A polymorphism in the oestrogen receptor gene explains covariance between digit ratio and mating behaviour

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In vertebrates, including humans, the relative length of the second to the fourth digit correlates with sex hormone-dependent behavioural, psychological and physiological traits. However, despite a decade of research, the underlying mechanism linking digit ratio to these sex hormone-dependent traits remains unclear. Previous work suggests that during embryo development, circulating levels of plasma androgens or oestrogens may act through their receptors to affect transcription levels of posterior HOX genes in the developing digits, thereby possibly influencing their relative length. The correlation between digit ratio and sex hormone-dependent traits might thus stem from variation in expression or sensitivity of the sex hormone receptors, or from variation in sex hormone levels in the embryo. Here, we show that in a population of 1156 zebra finches *Taeniopygia guttata*, a polymorphism in the oestrogen receptor α gene (ESR1) explains 11.3 per cent of the variation in digit ratio, and is also associated with male and female-mating behaviour. By contrast, we found no associations between digit ratio or mating behaviours and polymorphisms in the androgen receptor gene. Thus, our results (i) provide an explanation for the observed significant genetic covariance between digit ratio and male and female mating behaviour and (ii) strongly confirm the indicator function of digit ratio through the oestrogen pathway. Finally, we note that the commonly invoked effect of foetal testosterone on human digit ratio seems to be substantially weaker than the effect described here.

Keywords: 2D : 4D; finger length; HOX genes; sexual behaviour; sex hormones

1. INTRODUCTION

In humans, a particular measure of finger morphology—the relative length of the index to ring finger (D2 : D4)—shows unexpected correlations with a multitude of physiological and psychological traits that seem to have in common a dependence on sex hormones (Manning 2002). The fact that similar relationships between relative digit lengths (digit ratio) and sex hormone-related traits have also been found in various species of mammals, birds, reptiles and amphibians (e.g. Brown *et al.* 2002*a*; Burley & Foster 2004; Leoni *et al.* 2005; Chang *et al.* 2006; Rubolini *et al.* 2006; Navarro *et al.* 2007; Saino *et al.* 2007; Brana 2008; Chang 2008; Dreiss *et al.* 2008), points to a shared ancient molecular mechanism that is more than 300 Myr old. In an early seminal study Manning *et al.* (1998) suggested that perinatal testosterone and oestrogen reflect digit ratio through an unknown mechanism. Subsequent work has neglected oestrogen and emphasized the role of testosterone, leading to the suggestion that digit ratio could be used as a proxy for testosterone levels during embryo development. Despite a decade of research on digit ratio as a marker for perinatal sex hormone action, we are still only beginning to understand the underlying mechanisms and hence the kind of variation (genetic or environmental, of maternal, fraternal

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or embryonic origin) that is actually reflected by this morphological marker (McIntyre 2006).

Most published work on digit ratio has remained relatively vague with regard to pinpointing the suspected causal mechanisms. Here, we summarize much of the existing evidence into a simplified working hypothesis (figure 1) that is open to further empirical testing. Previous work suggests that during a relatively early stage of development, circulating levels of plasma androgens and oestrogens (presumably testosterone and oestradiol) act through their respective receptors (androgen receptor (AR) and oestrogen receptor (ER) α or β) to affect the levels of transcription of posterior HOX genes (e.g. HOXA10 or HOXA11) in the phalangeal anlagen or the growing digits and thereby influence their relative length. Sex hormone-related traits expressed in adulthood might be correlated with digit ratio for three reasons: (i) owing to a population polymorphism in sex hormone receptor genotypes with pleiotropic effects on digit ratio and other sex hormone-related traits, (ii) owing to organizational effects of foetal sex hormone levels, and (iii) owing to activational effects of adult sex hormone levels, which potentially are correlated with foetal sex hormone levels.

