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An examination of the influence of prenatal sex hormones on handedness: Literature review and amniotic fluid data

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ABSTRACT

Competing theories have posited roles for foetal androgen exposure in the development of human handedness. However, due to practical and ethical considerations, few studies have used hormonal measures to examine this possibility. The current paper reviews this literature and reveals a generally inconsistent pattern of results. We also present data from a longitudinal study of prenatal sex hormone exposure and subsequent handedness. More specifically, we examine correlations between testosterone and estradiol measured from second trimester amniotic fluid and hand preference (Dutch language version of the Edinburgh Handedness Inventory) and hand skill asymmetry (pegboard task) measured at 15 years of age. Prenatal sex hormone exposure was not associated with the direction of hand preference in either males or females. However, in females, high levels of prenatal testosterone were associated with weaker lateralisation of hand skill, and high levels of prenatal estradiol were associated with weaker hand preference. In addition, high levels of prenatal testosterone were associated with increased task duration (i.e., slow hand speed) for the right and left hands of males. The pattern of results observed here is not entirely consistent with any of the main theories linking sex hormones with handedness, suggesting that an association between these variables may be more complex than initially thought.

1. Introduction

An enduring topic in cognitive neuroscience concerns functional (and anatomic) differences between the cerebral hemispheres and the relationship of cerebral asymmetry to a wide range of cognitive, emotional, social and behavioural phenomena (Ocklenburg and Güntürkün, 2018). The most salient aspect of human cerebral asymmetry is handedness (Beaton, 2003; Ocklenburg and Güntürkün, 2018): the preference most humans show for using one hand, usually the right, over the other. A recent meta-analysis of k = 262 datasets (n = 2,396,170) estimated the prevalence of left-handedness at 10.60% (95% CI = 9.71%, 11.50%) (Papadatou-Pastou et al., 2020). This species-level right hand preference has existed at least from the time of the Neanderthals (Estalrrich and Rosas, 2013; Fiore et al., 2015) and possibly earlier (Cashmore et al., 2008; Frayer et al., 2016; Lozano et al., 2009; Steele, 2000). No hominin species, or group within the human species, has ever been shown to have a predominance of individuals preferring to use the left hand for unimanual actions. The very ubiquity of this phenomenon demands explanation as does the existence of individuals who consistently prefer the left hand. Although the subject may be considered interesting enough to study in its own right, arguably the most important reason for doing so is that gaining a full understanding of handedness and how it relates to various developmental conditions and brain organisation patterns can provide insights into the genetics of language/

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language pathology and aspects of psychiatric illness (Cuellar-Partida et al., 2020; Ocklenburg et al., 2020; Ocklenburg and Güntürkün, 2018).

Most people show some degree of preference for using one hand over the other (hand preference) as well as greater skill in one hand relative to the other (hand skill asymmetry). Although these traits are moderately correlated (e.g., Annett, 1970a, 1970b, 2002; Beaton et al., 2012), they index separate facets of the same phenomenon (i.e., handedness). Direction of hand preference is formed early in life (Michel and Harkins, 1986; Nelson et al., 2014), possibly during gestation (Hepper, 2013; Hepper et al., 1991, 2005, but see Reissland et al., 2015) and certainly before puberty (Annett, 1970b; Longoni and Orsini, 1988; McManus et al., 1988; Scharoun and Bryden, 2014). Considered alongside a small though robust sex difference in which males are more likely than females to be left-handed, mixed-handed, or non-right-handed (Cuellar-Partida et al., 2020; Papadatou-Pastou et al., 2008, 2020) it is possible that variations in prenatal exposure to sex steroids such as testosterone and estradiol influence the direction and/or strength of handedness observed later in life.

Three main theories have dominated the literature that links prenatal androgen exposure with hand preference. First, the sexual differentiation hypothesis (Hines and Shipley, 1984; Levy and Gur, 1980) considers sex differences in lateralised functions to be intrinsically linked to processes of sexual differentiation, which can themselves be accounted for by exposure to prenatal sex hormones. As males are on average less dextral than females (Cuellar-Partida et al., 2020; Papadatou-Pastou et al., 2008, 2020), this theory predicts that high levels of foetal testosterone will be associated with left-handedness. Furthermore, as males may also have less strong lateral preferences than females, the sexual differentiation hypothesis can be extended to predict that foetal exposure to high levels of testosterone will associate with weak lateralisation of handedness.

Second, the Geschwind-Behan-Galaburda model (hereafter GBG) (Geschwind and Behan, 1982; Geschwind and Galaburda, 1985a, 1985b, 1985c, 1987) suggests that exposure to high levels of testosterone during gestation slows development of the left cerebral hemisphere, which can result in 'anomalous dominance', a term that is used to cover a range of phenomena including right hemisphere (or bilateral) speech representation, and left-handedness. Although the theory has at times been heavily criticised (see Bryden et al., 1994), many researchers have continued to test hypotheses derived from it (e.g., Beaton et al., 2011; Beaton et al., 2012; Kalmady et al., 2013; Stoyanov et al., 2011; Tran et al., 2014). Analogous with the sexual differentiation hypothesis, GBG theory predicts that high levels of foetal testosterone exposure will associate with left-handedness. Furthermore, assuming that mixed-handedness and/or ambidexterity can be considered within the construct of anomalous dominance, it may be inferred that the GBG theory would predict high levels of foetal testosterone to associate with weakly lateralised handedness.

Third, the callosal hypothesis (Witelson and Nowakowski, 1991; Witelson and Goldsmith, 1991) suggests that lateralisation of the brain occurs due to cell death and axonal pruning in the corpus callosum and temporo-parietal cortex during early development, and that these processes are affected by foetal testosterone. Exposure to low levels of this hormone are suggested to reduce the magnitude of these regressive events, resulting in increased callosal size, less lateralisation of cognitive functions, and increased likelihood of left-handedness (Witelson, 1989; Witelson and Goldsmith, 1991). This theory is therefore diametrically opposite to the GBG theory and sexual differentiation hypothesis in that it predicts high levels of foetal testosterone to lead toward right-handedness rather than left-handedness. However, in its initial formulation the callosal hypothesis makes this prediction in males only, with Witelson (1991, p. 144) stating '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.' Additionally, an extension to the theory proposed by Lust et al. (2011) suggested that high levels of prenatal testosterone may instead predict relatively strong lateral preferences, regardless of their direction. However, this appears unlikely, as the data presented by Lust et al. (2011) showed stronger handedness to be associated with *lower* prenatal testosterone in females, and that there was no effect in males.

