentrations on estrus cycle phase [7,34] was examined by general linear models, where estrus cycle was treated as a four-level fixed effect (proestrus, oestrus, oestrus–metoestrus, and metoestrus). Female testosterone level tended to be associated with estrus cycle phase ($F_{1,24}=2.77$, $P=0.064$), while corticosterone level was unrelated to estrus cycle ($F_{1,24}=0.48$, $P=0.70$). Hence, variation due to estrus cycle was removed from the maternal testosterone level and the residual values of this variable were used in the subsequent analyses.

We estimated the effects of maternal testosterone and corticosterone levels, left and right paws (laterality) and sex on the 2D:4D of pups using a Generalized Linear Mixed Model (GLMM) with REML estimation [23]. We also included all two-way interactions between maternal testosterone and corticosterone levels, laterality and sex in the model. Moreover, both individual and brood identities were included in the model as random factors in order to control for covariance in digit ratios within individuals (the left and right paws) and within broods. Hence, the Kenward–Roger method was used to estimate the denominator degrees of freedom of fixed effects [23]. The statistical significance of random factors was assessed with one-tailed likelihood ratio tests with chi-square mixture distributions [48]. However, the variance component for individual identity was estimated as negative. We therefore modelled this covariance as an intra-class correlation using a repeated measures approach with compound symmetry covariance structure [23]. This approach properly controls for type I error rate and resumes the power of the analysis. No stepwise model reduction was applied, because such methods dramatically increase the rate of type I errors [35] and because our aim here was to obtain accurate parameter estimates and their standard errors, and thus valid P-values [13]. The model residuals were normally distributed (Kolmogorov–Smirnov test, $D=0.048$, $P>0.15$). The association between maternal 2D:4D and circulating testosterone and corticosterone levels were examined using Pearson correlations, since all variables were normally distributed (maternal corticosterone level after natural log-transformation, Kolmogorov–Smirnov test, $P>0.08$). All analyses were done with SAS 9.2 software (SAS Institute Inc, Cary, NC, USA).

3. Results

3.1. Maternal pre-pregnancy testosterone, corticosterone and pup 2D:4D

There was a significant interaction between maternal corticosterone level and laterality, indicating that maternal corticosterone level had a differing effect on 2D:4D in the left and right paws (Table 1). In the right paw high maternal corticosterone level was related to a lower 2D:4D [β (95% CI) $=-0.018$ (-0.033, -0.003)], whereas in the left paw maternal corticosterone level did not correlate with 2D:4D [β (95% CI) $=0.002$ (-0.017, 0.020)] (Fig. 1). Maternal testosterone level was not associated with the 2D:4D of pups and this effect was independent of sex or laterality (Table 1).

Table 1
The effects of maternal pre-pregnancy testosterone and corticosterone levels on the 2D:4D of field vole pups.

	Estimate	SE	$df_{den,nom}$	χ^2 / F	P
Random effects					
Brood identity	0.0002	0.0001	1	4.23	0.02
Fixed effects					
Sex (S)	−0.0011	0.0100	1,100.6	0.01	0.92
Laterality (L)	0.0034	0.0103	1,103.7	10.64	0.0015
Corticosterone (C)	0.0036	0.0090	1,21.7	2.19	0.15
Testosterone (T)	−0.00002	0.0002	1,23.2	0.27	0.61
C×S	−0.0064	0.0107	1,100.6	0.36	0.55
T×S	−0.0001	0.0002	1,100.5	0.12	0.73
C×L	−0.021	0.011	1,103.7	3.89	0.051
T×L	0.0001	0.0002	1,103.7	0.62	0.43

3.2. Pre-pregnancy testosterone, corticosterone and 2D:4D of adult females

There were no significant correlations between pre-pregnancy testosterone (left paw: $r_{Pearson}$ (95% CI) $=-0.14$ (-0.59, 0.38), $P=0.15$; right paw: $r_{Pearson}$ (95% CI) $=-0.30$ (-0.62, 0.12), $P=0.60$)

