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A longitudinal examination of perinatal testosterone, estradiol and vitamin D as predictors of handedness outcomes in childhood and adolescence

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ABSTRACT

The developmental origins of handedness remain elusive, though very early emergence suggests individual differences manifesting *in utero* could play an important role. Prenatal testosterone and Vitamin D exposure are considered, yet findings and interpretations remain equivocal. We examined n = 767 offspring from a population-based pregnancy cohort (The Raine Study) for whom early biological data and childhood/adolescent handedness data were available. We tested whether 18-week maternal circulatory Vitamin D (25 [OH]D), and testosterone and estradiol from umbilical cord blood sampled at birth predicted variance in direction of hand preference (right/left), along with right- and left-hand speed, and the strength and direction of relative hand skill as measured by a finger-tapping task completed at 10 (Y10) and/or 16 (Y16) years. Although higher concentrations of Vitamin D predicted more leftward and less lateralized (regardless of direction) relative hand skill profiles, taken as a whole, statistically significant findings typically did not replicate across time-point (Y10/Y16) or sex (male/female) and were rarely detected across different (bivariate/multivariate) levels of analysis.

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Considering the number of statistical tests and generally inconsistent findings, our results suggest that perinatal testosterone and estradiol contribute minimally, if at all, to subsequent variance in handedness. Vitamin D, however, may be of interest in future studies.

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KEYWORDS Estradiol; Handedness; Testosterone; Vitamin D; Prenatal development

Introduction

Consistent limb preferences have been reported in a range of non-human species (Ocklenburg, Isparta, Peterburs, & Papadatou-Pastou, 2019; Ströckens, Güntürkün, & Ocklenburg, 2013), but the distribution of handedness observed in *Homo sapiens* is striking. The vast majority of people show preference for the right hand for most tasks, with a recent meta-analysis (k = 262; n = 2,396,170) estimating the prevalence of left-handedness at 10.6% (Papadatou-Pastou et al., 2020). As the right hand is primarily controlled by the left motor cortex and the left hand by the right motor cortex, handedness can be considered the most salient and easily measurable estimate of cerebral lateralization (Beaton, 2003; Ocklenburg & Güntürkün, 2018). The phenomenon is therefore of interest to researchers working in various disciplines, and studies in this area are often motivated by the potential for advancing our understanding of developmental and psychiatric conditions associated with atypical patterns of cerebral asymmetry (Cuellar-Partida et al., 2021; Ocklenburg & Güntürkün, 2018; Ocklenburg, Berretz, Packheiser, & Friedrich, 2020).

Behavioural genetics studies suggest that approximately 25% of variance in handedness is explained by genetic factors, and that little, if any, is explained by shared environment (Medland, Duffy, Wright, Geffen, & Martin, 2006, 2009; Schmitz et al., 2021; Somers et al., 2015). Much of the residual variance likely reflects random developmental noise (McManus, 2021) and a certain amount of measurement error. However, as the direction of hand preference (i.e., right or left) is observable from infancy (Campbell, Marcinowski, & Michel, 2018; Michel & Harkins, 1986; Nelson, Campbell, & Michel, 2014), and perhaps even from the prenatal period (Hepper, 2013), it is plausible that individual differences *in utero* also play a role. Indeed there are reports that handedness is associated with birth weight, multiple births, maternal anxiety, birth order, birth stress, and the presence/absence of breastfeeding, as well as with the country, year, and season of birth (Beaton, 2003; de Kovel, Carrión-Castillo, & Francks, 2019). Together these data suggest that examining the environment present *in utero* may provide critical insight into the development of handedness.

There has been much interest in the association between handedness and season of birth, with results from this research potentially indicating a role for early Vitamin D exposure. However, findings from individual studies are inconsistent, (e.g., de Kovel et al., 2019; Leviton & Kilty, 1979; Martin & Jones, 1999; Nicholls, 1998; Stoyanov, Nikolova, & Pashalieva, 2011; Tran, Stieger, & Voracek, 2014) and some have observed no association between handedness and birth month (e.g., Cosenza & Mingoti, 1995; McManus, 1980; Milenković, Rock, Dragović, & Janca, 2008; Tonetti, Adan, Caci, Fabbri, & Natale, 2012). A meta-analysis by Jones and Martin (2008) ($k = 9$; $n = 39{,}379$) reported a relatively high incidence of left-handedness in men born in the spring and early summer (March–July in the northern hemisphere; September–January in the southern hemisphere), though this effect could have been confounded by the small number of samples included as well as by the heterogeneous methods used for classifying handedness (see Beaton, 2008). More recently, de Kovel et al. (2019) utilized a very large sample ($n = 421{,}667$) from the British Biobank and reported a slight excess of left-handedness in women born in summer; the peak for males was smaller and appeared in the autumn months. It is unclear why such effects occur, though seasonal variations in temperature and exposure to infectious agents (Leviton & Kilty, 1979), maternal anxiety (Jones & Martin, 2008), or Vitamin D could potentially act as mediators. Vitamin D is of interest here because relatively low concentrations in early life have been linked to autism (Kočovská, Fernell, Billstedt, Minnis, & Gillberg, 2012), language impairment (Whitehouse et al., 2012a) and schizophrenia (Cui, McGrath, Burne, & Eyles, 2021), all conditions that are associated with increased left-handedness (autism: Markou, Ahtam, & Papadatou-Pastou, 2017; language impairment: Abbondanza et al., 2022; schizophrenia: Hirnstein & Hugdahl, 2014). Additionally, Vitamin D levels correlate positively with testosterone (Wehr, Pilz, Boehm, März, & Obermayer-Pietsch, 2010), another possible mediator of the association between handedness and birth month (Geschwind & Galaburda, 1987; Nicholls, 1998), albeit the association is weak (D'Andrea et al., 2021).

The possibility that prenatal testosterone exposure plays a causal role in the development of handedness has been discussed in the scientific literature for nearly half a century, with several influential theories making competing predictions. The sexual differentiation hypothesis (Hines & Shipley, 1984; Levy & Gur, 1980) and the Geschwind-Behan-Galaburda (GBG) theory (Geschwind & Behan, 1982; Geschwind & Galaburda, 1985a, 1985b, 1985c, 1987) both posit that high levels of prenatal testosterone increase the likelihood of left-handedness. Predictions can also be derived from these theories that elevated prenatal testosterone will reduce the strength of lateralization, regardless of its direction (see Richards et al., 2021a). On the other hand, the callosal hypothesis (Witelson & Goldsmith, 1991; Witelson & Nowakowski, 1991) predicts that elevated prenatal testosterone increases the likelihood of right-handedness (at

least in males). Lust et al. (2011) extended this theory to predict that high levels of prenatal testosterone will result in relatively strong lateralization patterns (regardless of their direction), though the results obtained from their empirical study directly contradicted this idea (see also Richards et al., 2021a).

A range of methods has been applied to examine the effects of prenatal testosterone exposure on handedness. These include studying congenital adrenal hyperplasia (CAH; a suite of autosomal recessive conditions characterized by elevated prenatal androgen production), comparing same-sex (SS) and other-sex (OS) twins (the idea being that testosterone transferred via the amniotic fluid results in elevated exposure in females with a male as opposed to a female co-twin), retrospective examination of the effects of synthetic oestrogen administered during pregnancy (e.g., diethylstilbestrol [DES]), and correlating naturally varying testosterone levels assayed from amniotic fluid with subsequent handedness measures. A recent review of such research revealed a confusing literature replete with inconsistent findings and replication failures (Richards et al., 2021a), an observation that corroborates the non-significant effect size estimate determined by an earlier meta-analytic study (Pfannkuche, Bouma, & Groothuis, 2009). Similarly, a meta-analysis by Richards et al. (2021b) of studies utilizing the second to fourth digit length ratio (2D:4D) as a proxy for prenatal testosterone exposure (see Manning, 2002; Manning, Scutt, Wilson, & Lewis-Jones, 1998) reported very small effect size estimates. Notably, the direction of correlation between left-handedness and right hand 2D:4D was negative whereas that between left-handedness and left hand 2D:4D was positive, diametrically opposite in terms of the implied effects of prenatal androgens.

Although the pattern of findings observed in the literature may suggest that prenatal testosterone is not a significant predictor of subsequent handedness outcomes, there are considerable validity issues regarding 2D:4D (e.g., Hickey et al., 2010; Hollier et al., 2015; Richards, Browne, & Constantinescu, 2020; Richards, Medland, & Beaton, 2021a), and studies using more direct methods have typically utilized sample sizes that lack the statistical power required to detect small or even medium sized effects. Furthermore, research relating to rare medical conditions (e.g., CAH), prenatal exposures (e.g., DES), complicated pregnancies (OS/SS twins) and samples that are not fully representative (e.g., children whose mothers underwent amniocentesis) may lack wider generalizability. One method that may be able to overcome these concerns, however, is to measure testosterone from umbilical cord blood sampled immediately after birth (Hollier, Keelan, Hickey, Maybery, & Whitehouse, 2014).