1.1. Studies of prenatal testosterone and handedness

Ideally, researchers would experimentally manipulate prenatal sex hormone levels to observe their effects on handedness. However, as it is unethical to do this in humans, or indeed to obtain foetal blood samples purely for research purposes, investigators have developed several other techniques. For instance, Tan and Tan (2001) analysed umbilical cord blood (taken from the umbilical artery) sampled shortly after birth from 116 full-term neonates (55 male, 61 female), and measured grasp-reflex 3–5 days later. Neonates were considered right-handed if their right grasp-reflex was stronger than their left or left-handed if their left grasp-reflex was stronger than their right. Right-handed males ($n = 39$) and right-handed females ($n = 32$) had higher free[2] testosterone compared with left-handed males ($n = 16$) and left-handed females ($n = 29$), respectively. Examination of the relative grasp-reflex strength of the two hands (i.e., right-left) showed that right-handedness increased with higher free testosterone whereas left-handedness increased with lower free testosterone, a finding that is consistent with the callosal hypothesis. Conversely, free testosterone correlated negatively with grasp-reflex strength for the left hand in males, and for both hands in females. A negative correlation between perinatal testosterone and left hand strength (or positive correlation with right hand strength) could be interpreted as consistent with the callosal hypothesis, whereas a negative correlation between perinatal testosterone and right hand strength (or positive correlation with left hand strength) may be interpreted as consistent with the GBG or sexual differentiation hypothesis. However, it should be noted that Tan and Tan (2001) reported that these relationships were not statistically significant when examining total testosterone rather than free testosterone. This finding is difficult to interpret considering that the two metrics are generally very strongly correlated (e.g., men $r = 0.97$; women, $r = 0.94$; Winters et al., 1998). Moreover, no published research has yet examined associations between sex hormones present in umbilical cord blood and handedness measured at a stage of development by which its strength and direction have been firmly established.

Some researchers have examined handedness in individuals diagnosed with one of a group of autosomal recessive genetic conditions known as congenital adrenal hyperplasia (CAH). These conditions are characterised by an enzymatic deficiency within the cortisol synthesis pathway: most commonly it is the 21-hydroxylase, with enzyme that is affected, and this results, amongst other things, in elevated androgen exposure starting during gestation. Some studies have reported increased occurrences of left hand preference (or decreased right hand preference) in males and females with CAH compared with controls (Kelso et al., 1999, 2000; Mathews et al., 2004; Nass et al., 1987; Somajni et al., 2011; Tirosh et al., 1993). However, others have found no difference in hand preference (Hampson, 2016; Helleday et al., 1994; Malouf et al., 2006; Plante et al., 1996; Ripa et al., 2003) or hand skill asymmetry measured by a finger-tapping task (Helleday et al., 1994), and it should be noted that, due to the condition's rarity, CAH studies typically rely on small sample sizes and so often lack sufficient statistical power to reliably detect effects of small magnitude (Richards et al., 2020a, 2020b). Although aspects of upbringing/socialisation may provide alternative explanations for why CAH patients and controls differ

[2] Note that whereas 'free' testosterone is generally used to refer to the fraction that is unbound to sex hormone-binding globulin (SHBG) or albumin (Shea et al., 2014), the measure referred to thusly by Tan and Tan (2001) appears to represent the fraction unbound to SHBG only.

for certain characteristics (Hines, 2004; Jordan-Young, 2012), the studies that reported significant associations between CAH and left-handedness could be taken as evidence for GBG or the sexual differentiation hypothesis.

A meta-analysis of most of the CAH studies discussed above (Pfannkuche et al., 2009) showed no overall correlation between prenatal androgen exposure and handedness. However, the authors included both male and female CAH samples in the analysis, as well as the study of amniotic fluid by Grimshaw et al. (1995) (for further details of the findings of that study, see the below subsection 'Amniotic testosterone and handedness'). This could be problematic for several reasons: first, the callosal hypothesis (at least as initially formulated) does not predict an association between prenatal testosterone and handedness in females; second, the amniotic testosterone levels assayed in the study by Grimshaw et al. (1995) relate specifically to a single measurement taken during the second trimester of typically developing pregnancies, whereas CAH (at least in females) is associated with elevated androgen levels from the initial onset of their production, and third: it remains unclear whether prenatal androgen levels of male foetuses developing with CAH are distinguishable from those of typically developing male foetuses (Pang et al., 1980; Wudy et al., 1999). This last point is particularly important as the elevated adrenal androgen levels associated with CAH may be normalised, at least partially, in males via a downregulation of testicular production (Pang et al., 1979). This consideration therefore increases the difficulty with which reliable inferences can be drawn from studies of males (as opposed to females) with CAH.

Another approach has been to examine hand preference in the offspring of women administered the synthetic estrogen diethylstilbestrol (DES) during pregnancy. Geschwind and Galaburda (1985b) proposed that DES resembles testosterone in that testosterone may exert its masculinising effects on the brain after conversion to estradiol via the enzyme aromatase. However, whereas much circulating estradiol is rendered inactive due to being bound to other chemicals in the blood, DES is not, which allows it to cross the blood-brain barrier and, potentially, to exert masculinising effects on the brain without first having to be metabolised. Consistent with GBG or the sexual differentiation hypothesis, a study of 77 daughters of women administered DES (Geschwind and Galaburda, 1985b, p. 545; Schachter, 1994) found the handedness distribution to be shifted away from strong right preference in comparison to controls. A similar observation was made for 175 Dutch women (Scheirs and Vingerhoets, 1995), and a third study (Smith and Hines, 2000) found increased left hand preference for writing (but not for overall handedness on an 18-item inventory) as well as increased strength of hand preference (regardless of direction). Although, the largest study in this area (Titus-Ernstoff et al., 2003) observed no difference in hand preference for writing between DES exposed females (n = 3941) and unexposed females (n = 1758), it did find that males who had been exposed to DES (n = 1336) were significantly more likely to be left-handed or ambidextrous for writing when compared with unexposed male controls (n = 1338). These studies imply that elevated prenatal exposure to estradiol may induce a shift to left-handedness (consistent with GBG theory and the sexual differentiation hypothesis) in males and/or females. However, it should also be noted that DES is a highly teratogenic chemical (Giusti et al., 1995), and so may exert its effect in other than endocrinological pathways.

Yet another approach has been to examine handedness in same-sex (SS) and opposite-sex (OS) twin pairs. This idea came from animal studies showing that in several species with large litter sizes prenatal transfer of testosterone between siblings can occur, and that females gestated between two males typically exhibit increased masculinisation of behavioural, physiological, and anatomic traits (Ryan and Vandenbergh, 2002). There is some evidence to suggest that similar processes may arise in human twin pregnancies (Ahrenfeldt et al., 2020; Tapp et al., 2011), though Ryan and Vandenbergh (2002, p. 673) commented that they "do not appear to cause the same level of modifications in humans as they do in the other mammals with larger litters", and the literature is highly inconsistent (Ahrenfeldt et al., 2020). Geschwind and Galaburda (1985b) suggested females of OS twin pairs should more frequently be left-handed compared with females from SS pairs. Likewise, this idea could extend to SS males showing increased left-handedness relative to OS males (Elkadi et al., 1999).

Elkadi et al. (1999) found no difference in hand preference between 59 OS twin pairs and 61 SS twin pairs (FF = 40, MM = 21). Likewise, Ooki (2006) observed no differences in hand preference between male SS (n = 150) and OS (n = 125) or female SS (n = 138) and OS (n = 125) twins, and replicated these effects in a second cohort (male SS, n = 182; male OS, n = 203; female SS, n = 209; female OS, n = 203). In a very large scale analysis of 54,270 twins and their non-twin siblings (n = 25,732), Medland et al. (2009) also failed to find any difference in parent-reported handedness between opposite- and same-sex twins (testing separately for males and females, although the relevant numbers of females from opposite- and same-sex pairs is not given). Contrariwise, Vuoksimaa et al. (2010) examined a sample of 4736 participants (about 70% of all Finnish twins born in the period 1983–1987), and found a higher prevalence of left hand preference in SS female twins (n = 1578) than OS female twins (n = 737); however, they did not observe any such difference in males (SS, n = 1584; OS, n = 706). The current review shows that the studies are consistent in that there is no OS handedness effect in human twins. However, as the literature that relates to the twin testosterone transfer effect in humans more generally is somewhat mixed (Ahrenfeldt et al., 2020; Tapp et al., 2011), this does not necessarily imply that testosterone has no early effect on handedness.