The evidence for this working hypothesis is still patchy and requires further empirical study. The posterior HOX genes (HOXA9 to 13 and HOXD9 to 13) are known to be important in the formation of digits (e.g. Kondo *et al.* 1997), but it is neither known how their expression is regulated in the respective tissues, nor how their expression levels affect the relative length of digits.
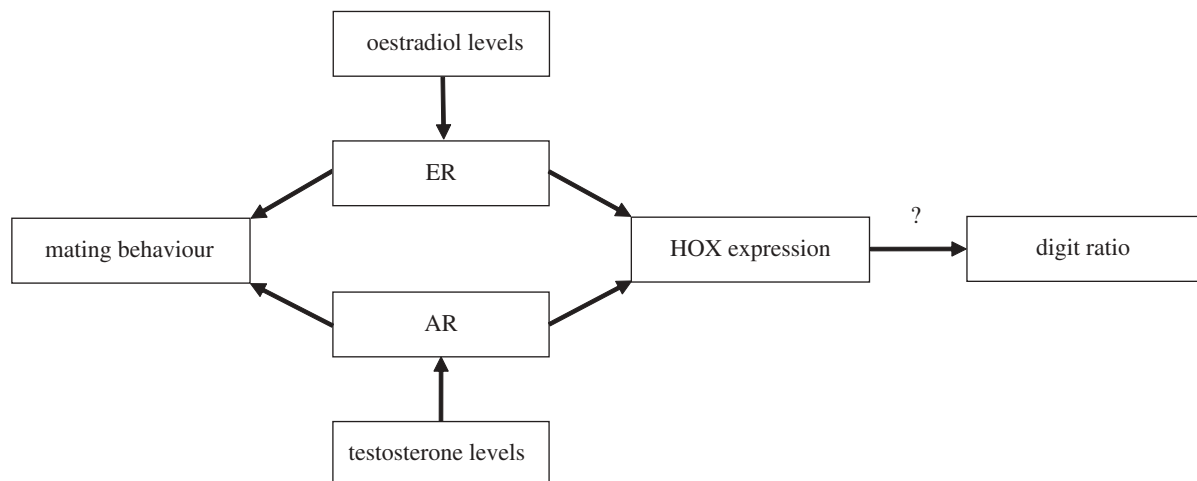
Figure 1. Putative mechanisms leading to covariance between digit ratio and mating behaviour. Oestradiol and testosterone, acting via the respective oestrogen and androgen receptors (ER and AR), are thought to influence both, mating behaviour and HOX gene expression levels, the latter of which in turn is hypothesized to influence the relative length of digits.

However, much research has been carried out on the regulation of HOX gene expression in other tissues like the female uterus (reviewed by Daftary & Taylor 2006), and there, among others the expression is affected by oestradiol and testosterone. For instance in the human endometrium, HOXA10 mRNA levels are enhanced by oestradiol acting through the ERs (Taylor et al. 1998; Akbas et al. 2004) but repressed by testosterone acting through the AR (Cermik et al. 2003). In line with this, it has been found that digit ratio correlates with perinatal sex hormone levels in humans (Lutchmaya et al. 2004; but see also Medland et al. 2008) and, in birds and rats, is affected by experimental sex hormone treatment (Saino et al. 2007; Talarovičová et al. 2009; but see Romano et al. 2005). Also, men with complete androgen insensitivity owing to non-functional ARs show digit ratios typical of women (Berenbaum et al. 2009). Furthermore, based on a sample of 50 male human subjects, Manning et al. (2003) found that digit ratio was associated with the length of a coding polymorphism in the AR gene ($r = 0.29$, $p = 0.04$). Taken together, this evidence provides some support for the scenario outlined in figure 1. However, we are still far from answering what kind of variation is reflected by digit ratio and to what extent. There may be heritable and non-heritable differences in perinatal oestradiol and testosterone levels but also genetic variation in ERs and ARs, and each of these four factors could potentially be responsible for the association between digit ratio and other sex hormone-related traits.