Tan and Tan (2001) examined testosterone (both as total testosterone and as free testosterone, i.e., that which is unbound to sex hormone binding globulin [SHBG]) sampled from the umbilical artery in relation to neonatal grasp-reflex strength measured at 3–5 days post-partum in 116 full-term neonates

(55 male, 61 female). Handedness (right or left) was defined in terms of which hand produced the stronger grasp-reflex. The study reported that right-handed males (n = 39) and right-handed females (n = 32) had higher concentrations of unbound testosterone than left-handed males (n = 16) and left-handed females (n = 29), respectively. Examination of the relative grasp-reflex strength revealed that stronger right-handedness increased with higher unbound testosterone levels; conversely, unbound testosterone levels correlated negatively with grasp-reflex strength for the left hand in males, and for both hands in females. This pattern of findings is not wholly consistent with any of the main theories linking testosterone with handedness (i.e., sexual differentiation, GBG, callosal), and indeed, no statistically significant associations were observed when examining total testosterone rather than free testosterone. It is also difficult to infer the meaning of these results. Considering that grasp-reflex strength is rarely examined in this literature, its relationship with other handedness measures is not well established, and handedness in general remains labile at such an early stage of infant development.

Although most research has focussed primarily on testosterone, other sex steroid hormones may play some role in the development of handedness. Three decades ago, Witelson (1991, p. 144) noted

> "It is not yet evident what the neuroanatomical substrate of handedness is in women and what role estrogen or other sex hormones may have in women in determining variations in structure related to handedness and other aspects of functional asymmetry",

and this is still true now. However, there has been recent interest in the role that estradiol could play in neurodevelopment (Baron-Cohen et al., 2020; Schultheiss, Köllner, Busch, & Hofer, 2021; Tsompanidis et al., 2021), and recent studies have linked amniotic estradiol concentrations with aspects of functional asymmetry (Beking, 2018, Chapter 4) and handedness (Richards et al., 2021a). More specifically regarding handedness, high levels of estradiol in the amniotic fluid predicted relatively weak hand preference in females assessed at age 15 years with a modified Dutch language version (van Strien, 2002) of the Edinburgh Handedness Inventory (Oldfield, 1971). However, no such effect was observed for males, and estradiol did not correlate with relative hand skill as assessed via the Annett pegboard task (Annett, 1970).

Considering the inconsistent pattern of findings from studies examining early hormone exposure in relation to handedness, in the present investigation we used data from an Australian longitudinal pregnancy cohort study, The Raine Study, to test whether testosterone and estradiol measured in perinatal umbilical cord blood, as well as the level of Vitamin D present in the maternal circulation at 18-weeks' gestation, were predictive of a range of handedness outcomes measured at 10- and/or 16-years of age. We pre-registered our analysis plan on the Open Science Framework (https://osf.io/rngq8)

and hypothesised that perinatal testosterone, estradiol and Vitamin D would each be significant predictors of handedness. However, due to the equivocal pattern of findings in this literature, and due to the competing predictions posited by various theories, we did not specify directions of effect.

Method

Participants

Participants were part of The Raine Study (McKnight et al., 2012), which is an ongoing longitudinal, population-based examination of women and their offspring recruited from the public antenatal clinic at King Edward Memorial Hospital or surrounding private clinics in Perth, Western Australia. Between May 1989 and November 1991, 2,900 women were enrolled into the study if they were between 16 and 20 weeks pregnant with sufficient command of the English language to comprehend the study demands, a plan to deliver at King Edward Memorial Hospital, and an intention to continue residence in Western Australia (to enable future study of their child). By the end of the recruitment period, 2,868 live births (96%) were available for follow-up. Informed written consent was obtained from parents during initial recruitment and at each follow-up on behalf of their participating child who then provided their own consent after they turned 18 years of age. The study protocols were approved by the Human Research Ethics Committee at King Edward Memorial Hospital and/or Princess Margaret Hospital in Perth, Western Australia.

Measures

Maternal 25(OH)-Vitamin D

Venous blood was collected from 929 randomly selected women who were 18 weeks pregnant. Blood specimens were centrifuged, and serum was collected and stored at −80°C. In June 2011, serum 25(OH)-Vitamin D concentrations were measured using a commercial enzyme immunoassay kit (Scottsdale, AZ). Twenty-eight serum samples were also measured using isotope-dilution liquid chromatography tandem mass spectrometry by RMIT Drug Discovery Technologies (Melbourne, Australia). Levels of 25(OH)-Vitamin D measured using immunoassay strongly correlated with those of mass spectrometry, $R^2 = 0.87$ (Whitehouse et al., 2012a) thus, confirming the low likelihood of molecules in the blood serum samples that may have interfered with the immunoassay of 25(OH)-Vitamin D.

Perinatal sex steroids

Mixed arterial and venous umbilical cord blood was obtained at the birth of n = 861 randomly selected deliveries. Blood samples were immediately

centrifuged, plasma isolated, and then stored at −80°C. Detailed sequence analysis of DNA obtained from 10 mother–child pairs confirmed that the cord blood samples were not contaminated by maternal blood (Keelan et al., 2012). In January 2010, cord serum samples were thawed and aliquoted. Androgens including total testosterone (TT), Δ4-androstenedione (A4), and dehydroepiandrostenedione (see Keelan et al., 2012), and estrogens including estrone (E_1), estradiol (E_2), estriol (E_3) and estetrol (E_4) (see Hickey, Hart, & Keelan, 2014) were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) after solvent extraction. SHBG was assayed by ELISA using a commercial kit (IBL International, Hamburg, Germany) according to the manufacturer's instructions. All samples were measured in duplicate by a single operator using assay kits from the same batch. Samples with an initial replicate coefficient of variability (CV) of >10% were reanalysed. The inter-assay imprecision (CV) was <4.5% (n = 25) and intra-assay CV was 5.2% (n = 861) (Keelan et al., 2012). In the current study we examined specifically free testosterone and total estradiol. Free testosterone was calculated for participants in The Raine Study (Hollier et al., 2015) as that un-sequestered by SHBG using the method described by Keelan et al. (2012; see also Sartorius, Ly, Sikaris, McLachlan, & Handelsman, 2009).

Hand preference and relative hand skill

The McCarron Assessment of Neuromuscular Development (MAND; McCarron, 1997) is a standardized, quantitative, and reliable method of assessing motor proficiency designed for use with children between 3 and 16 years (Hands, Larkin, & Rose, 2013). A trained assessor administered the MAND to The Raine Study cohort at 10, 14, and 16 year follow-ups (Hands et al., 2013). Children's hand preference was recorded via a single item measure, "Preferred Hand", with response options being "Right" and "Left" (McCarron, 1997, p. 28). The assessment also involved 10 tasks examining children's fine motor skills (beads in box, beads on rod, finger tapping, nut and bolt, and rod slide) and gross motor skills (hand strength, finger-nose-finger, jumping, heel-toe walk, and standing on one foot). We chose finger tapping from the 10 year (Y10) and 16 year (Y16) follow-ups as an index of relative hand skill because many previous studies in the laterality literature (e.g., Parker, Woodhead, Thompson, & Bishop, 2021; Peters & Durding, 1978; Tzourio Mazoyer et al., 2015) have utilized similar measures, whereas the other tasks that comprise the MAND have rarely (if at all) been examined in relation to handedness. We derived a laterality index (LI) using the following formula:

$$\mathrm{LI} = \frac{R - L}{0.5 \times (R + L)}$$

in which R and L are the number of finger taps made by the right and left index fingers, respectively, during a 10-second interval. The resulting values

(signed LI) indicate both the direction and strength of relative hand skill; scores > 0 indicate faster right hand (relative to left hand) speed and scores further from 0 (either positive or negative) indicate stronger lateralization of relative hand skill. We also examined the unsigned (absolute) LI, which indicates the strength of relative hand skill regardless of its direction (higher scores indicating stronger lateralization). Additionally, right- and left-hand speed (measured by finger tapping scores for each hand) was also of interest (see Richards et al., 2021a).

Statistical analyses

In our pre-registered analysis plan (https://osf.io/rngq8) we hypothesised that handedness outcomes would be stable across the Y10 and Y16 follow-ups. We conducted a chi-square test to determine the level of association between self-reported hand preference (right or left), and used bootstrapped (10,000 resamples) Pearson's correlations and paired-samples *t* tests to determine the level of association and difference for the continuous handedness outcomes (right-hand speed, left-hand speed, direction of relative hand skill, and strength of relative hand skill). We predicted that each of these measures would correlate positively across the time-points, that there would be no difference between them for direction or strength of relative hand skill, and that right- and left-hand speed would be faster at Y16 than Y10.

We transformed testosterone scores via natural logarithm (ln). Although bootstrapping does not assume a normal distribution of the error terms, there were some values that would be considered large outliers within the context of a normal distribution, which might still have exerted an undue influence (see Figure 1 for histograms of raw scores and log transformed scores). Although not pre-registered, we computed bootstrapped (10,000 samples) Pearson's correlations, stratified by sex, between hormonal predictors (testosterone, estradiol, and Vitamin D) and handedness outcomes (hand preference [right or left], right-hand speed, left-hand speed, direction of relative hand skill, and strength of relative hand skill). (Note that when hand preference is the outcome, correlations are point-biserial.) Our reasoning for including these exploratory analyses is that they may be more directly comparable to findings from other studies that have not controlled for covariates statistically.