Further exploration of handedness and foetal sex hormone exposure has relied on studies of the ratio of index to ring finger length (2D:4D). The 2D:4D ratio exhibits a sex difference, with males typically having a relatively longer fourth finger compared to females (Hönekopp and Watson, 2010). It has been proposed that this indicates the level of exposure (and/or sensitivity) to prenatal testosterone (Brown et al., 2002; Manning et al., 1998; Ventura et al., 2013) or the ratio of prenatal testosterone to estradiol (Lutchmaya et al., 2004; Manning, 2011; Zheng and Cohn, 2011). Some studies have reported left-handedness to be associated with male-typical patterns of digit ratio (e.g., Beaton et al., 2011; Fink et al., 2004; Manning and Peters, 2009; Manning et al., 2000; Nicholls et al., 2008; Stoyanov et al., 2011; Stoyanov et al., 2009; Swami et al., 2013; Voracek et al., 2006), others with female-typical patterns (e.g., Baker et al., 2013; Gillam et al., 2008; Jackson, 2008; Kalichman et al., 2014; Ypsilanti et al., 2008), and yet others with no effect at all (e.g., Bescós et al., 2009; Boets et al., 2007; Papadatou-Pastou and Martin, 2017). A meta-analysis of published and unpublished literature (Richards et al., in press) reported negligible effect size estimates (all unsigned point estimates $r < 0.060$) that were not consistent in direction. More specifically, left-handedness was associated with low right hand 2D:4D and a relatively low right hand 2D:4D relative to left hand 2D:4D ($D_{[R-L]}$), both of which have been proposed to indicate high prenatal testosterone (Manning, 2002), but also with high left hand 2D:4D, which is considered to indicate low prenatal testosterone. However, the value of 2D:4D as a proxy for prenatal testosterone has been questioned (e.g., Beking et al., 2017; Berenbaum et al., 2009; Putz et al., 2004; Richards, 2017; Richards et al., 2020b; Richards et al., 2020b; Wallen, 2009; Wong and Hines, 2016) and using more direct measures of hormones may result in greater consistency of findings.

1.2. Amniotic testosterone and handedness

Amniocentesis is an invasive procedure in which fluid is extracted from the amniotic sac surrounding the developing foetus. This is most commonly performed in the second trimester of pregnancy, during the time at which testosterone levels are maximally differentiated between male and female pregnancies (Hines, 2004; Reyes et al., 1974). Predictably, there is a large and robust sex difference, with amniotic

testosterone being higher when the foetus is male (Auyeung et al., 2009; Bergman et al., 2010; Finegan et al., 1992; Judd et al., 1976; Rodeck et al., 1985; Ventura et al., 2013), although there is a certain amount of overlap (Lust et al., 2010, 2011); a recent meta-analysis reported an effect size estimate of $d = 1.71$ (Baron-Cohen et al., 2015, see supplementary materials of that paper). Some researchers (e.g., Baron-Cohen et al., 2004; Beking et al., 2017; van de Beek et al., 2004) have suggested that examining testosterone concentrations present in amniotic fluid is the best method for investigating the effects of foetal androgens on subsequent phenotype.

Only two previously published studies have examined amniotic testosterone in relation to handedness. The first (Grimshaw et al., 1995) assessed hand preference in 28 boys and 25 girls aged 10 years. For girls, the strongest right hand preference was associated with the highest levels of foetal testosterone, which is consistent with the callosal hypothesis (if considered to extend to females). For boys, there was no relationship between prenatal testosterone and hand preference. More recently, Lust et al. (2011) examined amniotic testosterone in relation to hand preference in 31 girls and 34 boys at age 6, but no significant relationship emerged for either sex. However, considering only strength of hand preference (i.e., regardless of direction), a significant negative relationship emerged, increased strength of hand preference being associated with lower foetal testosterone levels. This finding was therefore inconsistent with the earlier findings of Grimshaw et al. (1995), though may arguably be in line with predictions of the GBG and sexual differentiation theories.

Amniocentesis is now seldom performed due to the advancement of less invasive techniques (see Akolekar et al., 2015). Consequently, it is unlikely that many more studies of amniotic testosterone will be conducted, making the analyses of existing data even more relevant. The current paper therefore reports on a longitudinal study in which sex hormones were assayed prenatally from amniotic fluid and then related to handedness measures obtained during adolescence. The cohort examined is that of Lust et al. (2011) (see also Beking et al., 2018; Beking, 2018; Lust et al., 2010; van de Beek et al., 2004; van de Beek et al., 2009). However, whereas Lust et al. (2011) examined prenatal testosterone levels in relation to hand preference in these children at 6 years of age (when handedness may remain somewhat labile; McManus et al., 1988), the current study examines hand preference at 15 years (an age by which handedness is certain to resemble that of adults; Scharoun and Bryden, 2014). Furthermore, the current study additionally includes a measure of hand skill asymmetry. Although moderately correlated with hand preference, hand skill asymmetry may be a more accurate index of lateralised manual function, as it is not subject to misinterpretation and response bias associated with self-report. Whereas previous research has typically only considered the effects of testosterone (Beking et al., 2018), the current study also examines estradiol. This is because estradiol is metabolised from testosterone via aromatisation, and estrogens have been shown to play an important role in the masculinisation of brain functions in many animal models (e.g., McEwen et al., 1977; Whalen and Olsen, 1978).

Due to inconsistent findings in the extant literature, it is difficult to make specific hypotheses. We therefore proceeded with an exploratory study to determine which theory (i.e., sexual differentiation, GBG, or callosal), if any, best accounts for the current data. Although making theory-informed predictions beyond the feasibility of an association between handedness and estradiol was not possible, we would expect high levels of testosterone to associate with left-handedness (and possibly with weak lateral preferences, regardless of direction) if either the GBG theory or sexual differentiation hypothesis were correct, and with right-handedness (at least in males) if the callosal hypothesis were correct.

2. Material and methods

2.1. Participants

Thirty boys (M age = 15.0 years, $SD = 0.6$, range = 14.0–16.1) and 30 girls (M age = 15.1 years, $SD = 0.6$, range = 14.0–16.1) took part in the current study. Each was part of an initial cohort of 196 children born in 2000 whose mothers underwent amniocentesis during pregnancy (van de Beek et al., 2004). Invitation letters were sent to those who participated at the 6-year follow-up (see Lust et al., 2010, 2011) because their contact details were most up to date. Of $n = 90$ that were invited, $n = 28$ could not be contacted, and $n = 60$ agreed to participate. We aimed for equal numbers of boys and girls and an approximately flat distribution of free testosterone values; that is, in the selected sample there is an overrepresentation of low and high values per sex. Extra effort was made to contact participants with relatively low or high prenatal testosterone levels, a process that resulted in the inclusion of 1 boy with high prenatal testosterone, 1 girl with high prenatal testosterone, and 1 girl with low prenatal testosterone. The purpose of this was to provide enough overlap between the sexes to disentangle observable hormonal effects from those of sex.