In a population of captive zebra finches, we previously showed that individual differences in digit ratio (the relative length of the second to fourth toe, corresponding to the same toes in mammals, Campbell & Lack 1985) were highly heritable ($h^2 = 0.71$; Forstmeier 2005). Moreover, the trait was genetically (rather than only phenotypically) correlated with measures of male and female courtship activity during encounters with potential mates (Forstmeier 2005), suggesting the existence of pleiotropic gene loci with correlated effects on both digit ratio and aspects of mating behaviour. The aim of the present study was to test whether the androgen or the oestrogen

receptor α genes (AR and ESR1) are among those loci with pleiotropic effects. The identification of those pleiotropic genes is a first step towards understanding why digit ratio is correlated with individual differences in mating behaviour and physiology.

2. MATERIAL AND METHODS
(a) *Study population*
We carried out this study on the same population of domesticated zebra finches as described in Forstmeier (2005), with the only difference that the population now spans over four successive generations and comprises 1209 individuals. The genetic relationships in the entire pedigree are known with complete certainty, as confirmed by genotyping all individuals for 10–18 microsatellite markers (Forstmeier et al. 2007), and 90 per cent of the individuals for 1424 polymorphic single nucleotide polymorphisms (Backström et al. 2010).

(b) *Phenotypic measurements*
W.F. measured digit ratio of the right foot for 1156 individuals as previously described (Forstmeier 2005). For the remainder of the population (4.4%) digit ratio could not be measured, mostly owing to toe swellings presumably caused by infections. In this population, digit ratio (mean ± s.d. = 0.928 ± 0.0347) is sexually monomorphic ($t_{1154} = 0.56$, $p = 0.58$), and unrelated to body size measured as tarsus length ($n = 1{,}156$, $r = -0.012$, $p = 0.68$). Digit ratio is not entirely independent of average toe length (mean of the second and fourth toe, $r = -0.186$, $p < 0.001$) since the highest digit ratios are found in birds with rather short toes. However, we ignore this phenomenon because average toe length is not related to any of the traits of interest in this study (details not shown).

We also measured the amount of male courtship song directed towards females during standardized brief pairwise encounters. This is essentially a measure of male sex drive, because it is strongly correlated with the number of male copulation attempts. This measure of amount of display song has been found to correlate positively with digit ratio (Forstmeier 2005). Altogether, 586 males participated in a total of 4198 encounters, lasting either five ($n = 3292$) or

2 min ($n = 906$), with the amount of directed song measured in seconds using a stop watch. To obtain a single average song rate per individual, we extracted random effect level estimates from a mixed model (lmer in R.2.8.0, Bates *et al.* 2008) of square-root transformed song rate controlling for measurement session (which also accounts for trial duration) and uni- versus mixed-sex rearing of the individual as fixed effects, and individual identity as the random effect. Uni- versus mixed-sex rearing during puberty was implemented for reasons beyond the scope of the present study, and mixed-sex rearing had a small but significantly positive effect on male song rate ($n = 586$ males, $t = 3.0$, $p = 0.003$).

In females, we measured the number of hops made in a four-way choice chamber during a 3 h choice trial. This measure of female activity during mate choice had been found to correlate negatively with digit ratio (Forstmeier 2005). To approach normality, we calculated the square root of the number of hops made per hour. A total of 552 females participated in 849 choice trials (1.54 ± 0.85 s.d. trials per female). Again individual means were extracted as random effect estimates from a mixed model accounting for measurement session, test order within sessions and time of day as fixed effects and individual female as random effect.