We next conducted bootstrapped (10,000 samples) regression models with handedness variables as outcomes. Binary logistic regression was used when the outcome was dichotomous (hand preference: right or left) and linear regression was used when the outcome was continuous (right-hand speed, left-hand speed, direction of relative hand skill, strength of relative hand skill). These models each controlled for sociodemographic information

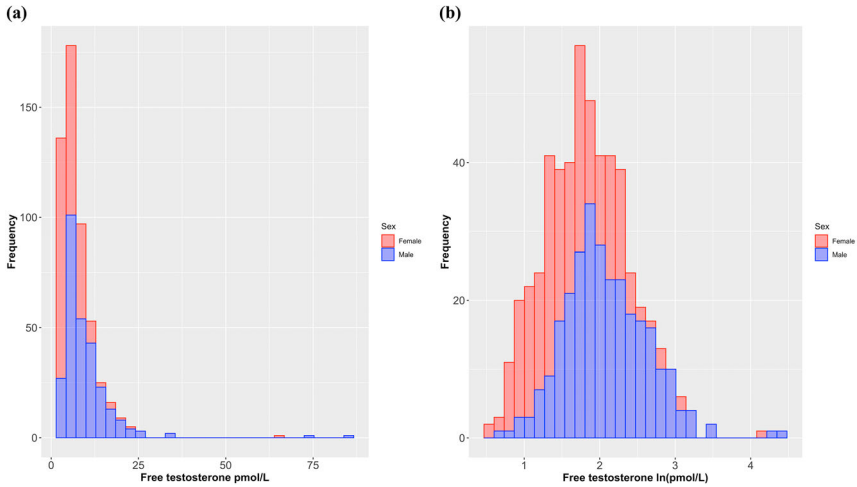

Figure 1. Histograms of the raw (**a**) and natural log transformed (**b**) scores for perinatal umbilical cord free testosterone.

recorded at 18 weeks' pregnancy, antenatal factors recorded at 34 weeks' pregnancy, and obstetric variables recorded at birth. We conducted hierarchical analyses with three steps (Step 1: enter covariates; Step 2: enter testosterone, testosterone × sex, estradiol, and estradiol × sex; Step 3: enter Vitamin D and Vitamin D × sex). The covariates included relate broadly to sociodemographic, obstetric, and antenatal variables. More specifically, these were delivery mode (presence or absence of labour), gestational age at delivery (number of days), maternal age at conception (years and months), birth weight (grams), maternal smoking during pregnancy (any vs. none), maternal alcohol consumption during pregnancy (any vs. none), maternal/paternal hand preference (one/both parent[s] vs. neither parent writes with left/either hand), family income ([1] less than AUD$7,000, [2] AUD$7,000-11,999, [3] AUD $12,000-23,999, [4] AUD$24,000-35,000, [5] more than AUD$36,000), parental education (average of maternal and paternal education: [0] none, [1] trade certificate or apprenticeship, [2] professional registration [non-degree], [3] college diploma or degree, [4] university degree), age at relevant assessment (years and months), sex (male or female), and Australian season of birth (spring [1; September, October, November], summer [2; December, January, February], autumn [3; March, April, May], or winter [4; June, July, August]). We also conducted exploratory multivariate analyses for Vitamin D. This was to increase statistical power, as the samples for which Vitamin D and testosterone and/or estradiol had been measured only overlapped partially. For these, Vitamin D and Vitamin D × sex were entered as predictors, along with the previously specified covariates, in a single-step simultaneous entry bootstrapped (10,000 samples) multiple regression model.

Statistical analyses were run using RStudio (Version 1.3.1073). All tests were two-tailed, and statistical significance was determined by $p < 0.05$ (non-bootstrapped analyses) or via bootstrapped 95% confidence intervals (CIs) that did not overlap with zero.

Results

Description of the sample and key variables

Of the original Raine Study cohort that was available for follow-up (N = 2,868), perinatal testosterone, estradiol, and Vitamin D data were measured for n = 827 (28.8%), n = 860 (30.0%), and n = 928 (32.4%), respectively. Both testosterone and estradiol were available for n = 827. However, of participants for whom Vitamin D had been assayed, testosterone and estradiol data were available for only n = 565 and n = 591, respectively. Handedness data (at least one outcome) were available for n = 1,644 (57.3%) at Y10 and n = 1,248 (43.5%) at Y16 (data from both follow-ups were available for n = 1,091).

Except where specified otherwise, we restricted our analyses to those participants for whom at least one perinatal hormone measure (testosterone, estradiol, or Vitamin D) and at least one handedness outcome (hand preference, right-hand speed, left-hand speed, direction of relative hand skill, strength of relative hand skill [from either or both of Y10 and Y16]) were available (n = 767). Descriptive statistics for this subsample, both as a whole and stratified by sex, are presented in Table 1. We did not compare this subsample with the wider cohort because those participants for whom testosterone and estradiol were assayed (Whitehouse et al., 2012b), as well as those for whom maternal Vitamin D samples were obtained (Whitehouse et al., 2012a), have already been shown to be fairly representative of the wider Raine Study cohort in terms of sociodemographic and obstetric characteristics.

Stability of handedness across childhood/adolescence

Of those who reported being right-handed at Y10, only 1 reported being left-handed at Y16; likewise, of those who reported being left-handed at Y10, only 3 reported being right-handed at Y16. Hand preference across these time-points therefore correlated almost perfectly, χ^2 (1, N = 464) = 424.875, $p < 0.001$, Cramér's V = 0.957. Correlations across time-points were small-to-moderate for right-hand speed, r (453) 0.259 (BCa 95% CI: 0.178–0.339), and left-hand speed, r = (456) = 0.296 (BCa 95% CI: 0.207–0.379), but small and non-significant for relative hand skill direction, r = (450) = 0.071 (BCa 95% CI: −0.095–0.183), and relative hand skill strength, r (450) = 0.079 (BCa 95% CI: −0.019–0.248). Given that these latter two variables are expected to correlate when measured for the same participants at different time-points, we

Table 1. Descriptive statistics for hormonal, sociodemographic, obstetric, antenatal, and handedness variables.

		Overall sample			Males			Females		
Continuous variables		*n*	*M*	*SD*	*n*	*M*	*SD*	*n*	*M*	*SD*
Free testosterone (pmol/L)		527	7.80	6.87	280	9.56	7.81	247	5.80	4.91
Estradiol (nmol/L)		548	28.25	17.15	283	28.95	16.58	265	27.49	17.74
Vitamin D (25[OH]D) (nmol/L)		596	57.78	18.80	249	57.55	17.96	347	57.94	19.39
Y10 right-hand speed		693	35.81	7.13	331	35.76	7.48	362	35.85	6.81
Y16 right-hand speed		526	39.43	7.56	248	41.82	7.63	278	37.29	6.84
Y10 left-hand speed		694	32.48	7.11	333	32.97	7.10	361	32.03	7.11
Y16 left-hand speed		527	37.43	7.09	248	39.69	6.94	279	35.42	6.61
Y10 LI (signed)		687	0.10	0.22	328	0.08	0.21	359	0.12	0.23
Y16 LI (signed)		526	0.05	0.16	248	0.05	0.17	278	0.05	0.15
Y10 LI (unsigned)		687	0.16	0.18	328	0.16	0.16	359	0.17	0.19
Y16 LI (unsigned)		526	0.12	0.12	248	0.12	0.13	278	0.11	0.11
Birth weight		549	3229.28	598.10	266	3233.50	558.12	283	3225.30	634.34
Gestational age at birth		747	274.31	15.38	358	274.73	15.12	389	273.92	15.63
Maternal age at conception		747	28.21	5.75	358	28.18	5.74	389	28.24	5.76
Age at Y10		748	10.59	0.18	358	10.60	0.17	390	10.58	0.20
Age at Y17		637	17.07	0.29	299	17.06	0.28	338	17.08	0.29
Nominal variables		*Overall n (%)*			*Male n (%)*			*Female n (%)*		
Y10 hand preference	*Right*	619 (88.05%)			296 (87.57%)			323 (88.49%)		
	Left	84 (11.95%)			42 (12.43%)			42 (11.51%)		
Y16 hand preference	*Right*	471 (89.20%)			222 (89.16%)			249 (89.25%)		
	Left	57 (10.80%)			27 (10.84%)			30 (10.75%)		
Sex	*Female*	400 (52.15%)			0 (0.00%)			400 (100.00%)		
	Male	367 (47.85%)			367 (100.00%)			0 (0.00%)		
Labour onset	*Present*	657 (87.95%)			311 (86.87%)			346 (88.95%)		
	Absent	90 (12.05%)			47 (13.13%)			43 (11.05%)		
Maternal smoking	*No*	114 (33.93%)			57 (34.34%)			57 (33.53%)		

(*Continued*)

Table 1. Continued.