Karyotyping of the amniotic fluid samples confirmed that all boys were XY and all girls were XX. Ethical clearance was granted by the local Psychology Ethical Committee (reg.no. ppo-013-120), and each participant provided written informed consent prior to taking part in the current study. The 15-year follow-up sample has already been described in detail by Beking et al. (2018).

2.2. Prenatal hormone assays

Due to advanced maternal age (36–42 years), participants' mothers had undergone amniocentesis between gestational weeks 15 and 18 at the University Medical Centre of Utrecht, the Netherlands. Testosterone, estradiol, progesterone, androstenedione, sex hormone binding globulin (SHBG), and dehydroepiandrosterone sulphate (DHEAS) were measured from amniotic fluid using radioimmunoassay (RIA). Details of the hormone measurement procedures for this cohort have already been reported by van de Beek et al. (2004): inter-assay coefficient of variation was 8.8% at a testosterone level of 0.75 nmol/l and 9.4% at 2.55 nmol/l; for estradiol, inter-assay variation was 5.1% at 1060 pmol/l.

There is some debate as to the most effective method for quantifying testosterone concentration: some measure the total amount present in circulation (total testosterone), others consider only the fraction that is not bound to SHBG or albumin (free testosterone), and yet others examine free testosterone in addition to that bound to albumin (bioavailable testosterone) because albumin binds with low affinity and so may not render the testosterone incapable of interacting with androgen receptors. In the current study we examine only the unbound (i.e., free) testosterone as it is certain to be metabolically active (for a discussion of quantification methods, see Shea et al., 2014).

2.3. Handedness

Hand preference was assessed via a modified Dutch language version (van Strien, 2002) of the Edinburgh Handedness Inventory (Oldfield, 1971), which was administered via online questionnaire. The measure consists of 11 questions each answered on a visual analogue scale ranging from −100 ("always with left hand") to +100 ("always with right hand") and provides an assessment of the strength and direction of hand preference. The range of the hand preference score is −1100 to +1100.

A pegboard task (Annett, 1970b) was administered to provide an indication of hand skill asymmetry. This task requires participants to move a series of 10 wooden pegs (individually) from a set of holes on one side of a board to a set of holes on the other side, as quickly as possible. Participants were instructed to complete two trials for each hand.

However, some participants made mistakes ($n = 7$); four of these completed a third set of trials (the times for the trials involving mistakes were not recorded), whereas the other three only completed one successful trial for each hand (all of which were included as the mean of available scores). Mean times for the right and left hands separately were calculated from the available scores, and a laterality index was calculated as follows: LI = (L-R) / (0.5 × (L + R)). LI was then multiplied by 100 (henceforth LI%), so that the resulting scores indicate the percentage difference in task duration between the hands (positive scores indicate a faster right hand relative to left hand).

2.4. Design and procedure

Participants initially completed an anonymous online survey (hosted by Qualtrics), which included the handedness questionnaire. One of the authors (TB) then visited participants' homes to administer the pegboard task and functional Transcranial Doppler Ultrasonography, and to collect saliva samples for pubertal hormone analysis (see Beking et al., 2018). For the current study, amniotic testosterone and estradiol were used as predictor variables; the direction and strength of hand preference and hand skill asymmetry, as well as task duration for the pegboard task (i.e., right hand speed and left hand speed) were used as outcomes.

2.5. Statistical analysis

As the sample size was relatively small, most of the study variables showed large deviations from the normal distribution that could not be corrected by transformation, and (biologically relevant) outliers were present, we used bootstrapping (10,000 samples) procedures for all inferential statistical tests. If there was discrepancy regarding the interpretation of statistical significance between the observed p value (i.e., that computed from the standard inferential statistical test) and the bias corrected accelerated 95% confidence intervals (BCa CI) (i.e., the confidence intervals calculated from the bootstrapping procedure), we interpret the effects in regard to the BCa 95% CIs.

Bootstrapped independent samples t-tests were used to examine whether there were sex differences in the amniotic hormone concentrations and handedness outcomes. We next used bootstrapped Pearson's correlations to determine the strength of association between hand preference and hand skill asymmetry, and to examine intercorrelations between prenatal and pubertal hormone levels. We then used bootstrapped Pearson's correlations to examine whether amniotic testosterone and estradiol concentrations were associated with direction of hand preference (signed hand preference scores), direction of hand skill asymmetry (signed hand skill asymmetry scores), and right hand and left hand speed for completion of the pegboard task. We then converted the hand preference and hand skill asymmetry scores to absolute values (so all scores were positive) to reflect strength of handedness regardless of direction (Luders et al., 2010). Both the unsigned hand preference score and the unsigned pegboard LI% score were treated as continuous variables (low scores indicating weak lateralisation), and examined in relation to amniotic testosterone and estradiol concentrations via bootstrapped Pearson's correlations.

3. Results

3.1. Sex differences

Amniotic testosterone levels were significantly higher in males than females (Beking et al., 2018), and prenatal estradiol levels were significantly higher in females than males; there were no statistically significant sex differences for any of the handedness measures (Table 1). Direction of hand preference (signed hand preference scores) and direction of hand skill asymmetry (signed pegboard LI%) were positively correlated, r (58) = 0.648, $p < 0.001$ (BCa 95% CI = 0.461, 0.770), which is consistent with previous findings (Annett, 1970a, 1970b, 2002; Beaton et al., 2012). Six females (20%) and 4 males (13.3%) reported writing with their left hand, which is notably higher than the general population estimate of 9.29% provided by Papadatou-Pastou et al. (2020). This is likely a chance effect associated with studying a relatively small sample. For graphical representations of the distributions of hand preference and hand skill asymmetry, see Figs. 1 and 2, respectively.

3.2. Hormonal intercorrelations

Intercorrelations between the prenatal and pubertal hormone concentrations are presented in Table 2. Prenatal testosterone did not correlate with pubertal testosterone in males or females, and, likewise, there were no associations between prenatal and pubertal estradiol. However, prenatal testosterone was positively correlated with pubertal estradiol in males, and positive correlations were observed between pubertal measures of testosterone and estradiol in both sexes.

3.3. Direction of handedness

Bootstrapped Pearson's correlations revealed no significant associations between amniotic sex hormone exposure and the direction of hand preference (signed) or hand skill asymmetry (signed) (Table 3). However, exposure to high levels of prenatal testosterone was associated with increased task duration (i.e., slow hand speed) for both the right hand (Fig. 3) and left hand (Fig. 4) in males. It should be noted that the latter effect would not be considered statistically significant (i.e., $p < 0.050$) if using the conventional parametric statistical approach; this effect (and those relating to similar instances reported below) is considered significant because the BCa 95% CIs derived from the bootstrapping procedure do not include 0. The observed pattern of effects was not materially affected by controlling for pubertal hormone levels (see **Table S1**).

Table 1
Descriptive statistics and sex differences for hormone and handedness measures.