(c) Genotyping, haplotype reconstruction and conceptual approach

First, we searched the zebra finch genome using the University of California, Santa Cruz genome browser (http://genome.ucsc.edu) for simple tandem repeats in the regions of the AR gene (located on chromosome 4a) and ER α gene (ESR1 on chromosome 3). There is also a non-annotated ER β gene homologue in the zebra finch (ESR2 on chromosome 5) but this was not clear by the time of study design. We then designed primer pairs to amplify two tetranucleotide microsatellites in the AR gene and three dinucleotide microsatellites in the ESR1 gene regions. Marker locations and details including polymerase chain reaction (PCR) conditions are given in the electronic supplementary material, table S1 (figure 1). The lengths of PCR products from five-locus multiplex PCRs were scored on an ABI Prism 3100. We checked allele calls for all 1209 individuals for Mendelian inheritance. This lead to the discovery of null alleles at three of the five markers (ARmicro1, ARmicro2, ESRmicro2), which, however, proved easy to infer owing to the pedigree information and owing to strong linkage disequilibrium with specific alleles at the neighbouring marker loci (see electronic supplementary material, table S2). To deal with the non-independence of closely located markers, we inferred the existing haplotypes (i.e. combinations of microsatellite alleles at neighbouring loci that are always inherited together) for the AR and ESR1 regions, respectively. We found 28 different AR haplotypes and 16 different ESR1 haplotypes in our population. However, eight AR and three ESR1 haplotypes were not further considered, because they were present in only one to four individuals of the parental generation and went to extinction in our pedigree. The remaining 20 AR and 13 ESR1 haplotypes (listed in electronic supplementary material, table S2) were analysed for association with digit ratio (see below). We found no evidence for a recombination event between neighbouring microsatellite markers within our pedigree. This would be rather unlikely too, given their close proximity (3.1 kb for AR and 62.7 kb for ESR1) and the low recombination rates (*ca* 0.1cM Mb^{-1}) in the central

regions of zebra finch chromosomes 3 and 4a (Backström *et al.* 2010). However, we detected five mutation events (always by a single tetra-nucleotide step) in ARmicro1, giving rise to three new haplotypes not previously recorded elsewhere in the pedigree (not listed in electronic supplementary material, table S2). In the subsequent analyses all newly arisen haplotypes were lumped with their respective ancestral haplotypes for the following reason. Considering the large size of the AR and ESR1 gene regions (electronic supplementary material, figure S1), it is likely that these genes contain a high number of polymorphisms. It thus seems rather unlikely that the lengths of the non-translated microsatellites *per se* affect the transcription rate or the receptor protein function by alternative splicing. Instead, our approach is based on the assumption that in our somewhat bottlenecked captive population, the identified haplotypes are in strong linkage disequilibrium with functionally relevant genetic variants, just as we find strong linkage disequilibrium between the neighbouring microsatellite markers, i.e. unique combinations of specific alleles (see electronic supplementary material, table S2).

(d) Statistical analysis

We tested the overall association of the AR and ESR1 haplotypes with digit ratio in a quantitative transmission disequilibrium test following Abecasis *et al.* (2000) using the program Quantitative Transmission Disequilibrium Test (QTDT) v. 2.6.1. Relatedness and linkage were accounted for by expected variance–covariance matrices of genetic and residual components. Probabilities of the proportion of alleles shared identical-by-descent for all pairs of individuals were calculated using the program SimWalk2 v. 2.91 following Sobel *et al.* (2001). For the overall test of each locus, haplotypes with frequencies of less than 0.05 were lumped. Associations of single haplotypes with digit ratio were tested in the same way (without lumping).

To quantify the genetic effect of each AR and ESR1 haplotype on digit ratio, we selected for each haplotype the corresponding subset of informative full-sibling families, i.e. families where the siblings varied in how many copies they carried of the respective haplotype (zero, one or two). We then regressed their phenotypic digit ratio on the number of copies carried as a continuous fixed effect (one degree of freedom, assuming no dominance deviation from additivity) or alternatively as a factor with three levels (d.f. = 2, to test for dominance deviations). In these models, full-sibling family identification (ID) was accounted for as a random effect (using the lmer function in R.2.8.0, Bates *et al.* 2008). We ran a separate model for each haplotype, owing to the changing informative families. The *p*-values resulting from this analysis were adjusted following a strict Bonferroni correction ($\alpha' = 0.05/k$, where k = number of tests).