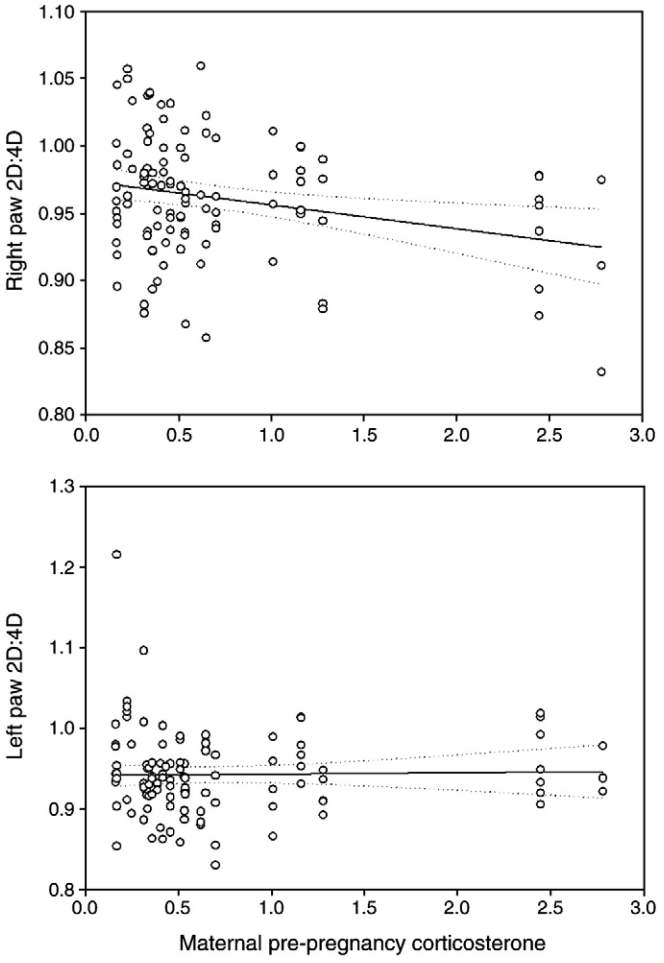

Fig. 1. The correlation between 2D:4D and maternal pre-pregnancy corticosterone levels (ng/mg) in the right and left paws of pups.

and corticosterone levels (left paw: $r_{Pearson}$ (95% CI) = −0.21 (−0.63, 0.33), $P = 0.45$; right paw: $r_{Pearson}$ (95% CI) = −0.03 (−0.42, 0.37), $P = 0.89$) and 2D:4D in adult females.

4. Discussion

Our results suggest that in field voles 2D:4D is not related to prenatal exposure to maternal testosterone, which contrasts both the founding assumption of the 2D:4D hypothesis and recent experimental evidence in other mammals [46] and birds [45], Therefore, if testosterone is a major factor contributing to 2D:4D formation [26], it is most likely the testosterone originating from the foetuses themselves, either from the focal individual or its siblings [18,43], and not from the mother. It should, however, be noted that the results of Hurd et al. [18] showed that mice gestated next to males had larger 2D:4D, not lower as expected by the theory, than those gestated next to females. However, we cannot state that maternal testosterone did not have any effects at all on pup 2D:4D. It may well be that in this species maternal circulating testosterone level correlates poorly with the testosterone level of the foetuses. For example, the enzyme aromatase in mammal placentas generally buffers high levels of testosterone [9], which suggests that maternal testosterone might have only a limited ability to influence foetal testosterone levels.

We found high maternal pre-pregnancy corticosterone levels, an index of maternal stress, to be associated with a lower 2D:4D, i.e., a more masculinized phenotype, in the right paw of both sexes. Previous research has established a rather consistent difference in the left and right hand 2D:4D in humans [26], and this is the case also in field voles [22]. In addition, in humans, it is common that the 2D:4D of the left and right hands is differently associated, for example, with performance in sports and exercise and to some degree, aggression [10]. It is typically the right hand 2D:4D that shows a stronger association with the traits studied [26,38], although the exact mechanism responsible for such laterality remains unknown. It was thus not surprising that maternal pre-pregnancy corticosterone level correlated with the 2D:4D of the right but not the left paw among the pups studied. This result is in agreement with studies on prairie voles (*Microtus ochrogaster*), in which experimental elevation of corticosterone in pregnant females resulted in pronounced masculine behaviour of the offspring [40]. It is however in contrast with the finding that high corticosterone levels in mothers inhibited masculinization in rats and mice [49]. It should be noted that our data are correlative and thus experimental studies exposing pregnant mothers to varying levels of stress are needed to confirm the relation between maternal stress and offspring digit ratio found here.

This study was the first to examine in a non-human species whether circulating testosterone levels in adult females were related to their own 2D:4D. In line with the current understanding in humans [19], we found no marked correlation between the two factors. Likewise, we observed no association between circulating corticosterone levels in females and their 2D:4D. This latter finding runs counter to a recent result in laboratory mice showing that mice having an approximately 2-fold higher level of circulating corticosterone compared to controls had a higher 2D:4D in the right paw [51]. The finding of Yan et al. [51] is somewhat unexpected since a previous research has generally linked high stress hormone levels to more masculinized phenotype. However, the result of Yan et al. [51] may be an artefact related to artificial selection for higher running rates in the mice in which higher corticosterone levels were observed.

To conclude, our results provide no evidence for 2D:4D being associated with individual prenatal exposure to testosterone. We did however find that maternal corticosterone (an indicator of maternal stress) is linked to asymmetry in the expression of digit ratios between paws of field vole offspring. More specifically, right paws exhibit a lower 2D:4D with increasing levels of maternal corticosterone. This suggests that digit ratios, or the degree of their lateral asymmetry, may be a useful phenotypic indicator of maternal stress during early development. This may have many potential effects on the phenotype of an individual.

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