	Males ($n = 30$)		Females (n = 30)		Sex difference		Mean difference [BCa 95% CI]	d
	M	*SD*	*M*	*SD*	*t*	*p*		
Amniotic testosterone (nmol/L)	1.50	0.55	0.70	0.38	6.651	**< 0.001**	**0.805 [0.573–1.039]**	**−1.692**
Amniotic estradiol (pmol/L)	839.33	262.87	1063.00	358.60	−2.755	**0.008**	**−223.667 [−377.040 – −75.872]**	**0.711**
Hand preference (signed)	782.33	671.64	653.40	715.29	0.720	0.475	128.933 [−208.013–469.041]	−0.186
Hand skill asymmetry (signed)	0.08	0.11	0.09	0.11	−0.565	0.574	−0.017 [−0.073–0.040]	0.091
Right hand task duration (seconds)	12.06	1.19	11.68	1.15	1.251	0.216	0.378 [−0.197–0.956]	−0.325
Left hand task duration (seconds)	13.05	1.67	12.81	1.16	0.650	0.518	0.241 [−0.429–0.962]	−0.167
Strength of hand preference (unsigned)	1006.33	191.38	931.80	234.74	1.348	0.183	74.533 [−36.068–182.879]	−0.348
Strength of hand skill asymmetry (unsigned)	0.11	0.08	0.12	0.08	−0.360	0.720	−0.007 [−0.046–0.031]	0.125

Note. Values for t and p are calculated from standard independent samples t-tests; BCa 95% CIs around the mean difference are calculated from the bootstrapping (10,000 samples) procedure. Effects in bold are considered statistically significant (i.e., the BCa 95% CIs do not include 0).

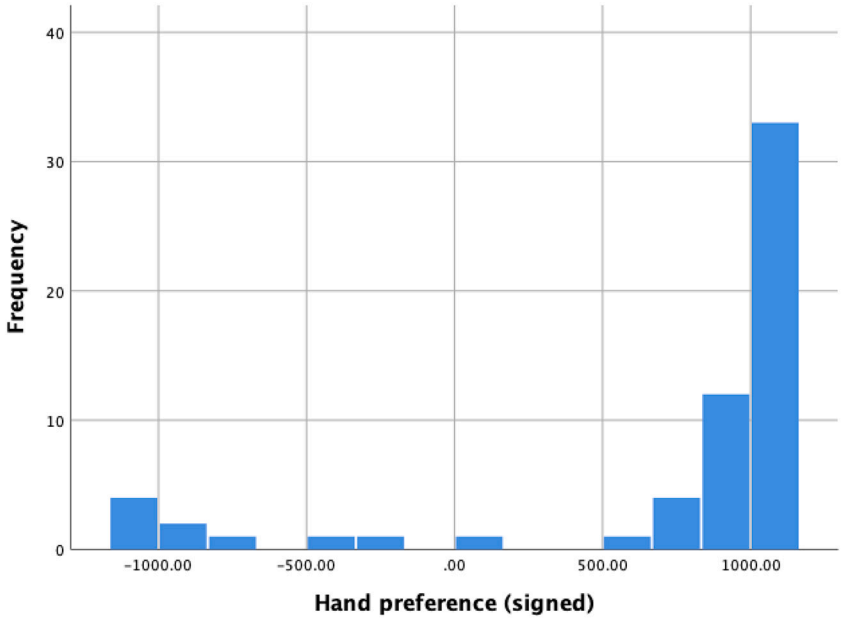

Fig. 1. Hand preference (signed) distribution.

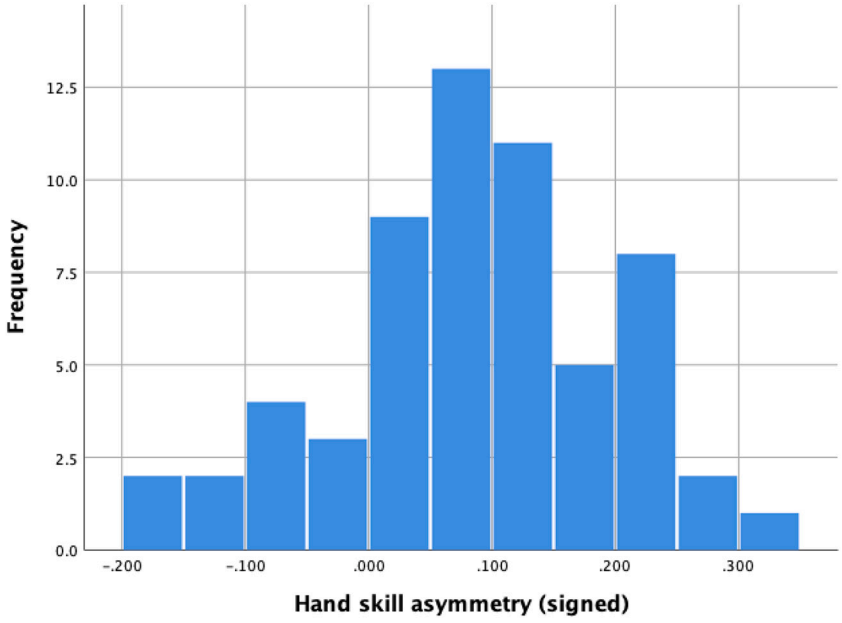

Fig. 2. Hand skill asymmetry (signed) distribution.

Table 2
Correlation matrix for prenatal and pubertal hormone levels.

	Amniotic T			Amniotic E			Pubertal T			Pubertal E		
	r	BCa 95% CI	*p*	*r*	BCa 95% CI	*p*	*r*	BCa 95% CI	*p*	*r*	BCa 95% CI	*p*
Amniotic T	–			0.109	−0.169–0.483	0.566	0.133	−0.147–0.433	0.484	**0.430**	**0.166–0.782**	**0.018**
Amniotic E	0.170	−0.127–0.623	0.370	–			0.094	−0.208–0.400	0.621	0.323	−0.080–0.612	0.082
Pubertal T	0.083	−0.291–0.372	0.664	0.004	−0.223–0.241	0.985	–			**0.393**	**0.081–0.651**	**0.032**
Pubertal E	0.092	−0.243–0.457	0.630	−0.126	−0.447–0.353	0.507	**0.623**	**0.260–0.846**	**< 0.001**	–		

Note. Correlations for males are above the diagonal; correlations for females are below the diagonal. E = estradiol; T = testosterone; BCa 95% CIs are calculated from the bootstrapping (10,000 samples) procedure. Effects in bold are considered statistically significant (i.e., the BCa 95% CIs do not include 0).

3.4. Strength of handedness

Bootstrapped correlations were used to test for associations between prenatal sex hormone exposure and strength of hand preference (unsigned hand preference score) and strength of hand skill asymmetry (unsigned pegboard LI%) (Table 4). In females, exposure to high levels

Table 3
Associations between amniotic sex hormone concentrations and hand preference, hand skill asymmetry, and right and left hand task duration.