		Overall sample			Males			Females		
Continuous variables		*n*	*M*	*SD*	*n*	*M*	*SD*	*n*	*M*	*SD*
	Yes	222 (66.07%)			109 (65.66%)			113 (66.47%)		
Maternal alcohol consumption	*No*	405 (54.29%)			187 (52.23%)			218 (56.19%)		
	Yes	341 (45.71%)			171 (47.77%)			170 (43.81%)		
Season of birth	*Spring*	180 (23.47%)			79 (21.53%)			101 (25.25%)		
	Summer	247 (32.20%)			118 (32.15%)			129 (32.25%)		
	Autumn	189 (24.64%)			90 (24.52%)			99 (24.75%)		
	Winter	151 (19.69%)			80 (21.80%)			71 (17.75%)		
Parental handedness	*Neither non-right-handed*	624 (83.76%)			304 (84.92%)			320 (82.69%)		
	Either/both non-right-handed	121 (16.24%)			54 (15.08%)			67 (17.31%)		
Average parental education	*None*	187 (25.03%)			86 (24.02%)			101 (25.96%)		
	Trade certificate/ apprenticeship	214 (28.65%)			100 (27.93%)			114 (29.31%)		
	Professional registration	167 (22.36%)			79 (22.07%)			88 (22.62%)		
	College diploma/degree	93 (12.45%)			50 (13.97%)			43 (11.05%)		
	University degree	86 (11.51%)			43 (12.01%)			43 (11.05%)		
Annual family income	*< AUD$7,000*	43 (5.94%)			23 (6.61%)			20 (5.32%)		
	AUD$7,000-11,999	61 (8.43%)			29 (8.33%)			32 (8.51%)		
	AUD$12,000-23,999	180 (24.86%)			82 (23.56%)			98 (26.06%)		
	AUD$24,000-35,000	189 (26.11%)			89 (25.58%)			100 (26.60%)		
	≥ AUD$36,000	251 (34.67%)			125 (35.92%)			126 (33.51%)		

Note. Age at Y16 was not recorded when handedness tasks were completed, so we used age at the Y17 follow-up as a proxy. Parental educational attainment was calculated as follows. First, those who responded with "Other" were recoded as "Trade certificate/apprenticeship" because this was the modal value for both males and females, and because "other" implies more than "none". Second, missing values were replaced with those of participants' partners when such information was available. (Prior to this imputation, maternal and paternal educational attainment were moderately correlated, r_s [650] = 0.361, $p < 0.001$). Finally, the average of maternal and paternal educational attainment was taken and rounded to the nearest integer (i.e., to the nearest attainment category).

performed additional (not pre-registered) analyses to determine whether this was the case for the whole Raine Study cohort. For this analysis we did observe statistically significant, though very small, positive correlations for both direction, r (1061) = 0.088 (BCa 95% CI: 0.002–0.156), and strength of relative hand skill, r (1061) = 0.067 (BCa 95% CI: 0.008–0.151).

The number of taps made with the right hand at Y16 (M = 39.73, SD = 7.35) was significantly greater than that recorded at Y10 (M = 35.77, SD = 7.22), t (454) = −9.535, d = −0.544 (mean difference = −3.963, percentile 95% CI: −4.776 – −3.147), and a comparable effect was observed for the left hand (Y16: M = 37.72, SD = 6.97; Y10: M = 32.47, SD = 7.24), t(457) = −13.350, d = −0.740 (mean difference = −5.260, percentile 95% CI: −6.028 – −4.463). The mean (signed) relative hand skill LI at Y10 (M = 0.10, SD = 0.22) was significantly higher than at Y16 (M = 0.05, SD = 0.15), t(451) = 3.900, d = 0.250 (mean difference = 0.048, percentile 95% CI: 0.024–0.072), reflecting a larger difference at Y10 between the performance of the two hands in favour of the right hand. This was due to participants' left-hand speed improving relatively more than their right-hand speed from Y10 to Y16. Likewise, the unsigned relative hand skill LI decreased from Y10 (M = 0.17, SD = 0.18) to Y16 (M = 0.11, SD = 0.11), t(451) = 5.670, d = 0.362 (mean difference = 0.055, percentile 95% CI: 0.036–0.074), suggesting that during adolescence participants became less lateralized overall. As would be expected, those reporting right hand preference had higher (more rightward) signed relative hand skill scores than did those reporting left hand preference (Figure 2 for boxplots). This was the case at Y10 (right handers: n = 602, M = 0.12, SD = 0.22; left handers: n = 84, M = −0.05, SD = 0.17), t(123.05) = 8.074, d = 0.790 (mean difference = 0.167, percentile 95% CI: 0.127–0.208) and Y16 (right handers: n = 470, M = 0.07, SD = 0.15; left handers: n = 56, M = −0.08, SD = 0.19), t (63.651) = 5.906, d = 0.995 (mean difference = 0.153, percentile 95% CI: 0.106–0.205).

Perinatal testosterone, estradiol, and Vitamin D as predictors of handedness at Y10 and Y16

We first present exploratory (not pre-registered) bivariate analyses in which hormonal predictors are correlated with handedness outcomes (Table 2). These are included to ensure our findings are comparable with those of other studies that have not controlled for covariates. We next performed the multivariate analyses specified in our pre-registration (Table 3) and followed these with sex-stratified models in cases where significant hormone × sex interaction effects were observed. Additionally, we report exploratory (not pre-registered) multiple regression models in which Vitamin D and Vitamin D × sex (but not testosterone, testosterone × sex, estradiol, or estradiol × sex) are included as predictors, along with the pre-specified covariates

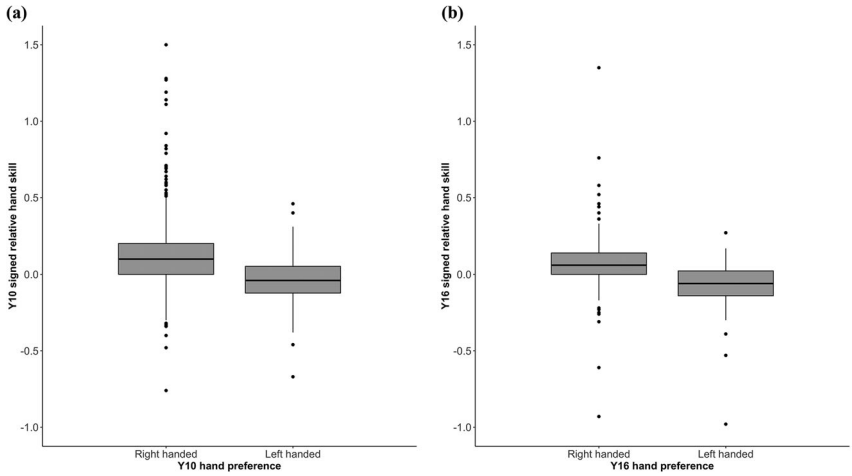

Figure 2. Boxplots for relative hand skill LI (signed) stratified by hand preference (right or left) at Y10 (**a**) and Y16 (**b**).

(Table 4). These were performed to explore whether effects of Vitamin D would be evident when examining a larger sample (i.e., we avoided listwise deletion of participants for whom Vitamin D was recorded but no data existed for testosterone and/or estradiol). For ease of comparison, we also summarise the findings from each stage of analysis qualitatively in Table 5.

Before conducting the multivariate analyses, we imputed values for several covariates to maximize sample size. We imputed birth weight for n = 101 males and n = 117 females with the means (male $M = 3233.50$; female $M = 3225.30$) present for the subsample in which both handedness and hormone data were available. As relatively few mothers reported whether they smoked during pregnancy, we recoded missing values (n = 431) to indicate non-smoking. Likewise, missing values for alcohol consumption during pregnancy (n = 21) were replaced to indicate non-consumption. In cases for which parental handedness data were unavailable for both parents (n = 22), we replaced the values as indicating that both parents were right-handed. Note that when only one parent reported their handedness (n = 20, all mothers), cases were coded as neither parent being non-right-handed if the respondent was right-handed, and as either or both parent(s) being non-right-handed if the respondent was non-right-handed. As already described in the note beneath Table 1, parental education was calculated as the average of maternal and paternal educational level. If the father's education was unknown but the mother's education was known, the former was imputed with the latter (n = 94); likewise, if the mother's educational level was not known but that of the father was known, the former was imputed with

Table 2. Bootstrapped (10,000 samples) Pearson's correlations between hormonal predictors and handedness outcomes, stratified by sex.