		Testosterone		Estradiol	
		r [BCa 95% CI]	*p*	*r* [BCa 95% CI]	*p*
Females	Hand preference (signed)	−0.227 [−0.706–0.283]	0.228	−0.096 [−0.474–0.182]	0.612
	Hand skill asymmetry (signed)	−0.273 [−0.546–0.038]	0.144	0.131 [−0.298–0.456]	0.489
	Right hand task duration	0.169 [−0.272–0.496]	0.373	−0.015 [−0.407–0.375]	0.938
	Left hand task duration	−0.167 [−0.447–0.178]	0.377	0.141 [−0.244–0.490]	0.457
Males	Hand preference (signed)	0.065 [−0.341–0.381]	0.732	0.127 [−0.210–0.385]	0.504
	Hand skill asymmetry (signed)	−0.042 [−0.365–0.320]	0.825	−0.056 [−0.366–0.295]	0.768
	Right hand task duration	**0.405 [0.110–0.725]**	**0.026**	0.100 [−0.283–0.444]	0.599
	Left hand task duration	**0.284 [0.049–0.563]**	0.129	0.026 [−0.254–0.297]	0.890

Note. Analyses presented are bootstrapped (10,000 samples) Pearson's correlations (two-tailed); r and p are calculated from standard Pearson's correlations whereas BCa 95% CIs are calculated from the bootstrapping procedure; effects in bold are statistically significant (i.e., the BCa 95% CIs do not include 0).

of prenatal testosterone was associated with weaker lateralisation of hand skill (Fig. 5), and exposure to high levels of estradiol was associated with weaker lateralisation of hand preference (Fig. 6). No significant correlations were observed for males and controlling for pubertal hormone concentrations did not noticeably affect the pattern of results observed (see **Table S2**).

4. Discussion

The current article reports on findings from a 15-year study of the associations between prenatal (amniotic) sex hormones and handedness outcomes in a cohort of Dutch youth. It is the first such study to examine the potential role of prenatal estradiol exposure in the development of handedness, and one of very few to examine the effects of amniotic testosterone. A previous study from this cohort (Lust et al., 2011) found no association between prenatal testosterone and direction of hand preference, but did report that high levels of prenatal testosterone predicted weaker lateralisation of hand preference (in either direction) at age 6. We broadly replicated these findings at age 15: prenatal testosterone exposure did not predict the direction of handedness (i.e., signed hand preference or hand skill asymmetry scores) but high concentrations of this hormone were associated with relatively weak lateralisation of hand skill in females. In addition, we found that high levels of prenatal estradiol were associated with weaker hand preference in females, and that high prenatal testosterone concentrations were associated with increased task duration (i.e., slow right hand and left hand speed) in males. Although they imply a role for prenatal sex hormones in the development of lateral preferences and skill, these findings are not wholly consistent with any of the GBG, callosal, or sexual differentiation theories.

4.1. Sex hormones and handedness

The lack of association between amniotic testosterone concentrations and direction of either hand preference or hand skill asymmetry observed here is broadly consistent with there being no hand preference differences between OS and SS twins, but is inconsistent with research observing elevated levels of left-handedness in people with CAH (see Introduction section) and with a study that reports a significant correlation between umbilical cord testosterone and the right-left difference in neonatal grasp-reflex (Tan and Tan, 2001). However, it is worth noting that within the extant literature only one significant correlation between amniotic testosterone and direction of hand preference has previously been reported. This was an association between high prenatal testosterone levels and right hand preference in females (Grimshaw et al., 1995). Grimshaw et al. (1995) also observed no significant association in boys, and the only other study (Lust et al., 2011) reported that amniotic testosterone was not associated with the direction of hand preference in either boys or girls. The current study also observed no significant effect for boys. We did however observe that high levels of prenatal testosterone were associated with high task duration for the right hand and left hand in boys. The former effect (i.e., slow right hand

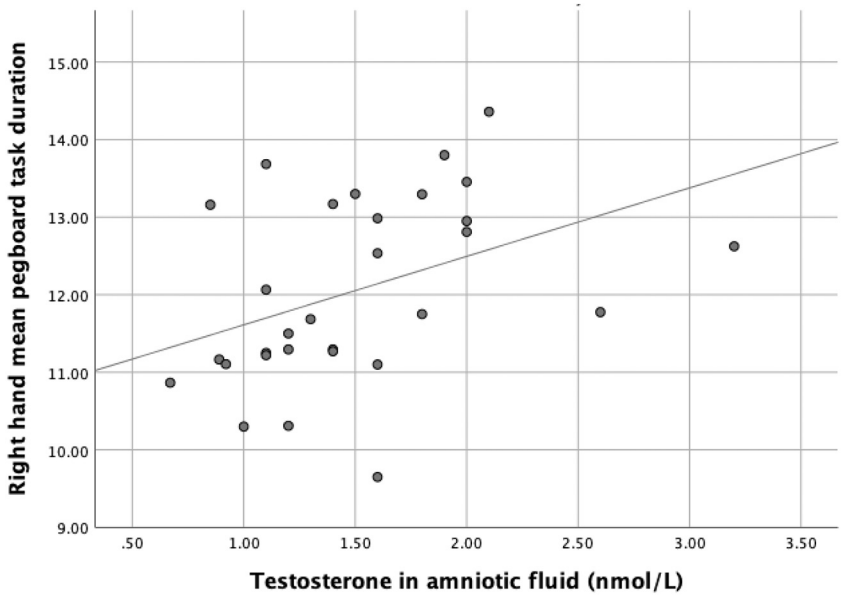

Fig. 3. Association between prenatal testosterone exposure and right hand task duration in males.

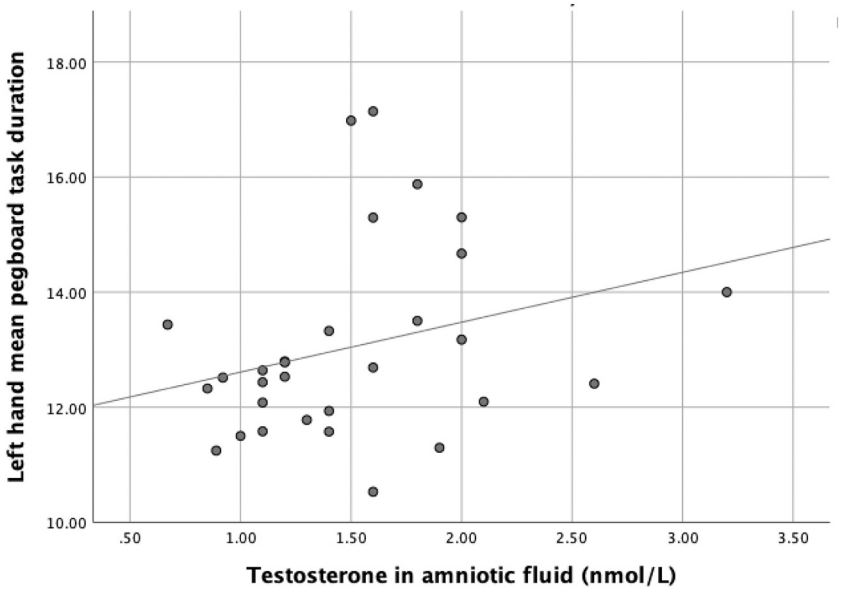

Fig. 4. Association between prenatal testosterone exposure and left hand task duration in males.

Table 4
Associations between amniotic sex hormone concentrations and strength of hand preference and hand skill asymmetry.