			Hand preference			Signed relative hand skill			Unsigned relative hand skill			Right-hand speed			Left-hand speed		
Sex	Age	Hormone	*n*	r_{pb}	BCa 95% CI	*n*	*r*	BCa 95% CI	*n*	*r*	BCa 95% CI	*n*	*r*	BCa 95% CI	*n*	*r*	BCa 95% CI
Male	*Y10*	Free testosterone (ln)pmol/L	255	0.102	−0.033–0.255	251	−0.059	−0.168–0.059	251	−0.035	−0.140–0.093	253	−0.136	−0.258 – −0.004	253	−0.059	−0.188–0.082
		Estradiol nmol/L	258	−0.060	−0.153–0.048	254	0.062	−0.106–0.199	254	0.191	0.057–0.328	256	0.059	−0.094–0.211	256	−0.020	−0.133–0.085
		Vitamin D nmol/L	230	0.051	−0.046–0.146	224	−0.042	−0.150–0.082	224	−0.097	−0.202–0.047	225	0.021	−0.113–0.156	227	0.051	−0.084–0.181
	Y16	Free testosterone (ln)pmol/L	–	–	–	191	−0.054	−0.196–0.082	191	0.004	−0.139–0.171	191	−0.104	−0.253–0.041	191	−0.086	−0.239–0.073
		Estradiol nmol/L	–	–	–	192	0.105	−0.031–0.230	192	0.008	−0.113–0.151	192	0.036	−0.094–0.157	192	−0.048	−0.172–0.082
		Vitamin D nmol/L	–	–	–	168	0.043	−0.085–0.154	168	−0.003	−0.139–0.128	168	0.038	−0.109–0.172	168	0.004	−0.150–0.156
Female	*Y10*	Free testosterone (ln)pmol/L	222	−0.050	−0.170–0.090	219	0.037	−0.112–0.212	219	−0.017	−0.167–0.194	219	0.015	−0.131–0.202	221	−0.050	−0.172–0.081
		Estradiol nmol/L	238	−0.065	−0.152–0.042	234	0.011	−0.100–0.120	234	−0.020	−0.125–0.084	235	0.078	−0.063–0.223	236	0.043	−0.065–0.154
		Vitamin D nmol/L	314	0.033	−0.077–0.148	309	−0.136	−0.216 – −0.045	309	−0.124	−0.202 – −0.038	312	0.024	−0.085–0.137	310	0.120	0.020–0.214
	Y16	Free testosterone (ln)pmol/L	–	–	–	175	0.032	−0.122–0.192	175	−0.049	−0.185–0.098	175	−0.064	−0.208–0.076	176	−0.077	−0.206–0.055
		Estradiol nmol/L	–	–	–	186	0.128	−0.006–0.255	186	0.008	−0.117–0.174	186	0.007	−0.107–0.127	187	−0.093	−0.216–0.035
		Vitamin D nmol/L	–	–	–	234	−0.077	−0.231–0.083	234	−0.184	−0.301 – −0.059	234	0.028	−0.096–0.150	235	0.101	−0.015–0.224

Note. Hand preference is coded as 0 = right handed, 1 = left handed.

Table 3. Bootstrapped (10,000 samples) multivariate regression models with hormones as predictors and handedness measures as outcomes.

Outcome	*Predictor*	Step 1	Step 2 β	BCa 95% CI	Step 3 β	BCa 95% CI
Y10 hand preference	Testosterone		−0.495	−2.761–1.664	−0.093	−2.760–2.416
	Testosterone × Sex		0.444	−0.890–1.758	0.327	−1.289–1.905
	Estradiol		−0.031	−0.114–0.037	−0.044	−0.150–0.042
	Estradiol × Sex		0.012	−0.029–0.057	0.021	−0.030–0.079
	Vitamin D				−0.002	−0.078–0.071
	Vitamin D × Sex				0.004	−0.039–0.046
	n	684	466		313	
	Model fit (McFadden Pseudo R^2)	0.03	0.03		0.04	
Y10 relative hand skill (signed)	Testosterone		0.067	−0.073–0.215	0.119	−0.031–0.275
	Testosterone × Sex		−0.051	−0.135–0.027	−0.086	−0.179–0.0002
	Estradiol		−0.0004	−0.004–0.003	−0.0005	−0.004–0.004
	Estradiol × Sex		0.001	−0.002–0.003	0.001	−0.002–0.004
	Vitamin D				−0.001	−0.005–0.002
	Vitamin D × Sex				0.0002	−0.002–0.003
	n	668	459		311	
	Model fit (adjusted R^2)	0.007	0.010		0.018	
Y16 relative hand skill (signed)	Testosterone		−0.003	−0.094–0.096	0.019	−0.085–0.130
	Testosterone × Sex		−0.002	−0.062–0.053	−0.016	−0.091–0.053
	Estradiol		0.002	−0.001–0.004	0.002	−0.001–0.005
	Estradiol × Sex		−0.0004	−0.002–0.001	−0.001	−0.003–0.001
	Vitamin D				−0.003	−0.006 – −0.001
	Vitamin D × Sex				0.002	−0.0002–0.004
	n	516	360		240	
	Model fit (adjusted R^2)	0.002	−0.004		0.024	
Y10 relative hand skill (unsigned)	Testosterone		0.005	−0.107–0.131	0.074	−0.044–0.207
	Testosterone × Sex		−0.005	−0.075–0.057	−0.051	−0.126–0.014
	Estradiol		−0.001	−0.004–0.001	−0.002	−0.005–0.001
	Estradiol × Sex		0.002	−0.0001–0.003	0.002	0.0000–0.004
	Vitamin D				0.0001	

(*Continued*)

Table 3. Continued.

Outcome	*Predictor*	Step 1	Step 2 β	BCa 95% CI	Step 3 β	BCa 95% CI
						−0.003–0.002
	Vitamin D × Sex				−0.0005	−0.002–0.001
	n	668	459		311	
	Model fit (adjusted R^2)	0.015	0.019		0.014	
Y16 relative hand skill (unsigned)	Testosterone		−0.023	−0.085–0.039	−0.030	−0.106–0.043
	Testosterone × Sex		0.008	−0.032–0.047	0.023	−0.025–0.077
	Estradiol		0.001	−0.0004–0.003	0.002	−0.0003–0.004
	Estradiol × Sex		−0.001	−0.002–0.001	−0.001	−0.002–0.001
	Vitamin D				−0.001	−0.003–0.002
	Vitamin D × Sex				0.0001	−0.001–0.002
	n	516	360		240	
	Model fit (adjusted R^2)	0.011	0.002		0.014	
Y10 right-hand speed	Testosterone		0.760	−4.038–6.591	0.295	−5.174–6.419
	Testosterone × Sex		−0.939	−4.266–1.933	0.089	−3.674–3.562
	Estradiol		0.022	−0.134–0.169	−0.008	−0.179–0.155
	Estradiol × Sex		−0.002	−0.102–0.099	0.022	−0.093–0.137
	Vitamin D				−0.087	−0.212–0.055
	Vitamin D × Sex				0.055	−0.030–0.140
	n	674	461		311	
	Model fit (adjusted R^2)	0.012	0.008		0.025	
Y16 right-hand speed	Testosterone		−1.090	−6.056–3.958	−1.601	−7.550–4.455
	Testosterone × Sex		0.262	−2.851–3.264	0.747	−3.256–4.582
	Estradiol		−0.043	−0.169–0.070	−0.018	−0.164–0.116
	Estradiol × Sex		0.033	−0.042–0.110	0.011	−0.089–0.102
	Vitamin D				−0.168	−0.290 – −0.021
	Vitamin D × Sex				0.112	0.021–0.197
	n	516	360		240	
	Model fit (adjusted R^2)	0.141	0.122		0.136	
Y10 left-hand speed	Testosterone		−2.472	−6.277–1.576	−4.126	−8.522–0.444
	Testosterone × Sex		1.422		3.151	

(*Continued*)

Table 3. Continued.

Outcome	*Predictor*	Step 1	Step 2 β	BCa 95% CI	Step 3 β	BCa 95% CI
				−1.192–3.966		0.102–6.344
	Estradiol		0.041	−0.064–0.142	0.024	−0.106–0.150
	Estradiol × Sex		−0.032	−0.100–0.033	−0.022	−0.113–0.060
	Vitamin D				−0.045	−0.157–0.079
	Vitamin D × Sex				0.045	−0.035–0.124
	n	675	463		312	
	Model fit (adjusted R^2)	0.021	0.012		0.039	
Y16 left-hand speed	Testosterone		−0.712	−5.390–3.891	−1.911	−7.323–3.435
	Testosterone × Sex		0.233	−2.526–3.125	1.179	−2.247–4.777
	Estradiol		−0.101	−0.211–0.003	−0.102	−0.226–0.011
	Estradiol × Sex		0.043	−0.022–0.111	0.031	−0.043–0.115
	Vitamin D				−0.032	−0.158–0.111
	Vitamin D × Sex				0.043	−0.045–0.130
	n	517	361		241	
	Model fit (adjusted R^2)	0.117	0.130		0.142	

Note. Analyses of hand preference used binary logistic regression; analyses of relative hand skill and right- and left-hand speed used multiple linear regression. All analyses used bootstrapping (10,000 resamples) and include the following covariates: delivery mode (presence [1] or absence of labour [2]), gestational age at delivery (number of days), maternal/paternal hand preference (neither parent writes with left/either hand [1] or one or both parents write with left/either hand [2]), family income (< AUD$7,000 [1], AUD$7,000-11,999 [2], AUD$12,000-23,999 [3], AUD$24,000-35,000 [4], ≥ AUD$36,000 [5]), parental education (average of maternal and paternal education: none [0] trade certificate or apprenticeship [1], professional registration [non-degree] [2], college diploma or degree [3], university degree [4]), maternal smoking during pregnancy (none [1] or any [2]), maternal alcohol consumption during pregnancy (none [1] or any [2]), maternal age at conception (years and months), birth weight (grams), age at relevant assessment (years and months), sex (female [1] or male [2]), and (Australian) season of birth (spring [September, October, November] [1], summer [December, January, February] [2], autumn [March, April, May] [3], or winter [June, July, August] [4]).

the latter (n = 1). In cases for which family income was not reported (n = 43), we replaced this with the modal value (≥ AUD$36,000).

As handedness outcomes (other than hand preference) were not strongly correlated across time-points, and because differences across time-points were observed (again, for outcomes other than hand preference), we correlated testosterone, estradiol, and Vitamin D with right-hand speed, left-hand speed, direction of relative hand skill, and strength of relative hand skill at both Y10 and Y16. For hand preference, we examined only Y10, as this yielded the larger sample size. Because we had specified our predictor variables *a priori*, and because the covariates in

Table 4. Bootstrapped (10,000 samples) multivariate regression models with Vitamin D and Vitamin D × sex as predictors and handedness measures as outcomes.

Outcome	Predictor	β	BCa 95% CI
Y10 hand preference	*Vitamin D*	0.008	−0.037–0.049
	Vitamin D × Sex	0.0004	−0.026–0.027
	n	527	
	Model fit (McFadden Pseudo R^2)	0.03	
Y10 relative hand skill (signed)	*Vitamin D*	−0.002	−0.005–0.0002
	Vitamin D × Sex	0.001	−0.001–0.003
	n	516	
	Model fit (adjusted R^2)	0.009	
Y16 relative hand skill (signed)	*Vitamin D*	−0.002	−0.005–0.001
	Vitamin D × Sex	0.001	−0.001–0.003
	n	393	
	Model fit (adjusted R^2)	0.005	
Y10 relative hand skill (unsigned)	*Vitamin D*	−0.001	−0.004–0.001
	Vitamin D × Sex	0.0001	−0.002–0.002
	n	516	
	Model fit (adjusted R^2)	0.015	
Y16 relative hand skill (unsigned)	*Vitamin D*	−0.002	−0.005 – −0.001
	Vitamin D × Sex	0.001	−0.0002–0.003
	n	393	
	Model fit (adjusted R^2)	0.034	
Y10 right-hand speed	*Vitamin D*	0.0002	−0.101–0.100
	Vitamin D × Sex	0.003	−0.066–0.076
	n	520	
	Model fit (adjusted R^2)	0.016	
Y16 right-hand speed	*Vitamin D*	−0.004	−0.112–0.111
	Vitamin D × Sex	0.003	−0.076–0.077
	n	393	
	Model fit (adjusted R^2)	0.147	
Y10 left-hand speed	*Vitamin D*	0.052	−0.037–0.150
	Vitamin D × Sex	−0.018	−0.084–0.047
	n	520	
	Model fit (adjusted R^2)	0.033	
Y16 left-hand speed	*Vitamin D*	0.069	−0.030–0.177
	Vitamin D × Sex	−0.037	−0.113–0.034
	n	394	
	Model fit (adjusted R^2)	0.114	

Note. Analyses of hand preference used binary logistic regression; analyses of relative hand skill and right- and left-hand speed used multiple linear regression. All analyses used bootstrapping (10,000 resamples) and include the following covariates: delivery mode (presence [1] or absence of labour [2]), gestational age at delivery (number of days), maternal/paternal hand preference (neither parent writes with left/either hand [1] or one or both parents write with left/either hand [2]), family income (< AUD$7,000 [1], AUD$7,000-11,999 [2], AUD$12,000-23,999 [3], AUD$24,000-35,000 [4], ≥ AUD$36,000 [5]), parental education (average of maternal and paternal education: none [0] trade certificate or apprenticeship [1], professional registration [non-degree] [2], college diploma or degree [3], university degree [4]), maternal smoking during pregnancy (none [1] or any [2]), maternal alcohol consumption during pregnancy (none [1] or any [2]), maternal age at conception (years and months), birth weight (grams), age at relevant assessment (years and months), sex (female [1] or male [2]), and (Australian) season of birth (spring [September, October, November] [1], summer [December, January, February] [2], autumn [March, April, May] [3], or winter [June, July, August] [4]).

our models were used specifically to control for processes that may be associated with perinatal hormone concentrations and/or handedness, we report only the effect size estimates (β) and BCa 95% CIs for predictors and not covariates.

Table 5. Qualitative summary of results from bivariate and multivariate analyses of association between perinatal hormone concentrations and handedness outcomes measured at Y10 and Y16.

		Analysis			
Outcome	Predictor	*Bivariate*	*Multivariate Step 2*	*Multivariate Step 3*	*Multivariate Vitamin D only*
Y10 hand preference	*Testosterone*	Null	Null	Null	N/A
	Estradiol	Null	Null	Null	N/A
	Vitamin D	Null	N/A	Null	Null
Y10 relative hand skill (signed)	*Testosterone*	Null	Null	Null	N/A
	Estradiol	Null	Null	Null	N/A
	Vitamin D	- (females)	N/A	Null	Null
Y16 relative hand skill (signed)	*Testosterone*	Null	Null	Null	N/A
	Estradiol	Null	Null	Null	N/A
	Vitamin D	Null	N/A	- (whole sample)	Null
Y10 relative hand skill (unsigned)	*Testosterone*	Null	Null	Null	N/A
	Estradiol	+ (males)	Null	+ (males)	N/A
	Vitamin D	- (females)	N/A	Null	Null
Y16 relative hand skill (unsigned)	*Testosterone*	Null	Null	Null	N/A
	Estradiol	Null	Null	Null	N/A
	Vitamin D	- (females)	N/A	Null	- (whole sample)
Y10 right-hand speed	*Testosterone*	- (males)	Null	Null	N/A
	Estradiol	Null	Null	Null	N/A
	Vitamin D	Null	N/A	Null	Null
Y16 right-hand speed	*Testosterone*	Null	Null	Null	N/A
	Estradiol	Null	Null	Null	N/A
	Vitamin D	Null	N/A	+ (males)	Null
Y10 left-hand speed	*Testosterone*	Null	Null	+ (males)	N/A
	Estradiol	Null	Null	Null	N/A
	Vitamin D	+ (females)	N/A	Null	Null
Y16 left-hand speed	*Testosterone*	Null	Null	Null	N/A
	Estradiol	Null	Null	Null	N/A
	Vitamin D	Null	N/A	Null	Null

Note. Non-significant effects (i.e., those in which the BCa 95% CIs include zero) are indicated by "Null"; "+" and "-" indicate statistically significant positive and negative correlations, respectively, and "N/A" indicates that no relevant effect size estimate was produced by that analysis. All effects indicated to be statistically significant in the male subsample were not statistically significant in the female subsample, and vice versa.

Hand preference

Hand preference (right or left) at Y10 was not associated with testosterone, estradiol, or Vitamin D in any of the bivariate or multivariate analyses, and in no case was a statistically significant hormone × sex interaction effect observed. For violin plots, see Figure 3.

Signed relative hand skill

The bivariate analysis revealed no significant correlations between the hormonal predictors and signed relative hand skill in males. A significant negative correlation was detected between Vitamin D and signed relative hand skill in females at Y10, indicating higher levels of Vitamin D to be associated with a reduction in the extent of right hand advantage. Although this effect was not observed at the multivariate level at Y10, Vitamin D was again negatively correlated with signed relative hand skill at Y16 in the multivariate analysis. However, the robustness of this effect is unconvincing because it was not detected when the larger sample was analysed in which participants were included regardless of whether testosterone and/or estradiol data were available.

Unsigned relative hand skill

In the bivariate analysis, unsigned relative hand skill was positively correlated with estradiol in males at Y10, implying that higher estradiol levels may lead to greater disparity in skill between the hands. A significant estradiol × sex interaction was subsequently observed for Y10 unsigned relative hand skill in the multivariate analysis. Sex stratified models (males: n = 143, adjusted $R^2 = 0.048$; females: n = 168, adjusted $R^2 = -0.034$) revealed the same pattern of findings as the bivariate analysis: specifically, there was a positive

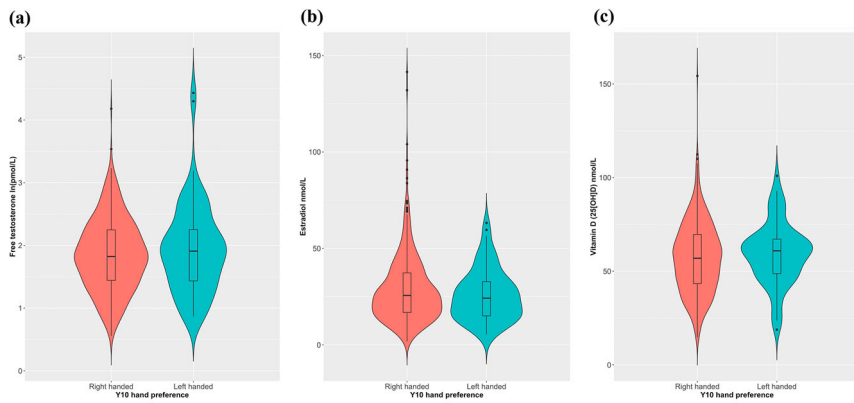

Figure 3. Violin plots for free testosterone (**a**), total estradiol (**b**), and Vitamin D (25 [OH]D) (**c**) stratified by Y10 hand preference (right or left).

correlation in males, β = 0.002 (BCa 95% CI: 0.0003–0.004) and no effect in females, β = 0.0001 (BCa 95% CI: −0.001–0.002). However, the robustness of this effect is questioned because it was detected at Step 3, but not at Step 2 when the sample size was larger. The bivariate analysis also revealed negative correlations between unsigned relative hand skill and Vitamin D in females at both Y10 and Y16. Although these effects were not observed in the pre-registered multivariate analyses (i.e., those which also included testosterone and estradiol as predictors), a negative correlation was again detected at Y16 (though as a main effect rather than an interaction effect) when the analysis was conducted on the larger sample that included participants for whom testosterone and/or estradiol data were unavailable (Figure 4). However, the effect was not observed in the equivalent analysis at Y10.