		Testosterone	*p*	Estradiol	*p*
		r [BCa 95% CI]		*r* [BCa 95% CI]	
Females	Hand preference strength (unsigned)	−0.156 [−0.570–0.156]	0.411	**−0.338 [−0.600 to −0.058]**	0.068
	Hand skill asymmetry strength (unsigned)	**−0.276 [−0.463 to −0.088]**	0.140	0.128 [−0.311–0.483]	0.499
Males	Hand preference strength (unsigned)	−0.028 [−0.358–0.392]	0.884	−0.087 [−0.330–0.151]	0.646
	Hand skill asymmetry strength (unsigned)	0.086 [−0.225–0.458]	0.650	−0.199 [−0.505–0.193]	0.291

Note. Analyses presented are bootstrapped (10,000 samples) Pearson's correlations (two-tailed); r and p are calculated from standard Pearson's correlations whereas BCa 95% CIs are calculated from the bootstrapping procedure; effects in bold are statistically significant (i.e., the BCa 95% CIs do not include 0).

speed) is broadly consistent with the GBG and sexual differentiation theories, but the latter (i.e., slow left hand speed) may be more consistent with the callosal hypothesis.

Considering the report by Lust et al. (2011) relates to the same cohort as the current study, it is reassuring that the overall pattern of results is similar despite there being considerable differences in the age at which participants were assessed, as well as the statistical methods used. Additionally, whereas Lust et al. (2011) observed children performing 10 different actions (Geuze et al., 2009), the present study used a self-report questionnaire for hand preference and a behavioural measure (i.e., pegboard task) for hand skill asymmetry. It is worth noting that not only may the handedness measures used be of importance, but that different results can sometimes be obtained from the same handedness questionnaire depending on the method used to classify hand preference (e.g., Beaton et al., 2015; Beaton et al., 2017; Hardie and Wright, 2014). In the present study we avoided classification of hand preference and analysed the raw continuous scores. Furthermore, the measure used by Lust et al. (2011) included items (e.g., 'stirring', 'grasping a glass', 'turning the hands of a clock') for which there exist few or no relevant data in the literature. Another important difference is that hand preference was measured at age 6 by Lust et al. (2011) and may have changed by the age of 15 in the current study. This could be important because, although the direction of hand preference appears to be largely fixed by age 3, strength of hand preference may not be fully determined until at least age 7, and perhaps even older (McManus et al., 1988; Scharoun and Bryden, 2014). However, it is noteworthy that we observed high levels of prenatal testosterone to be associated with weak lateralisation (i.e., low degree of hand skill asymmetry in either direction) in females, an effect that broadly replicates that initially reported by Lust et al. (2011). The theoretical meaning of this effect, however, remains elusive, although it is arguably most consistent with predictions of the GBG or sexual differentiation theories. We are also not aware of previous research reporting a direct measure of prenatal testosterone in relation to hand skill asymmetry, though three studies (Beaton et al., 2011; Fink et al., 2004; Manning et al., 2000) have observed an association between male-typical patterns of digit ratio (2D:4D) and strong left hand (relative to right hand) performance. However, Helleday et al. (1994) reported no difference between CAH patients and controls for hand skill asymmetry measured via a finger tapping task.

The current study also observed a significant association between exposure to high concentrations of prenatal estradiol and relatively weak hand preference in females. Although previously published studies have not examined amniotic estrogens in relation to hand preference, an association between elevated prenatal estradiol exposure and deviation from the typical pattern of strong right-handedness is consistent with studies reporting associations between DES exposure and left hand preference (Geschwind and Galaburda, 1985b, p. 545; Schachter, 1994; Scheirs and Vingerhoets, 1995; Smith and Hines, 2000; Titus-Ernstoff et al., 2003). However, the current finding is not consistent with Smith and Hines' (2000) observation that women exposed to DES during gestation showed increased strength of hand preference, and it should also be acknowledged that we observed a significant sex difference (F > M) for amniotic estradiol whereas other similar studies have not (Auyeung et al., 2010, 2012; Erdmann et al., 2019; Richards et al., 2020b). Although notably this effect was also reported for the larger cohort from which the current sample was derived (van de Beek et al., 2004), it is unexpected because the foetal ovaries are generally believed to be inactive during mid-trimester. Furthermore, if the hormone is of

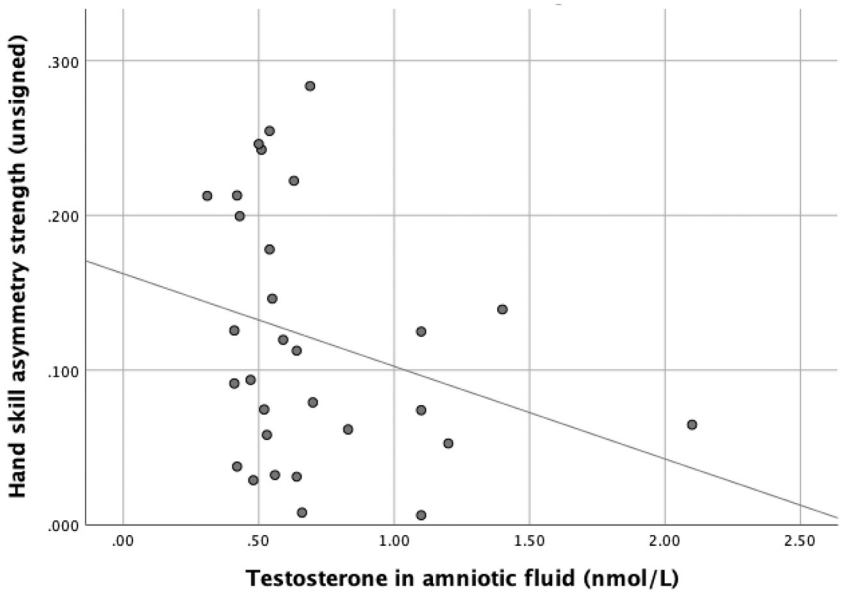

Fig. 5. Association between prenatal testosterone exposure and hand skill asymmetry strength (unsigned) in females.

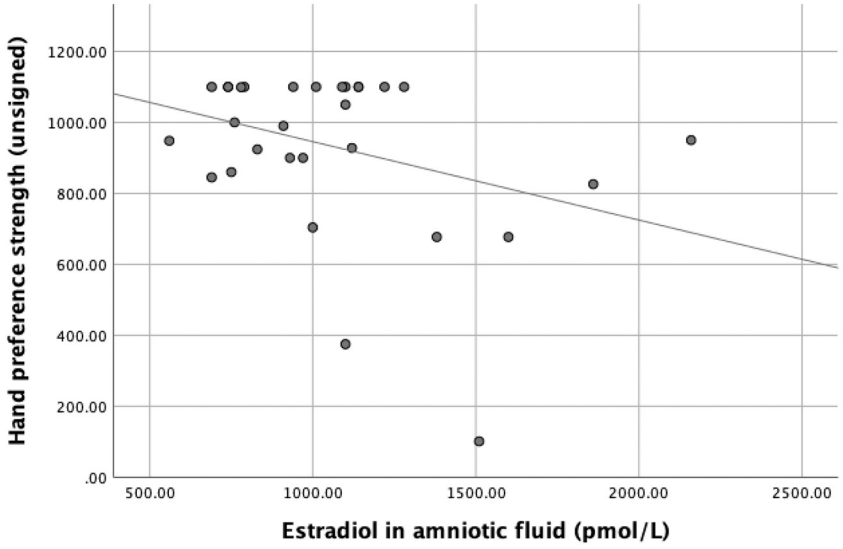

Fig. 6. Association between prenatal estradiol exposure and hand preference strength (unsigned) in females.

maternal origin one would expect no sexual differentiation in exposure unless there is a concomitant difference in placental function affecting its concentration. Considering these observations, we urge caution in interpreting our findings, but tentatively suggest that estradiol may represent an area of interest for future handedness research.