Right-hand speed

Right-hand speed correlated negatively with testosterone in males at Y10 in the bivariate analysis, though no such effect was detected at the multivariate level. However, Y16 right-hand speed was negatively correlated with Vitamin D at the multivariate level, and this effect was qualified by a significant Vitamin D × sex interaction. Sex-stratified models (males: n = 109, adjusted $R^2 = 0.091$; females: n = 131, adjusted $R^2 = 0.047$) revealed a positive correlation in males, β = 0.069 (BCa 95% CI: 0.001–0.148) and a negative, but non-significant, correlation in females, β = −0.053 (BCa 95% CI: −0.107–0.021). Importantly, however, this effect was not detected in the larger analysis of Vitamin D conducted without exclusion of those participants for whom testosterone and/or estradiol data were unavailable.

Left-hand speed

Left-hand speed was positively correlated with Vitamin D in females at Y10 in the bivariate analysis, but this effect was not observed at the multivariate level. A significant testosterone × sex interaction was detected in relation to Y10 left-hand speed in the multivariate analysis. Sex-stratified models (males: n = 143, adjusted $R^2 = 0.056$; females: n = 169, adjusted $R^2 = 0.007$) revealed a positive correlation in males, β = 2.523 (BCa 95% CI: 0.068–5.424), and a non-significant negative correlation in females, β = −1.094 (BCa 95% CI: −2.789–1.001). However, this effect was not detected in the larger analysis that included participants for whom Vitamin D data were unavailable, and no hormonal effects were detected in relation to left hand speed at Y16.

Discussion

The current study aimed to examine whether perinatal concentrations of testosterone, estradiol, and Vitamin D are predictive of handedness outcomes

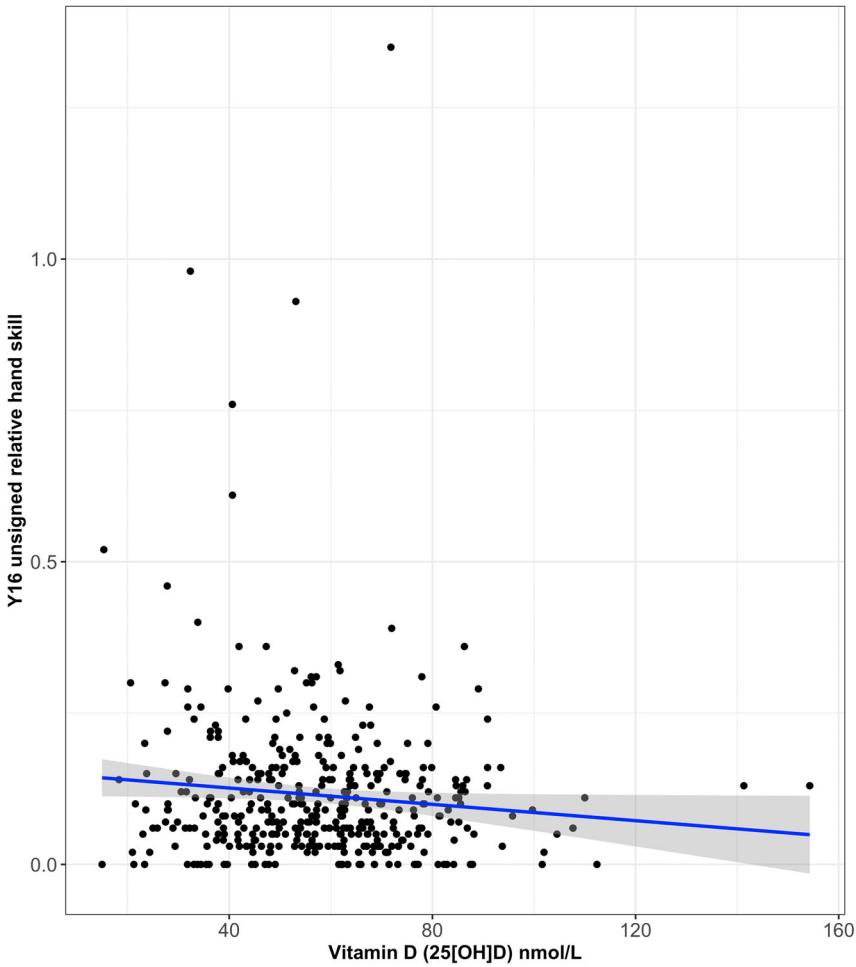

Figure 4. Scatterplot showing a negative correlation between maternal gestational Vitamin D (25[OH]D) concentration and relative hand skill (unsigned finger tapping LI) measured at Y16.

Note. Scatterplot shows all available (unadjusted) data points (i.e., it includes those of participants who were deleted listwise in the multiple linear regression models); higher unsigned relative hand skill values indicate greater difference in speed between the right and left hands.

measured during childhood/adolescence using a large dataset that includes perinatal (umbilical cord) sex steroid concentrations, second trimester maternal Vitamin D (25[OH]D) levels, and handedness outcomes measured at 10- (Y10) and/or 16-year (Y16) follow-up. Although some findings were statistically significant, most were not. Notably, of 54 bivariate correlations, only six were statistically significant.

Three effects could be considered to show a certain degree of consistency by having been replicated to some extent across different stages of analysis.

First, a positive correlation was observed between umbilical cord estradiol and Y10 unsigned relative hand skill in males in both the bivariate analysis and at Step 3 of the hierarchical regression analysis. However, this effect was not detected at Step 2 of the hierarchical analysis. This is noteworthy because sample sizes available at Step 3 (n = 240-313) were considerably smaller than those of Step 2 (n = 360-466) due to listwise deletion of participants missing either Vitamin D or testosterone/estradiol data. Higher powered multivariate tests were therefore available for testosterone and estradiol (i.e., Step 2 of the hierarchical regression analysis) and Vitamin D (i.e., the exploratory analysis in which testosterone and estradiol were not included as predictors [n = 393-527]). For this reason, we are reluctant to consider significant effects at Step 3 of the hierarchical regression analysis as anything other than Type 1 errors unless they are accompanied by a comparable observation in the respective higher-powered analysis. A negative correlation between Vitamin D and signed relative hand skill (in females, but not males) was observed at Y10 in the bivariate analysis, and a similar negative correlation (in absence of a significant Vitamin D × sex interaction) was observed at Y16 at the multivariate level. However, neither of these effects directly replicated across other stages of analysis, and the multivariate effect (i.e., that observed at Y16) related to the restricted sample size available at Step 3 of the hierarchical regression analysis and was not observed in the larger analysis relating specifically to Vitamin D. The only other effect that may be considered to have replicated across different stages of analysis was a negative correlation between Vitamin D and unsigned relative hand skill. This effect was observed in females at both Y10 and Y16 in the bivariate analysis, and then again for the whole sample (i.e., in absence of a significant Vitamin D × sex interaction) in the higher powered of the multivariate level analyses at Y16 (but not Y10). Other than these exceptions, statistically significant effects did not follow any obvious pattern, as they were not replicated across sex (male or female) or time-point (Y10 or Y16). Additionally, all five statistically significant effects (including interactions) detected in the preregistered analyses occurred at Step 3 of the hierarchical regression models. That is, they were detected in the lower-powered, but not in the higher-powered, multivariate analysis.

Our primary research aim was to test competing predictions from the sexual differentiation model (Hines & Shipley, 1984; Levy & Gur, 1980), GBG theory (Geschwind & Behan, 1982; Geschwind & Galaburda, 1985a, 1985b, 1985c, 1987) and callosal hypothesis (Witelson & Goldsmith, 1991; Witelson & Nowakowski, 1991) regarding associations between prenatal testosterone and handedness. Although no statistically significant hormonal differences were observed between right- and left-handed males and females, higher testosterone was associated with slower right hand speed (bivariate analysis) and faster left hand speed (Step 3, multivariate analysis) in males at Y10.

However, neither effect was detected in the higher-powered (i.e., Step 2) level of the hierarchical multivariate regression analysis. Our pattern of findings also differs from that of Tan and Tan (2001), who reported that unbound testosterone correlated negatively with grasp-reflex strength in males (left hand) and females (both hands). It also remains unclear whether, and, if so, how, the neonatal grasp reflex is related to child, adolescent, or adult patterns of handedness. This arguably makes it difficult to compare meaningfully the findings of Tan and Tan (2001) with those of the present study, which did not utilize this measure.

Testosterone is aromatised to estradiol, hence umbilical cord estradiol concentrations may be associated with handedness. Also, prenatal (amniotic fluid) concentrations of estradiol have previously been associated with handedness (Richards et al., 2021a). A positive correlation between estradiol and unsigned relative hand skill was observed in males at Y10 follow-up. However, this finding is directly opposite to that of Richards et al. (2021a) who reported that higher amniotic fluid estradiol concentrations in females (but not males) were associated with less lateralized hand preference profiles. Additionally, the effect observed in the current study was not observed in the more powerful (i.e., Step 2) level of the hierarchical regression model, and was not replicated at Y16, suggesting it may be a Type 1 error.

Vitamin D is thought to influence neurodevelopment but the hypothetical association between prenatal Vitamin D exposure and handedness has not previously been tested. Vitamin D may influence neurodevelopment, with recent research linking early life deficiency in this substance to autism (Kočovská et al., 2012), language impairment (Whitehouse et al., 2012a), and schizophrenia (Cui et al., 2021). Considering that Vitamin D is positively correlated with testosterone (D'Andrea et al., 2021) and exhibits similar seasonal fluctuations (Wehr et al., 2010), and that handedness has been widely (though often inconsistently) reported to correlate with month of birth (see de Kovel et al., 2019; Jones & Martin, 2008), we considered it plausible that prenatal Vitamin D exposure could play a role in the development of typical and/or atypical patterns of cerebral lateralization. As already noted, in females, Vitamin D was negatively correlated with signed relative hand skill at Y10; although this effect was not replicated in the multivariate analyses, a comparable negative correlation between signed relative hand skill at Y16 was observed (albeit this was in the whole sample and was not moderated by sex). Importantly, however, no significant association between Vitamin D and signed relative hand skill was detected at either Y10 or Y16 in our exploratory analyses of the larger sample in which participants without testosterone and/or estradiol were not subject to listwise deletion. These effects therefore do not appear to be robust. Vitamin D was also positively correlated with left-hand speed in females at Y10, although this effect was not detected in any subsequent multivariate level analysis. As already

noted, Vitamin D was negatively correlated with unsigned relative hand skill in females at Y10 and Y16; although these effects were not observed in the pre-registered multivariate analyses, a significant negative correlation (albeit a main effect rather than an interaction effect) was observed in our higher-powered exploratory multivariate analysis at Y16. However, as with other cases, this effect may not be deemed wholly convincing because no comparable effect was observed in the equivalent multivariate analysis at Y10 for which a larger sample was available for analysis (Y10, n = 516; Y16, n = 393). Taken together, our findings indicate that there could be a link between 18-week maternal gestational Vitamin D concentrations and subsequent measures of handedness, although the effects observed here appear far from robust. Considering the preliminary nature of our investigation, however, we suggest that exploring associations between early Vitamin D concentrations and neurodevelopmental outcomes remains a promising area for future research.

An unexpected finding was that correlations for participants' relative hand skill measured across timepoints were very low. More specifically, the correlations between Y10 and Y16 for both direction and strength of relative hand skill were negligible in size (Cohen, 1988) and only statistically significant when all participants with handedness data were considered rather than just the subsample for which hormonal measures had also been obtained. As hand preference (right or left) was essentially invariant, this suggests that finger tapping may be a noisy index of relative hand skill. This is consistent with the MAND's manual, which notes that this is "the most difficult test for the evaluator to administer" (McCarron, 1997, p. 30) and suggests that assessors should practice on at least 10 people prior to administering the task in an actual testing situation. However, it should also be noted that this task relates to a repetitive movement and does not measure the ability to intrinsically control the fine muscles of the hand like other such tasks tend to do, and that correlations between different measures of relative hand skill are often fairly weak, even when measured at similar stages of development (Buenaventura Castillo, Lynch, & Paracchini, 2020). It would therefore be useful for future studies to thoroughly examine the test-retest reliability of finger-tapping as well as other measures of relative hand skill, such as those derived from the Annett pegboard task (Annett, 1970), and the Tapley and Bryden dot-filling task (Tapley & Bryden, 1985; see also McManus, Van Horn, & Bryden, 2016). We additionally noted that relative hand skill was less rightward and less lateralized at Y16 than Y10, indicating that participants made greater improvements in terms of left-hand speed (relative to right-hand speed) between these two follow-ups. This could be because most participants reported right hand preference, and dominant hand performance on such tasks may be near to one's maximal ability, meaning there is more potential skill improvement to be gained via

practice with the non-dominant (in most cases, left) hand (McManus et al., 2018).

Strengths and limitations

This study has several strengths. It employed a pre-registered analysis plan and utilized the largest dataset to date to measure the association between perinatal sex steroid concentrations and handedness outcomes. We also implemented bootstrapping procedures, which do not assume a normal distribution (Diaconis & Efron, 1983). This may be advantageous when dealing with laterality indices (Wilke & Schmithorst, 2006) including those calculated for handedness (Richards, Beking, et al., 2021a), which typically show marked deviations from the normal distribution as well as the presence of datapoints that would be considered outliers within the context of a normal distribution. However, our study also has some limitations. First, although The Raine Study cohort is large, data relating to umbilical cord hormones and maternal circulation were only collected for relatively small, and not completely overlapping, subsamples. Additionally, although these subsamples are generally representative of the overall Raine Study cohort, some differences have been noted. For example, females were proportionally over-represented in a previous analysis of maternal Vitamin D and offspring neurocognitive development (Whitehouse et al., 2012a), and sample attrition is known to be biased by socioeconomic status, with those from lower socioeconomic backgrounds less likely to participate in follow-up studies (Whitehouse et al., 2012b).

Our analyses were limited to the available handedness data. Although it is clearly a strength to have relative hand skill (both in terms of strength and direction) rather than simply hand preference, it should be noted that the MAND is primarily intended for use as "a standardized and quantitative method of assessing psychomotor skills" (McCarron, 1997, p. 25). Although a composite handedness score is posited in the manual for the MAND, we did not compute this because it combines several different, albeit related, phenomena. More specifically, this metric includes finger-tapping in addition to hand strength and a task relating to putting beads in a bag (McCarron, 1997, p. 28). We decided against conflating these various measures into a single variable (see Buenaventura Castillo et al., 2020), and, because we wanted to minimize the number of statistical tests required, chose to examine only the finger tapping task. Whilst finger-tapping measures have been widely used in handedness research, the technical difficulty in administering this test could potentially explain the relatively low test-retest correlations. Furthermore, low measurement reliability could have obscured associations between the hormonal measures and handedness outcomes. That said, it should be recalled that predictions from the sexual differentiation model, GBG theory, and callosal hypothesis are primarily related to direction

of hand preference. Although Papadatou-Pastou, Martin, and Munafò (2013) noted that self-reported hand preference measures (i.e., right or left) misclassify 13.5% of left-handers (but only 0.4% of right-handers) when compared with the results of handedness questionnaires, the complete lack of correlation between this more reliable/stable outcome and any of the hormonal predictors raises serious doubt regarding the idea that these variables are related in any meaningful way.

Conclusion

This was the largest study to prospectively compare perinatal (umbilical cord) concentrations of testosterone and estradiol with handedness. Although higher estradiol was associated with stronger relative hand skill (regardless of direction) in males at Y10, the effect was in the opposite direction to that reported by Richards et al. (2021a) in relation to strength of hand preference in females. The effect was also not observed in the higher powered of the two multivariate analyses and did not replicate at Y16. Furthermore, testosterone was not consistently associated with any of the handedness outcomes at either time-point. Our findings therefore question the hypothesis that perinatal or prenatal sex steroid exposure is associated with handedness. The current study is also the first to examine the possible role of prenatal Vitamin D in the development of handedness. We observed that higher concentrations of Vitamin D (25[OH]D) in the mothers' circulation predicted more leftward and less lateralized (regardless of direction) relative hand skill profiles in their children. However, although there was a certain degree of consistency across analyses for these findings, those statistically significant effects were still accompanied by some null results. Even so, when considering recent findings from research on early Vitamin D exposure and neurodevelopment, we suggest this area remains worthy of further investigation.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author roles

GR, LL, DT, and AJOW obtained funding for the study. GR and DT wrote the manuscript. GR and ICM devised the statistical analysis plan, which was affirmed by the other co-authors prior to pre-registration. GR analysed the data. AAB, MH, MM, and ML revised the manuscript for important intellectual content. All authors read and approved the final version of the manuscript.

Data availability statement

The data that support the findings of this research are available from The Raine Study (https://rainestudy.org.au/information-for-researchers/). Restrictions apply to the availability of these data, which were used under license for this study. The R script that accompanies our analysis is available on the Open Science Framework (https://osf.io/yru2s/).

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