As our findings are not entirely consistent with any of the main hormonal theories (GBG, sexual differentiation, callosal), it is worth considering other factors that have been proposed to account for handedness. Monogenic models (e.g., Annett, 2002; McManus, 2002) have been refuted by a failure of molecular genetic studies to identify any single gene that explains a large amount of variance (Armour et al., 2014; McManus et al., 2013; Somers et al., 2015), and most researchers now accept the likelihood of polygenic effects (Brandler et al., 2013; Cuellar-Partida et al., 2020; de Kovel and Francks, 2019; McManus et al., 2013; Ocklenburg et al., 2014; Somers et al., 2015). Twin studies imply that additive genetic factors account for approximately a quarter of the variance in hand preference (Medland et al., 2006, 2009), and recent evidence (e.g., Schmitz et al., 2018) also suggests the involvement of epigenetic influences. Further to this, various early life events may affect the development of handedness, which include country, year and seasonality of birth, presence/absence of breastfeeding, birthweight, and being part of a multiple birth (de Kovel et al., 2019), as well as birth stress, birth order, position of the foetus in the womb, and maternal anxiety (for a review of proposed determinants of handedness, see Beaton, 2003). Postnatal learning (Provins, 1997) and chance factors (Annett, 2002; McManus, 2002) along with developmental instability (Yeo and Gangestad, 1993) have also been argued to play a role to a greater or lesser extent. It is of course possible that hormonal effects moderate and/or mediate some or all of these processes.

4.2. *Strengths and limitations*

There are several strengths to the current research. First, we analysed actual sex hormone concentrations, whereas many previous studies in this area have relied upon the indirect measure of second to fourth digit ratio (2D:4D) (Richards et al., in press). This is also the first study to

examine amniotic testosterone concentrations in relation to hand skill asymmetry. Additionally, whereas previous research has typically only examined testosterone (see Papadatou-Pastou et al., 2016), the current study also assessed the potential effects of estradiol, as estrogens have been suggested to play a role in the development of handedness (Witelson, 1991). Further, no previously published studies have assessed hand preference or hand skill asymmetry in relation to amniotic sex hormones at such an advanced stage of development (i.e., 15 years). This is a further advantage because by age 15, hand preference is certain to resemble that of adults (Scharoun and Bryden, 2014), whereas it can be somewhat labile in early childhood (McManus et al., 1988).

Although the findings from the current study may provide insights into the role that prenatal sex hormones could play in the development of strength and direction of lateral preferences, they should be considered in light of several limitations. First, we used a modified version of the EHI. Although widely used in the literature, the EHI has been criticised on a number of grounds, including the possibility that the majority of participants may misinterpret the instructions (Fazio et al., 2012), and that the existence of a vast array of modified versions makes direct comparison of results problematic (Edlin et al., 2015). Although using a modified Dutch language version of the EHI may not therefore be an improvement on previous research, including a measure of hand skill asymmetry (i.e., the peg moving task) alongside it certainly is. This is because hand skill asymmetry tasks provide a behavioural measure of manual lateralisation, which is not subject to misinterpretation or response bias associated with self-report measures of hand preference.

Second, the current sample was small (30 males, 30 females), so the analyses may have been underpowered. Although our sample size is comparable to those of the two previous studies of amniotic testosterone and hand preference (Grimshaw et al., 1995: 25 girls, 28 boys; Lust et al., 2011: 31 girls, 34 boys), it should be noted that the effect size for the sex difference in handedness is rather small (Papadatou-Pastou et al., 2008). This suggests that much larger samples may be required to observe robust and statistically significant effects (Mathews et al., 2004). However, this may be unrealistic for studies of amniotic fluid, and there are theoretically important reasons for examining prenatal sex hormone exposure in relation to handedness (Geschwind and Galaburda, 1987; Hines and Shipley, 1984; Levy and Gur, 1980; Lust et al., 2011; Witelson and Nowakowski, 1991; Witelson, 1991). As relevant data are scarce, it was thought useful to conduct these analyses even after considering the limitations.

Third, it should be noted that the antibody used for RIA may not only bind to the target hormone, but also to other substances by cross-reactions. These cross-reactivities, or "specificities", are usually reported by the manufacturer, though when using sample material for which the assay was not originally designed (e.g., amniotic fluid), this information may be inaccurate. Additionally, RIA is typically less precise than more modern techniques, such as liquid-chromatography tandem mass spectrometry (LC-MS) (e.g., Körner et al., 2019; Ventura et al., 2013), and it is not possible to accurately compare absolute values across studies that use these different methodologies. It also remains unclear exactly how the testosterone and estradiol levels present in amniotic fluid relate to those of the foetal circulation in human pregnancies. Rodeck et al. (1985) examined this question by measuring testosterone (but not estradiol) in amniotic fluid and the foetal circulation at the same time in a sample of patients referred for prenatal diagnosis of X-linked conditions ($n = 55$) and found no significant correlation. Further, at the time the original data were collected for the current study, amniocentesis was usually only offered when pregnancies were deemed high risk for genetic or chromosomal disorders (e.g., due to advanced maternal age). Therefore, although the children studied in this cohort are typically developing, they may not be representative of the general (Dutch) population. Another limitation is that prenatal hormone levels fluctuate considerably, and amniotic fluid is usually not sampled more than once during a single pregnancy. We also only examined hormone exposure and were unable to consider variation in the density of androgen and estrogen receptors in the brain (and whether they are asymmetrically distributed), or individual differences in sensitivity to the effects of sex hormones. However, these are general limitations of research involving amniotic hormone assays and are not therefore exclusive to the current study.

5. Conclusion

The current study examined whether testosterone and estradiol measured prenatally (from amniotic fluid) were associated with hand preference and hand skill asymmetry in a cohort of 15-year-old boys and girls from the Netherlands. Prenatal hormone exposure was not predictive of the direction of hand preference or hand skill asymmetry. However, in females, high prenatal testosterone concentrations predicted weak hand skill asymmetry scores, and high prenatal estradiol concentrations predicted weak hand preference. Furthermore, exposure to high levels of prenatal testosterone predicted slow right hand and left hand speed in males. The overall pattern of results is not entirely consistent with any of the main theories (i.e., GBG, callosal, sexual differentiation) in the area. However, previous research would suggest that the relationships observed between prenatal sex hormone levels and handedness outcomes depend on multiple moderating influences, such as the timing and method of measuring hormones, the age and sex of the participants studied, and the measures of handedness employed. As amniocentesis is now rarely performed, researchers should aim to examine associations between handedness measures and amniotic sex hormones in pre-existing cohorts because future opportunities may be limited at best and non-existent at worst.

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CRediT authorship contribution statement

Richards analysed the data and wrote the manuscript; Beking, Geuze, Kreukels, and Groothuis wrote the successful funding application and were involved in study design, data collection and analysis. Beaton revised the manuscript for important intellectual content, and Geuze and Groothuis supervised the project. All authors read and approved the final version of the manuscript prior to submission.

Declaration of competing interest

None.

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Appendix A. Supplementary materials

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2021.104929.

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