We also ran multiple regressions with as many predictors as there were haplotypes, whereby we omitted the most common haplotype since it provides only redundant information. Each predictor represented the allele count (zero, one or two) of one of the haplotypes. This time all individuals were included in the model, with family ID as the random effect. The overall model was compared with a null model with only the intercept and the random effect. These multiple regression models were run for digit ratio, but also for male and female mating behaviour. We also fitted those models as random-slope models (see Schielzeth & Forstmeier 2009), where the effect of a given haplotype is

allowed to vary among the different full-sibling families. Because these models yielded very similar results (e.g. *p*-value for ESR1_10 in electronic supplementary material, table S2 changed from $1.4 * 10^{-7}$ to $5.9 * 10^{-7}$) we omit them for brevity.

(e) Animal models

To estimate genetic correlations between digit ratio and male and female mating behaviour we used pedigree-based animal models (Lynch & Walsh 1998) performed by REML-VCE 6.0.2 (Groeneveld *et al.* 2008). This maximum-likelihood technique allowed us to decompose the phenotypic variance–covariance matrix into an additive genetic variance–covariance matrix (G-Matrix) and a residual, i.e. primarily environmental, variance–covariance matrix (E-Matrix). From these we estimated genetic and environmental correlations as well as the heritabilities (additive genetic variation divided by total phenotypic variation) of the traits. We ran a simple three-trait model, estimating the genetic and environmental variances in and covariances between (i) digit ratio, (ii) male song rate, and (iii) female hops. Because male song rate is also strongly affected by maternal effects in our population (Forstmeier *et al.* 2004), which would inflate the estimated genetic variance if unaccounted for, we included mother identity (250 levels) as a random effect for this single trait, hence estimating an M-Matrix that contained only the maternal variance for song rate, but not for other traits, and no covariances.

To test how the genetic covariances between the traits depend on the effect of the oestrogen-receptor locus, we re-ran the same model, but this time accounting for 'predicted digit ratio' (from a multiple linear regression of digit ratio over ESR1 genotypes, see above) as a fixed effect on each of the three traits. This was done to effectively remove any genetic covariance between digit ratio and the behavioural traits that is owing to correlated effects (using predicted digit ratio as a linear proxy for all haplotypic effects, d.f. = 1) of the ESR1 locus. A more complex model where the two alleles of an individual at the ESR1 locus were modelled as two random effects (with 13 levels each) failed to reach the convergence criteria for an obvious lack of power given the small effects of most ESR1 haplotypes on mating behaviour (see §3). The comparison between the two models, with and without accounting for 'predicted digit ratio', was used to assess how much of the genetic covariance between digit ratio and mating behaviour could be explained by the ESR1 locus. Given large standard errors of the genetic covariances (Forstmeier 2005), it was clear *a priori* that a 'significant' reduction in genetic covariance (by more than two standard errors) cannot be achieved. Nevertheless, the magnitude of change still provides information on whether the locus contributes substantially to the genetic covariances. The pedigree we used spans five generations (the first of which is without phenotypic data) and comprises 1378 individuals.

3. RESULTS

The overall association between haplotype and digit ratio was highly significant for the ESR1 gene (QTDT, d.f. = 6 owing to the pooling of rare alleles, $\chi^2 = 29.2$, $p = 0.00006$), but not significant for the AR gene (QTDT, d.f. = 5, $\chi^2 = 5.8$, $p = 0.34$). In single haplotype tests, the most significant were by the haplotypes ESR1_10 (QTDT, d.f. = 1, $\chi^2 = 14.5$, $p = 0.0001$), ESR1_7 (QTDT, d.f. = 1, $\chi^2 = 11.1$, $p = 0.0009$) and ESR1_13 (QTDT, d.f. = 1, $\chi^2 = 7.4$, $p = 0.007$). For male song rate, we also found a significant overall effect of the ESR1 locus (QTDT, d.f. = 6, $\chi^2 = 15.6$, $p = 0.016$), but not of the AR locus (QTDT, d.f. = 5, $\chi^2 = 6.2$, $p = 0.29$). The most significant single haplotype effects on song rate were by ESR1_10 (QTDT, d.f. = 1, $\chi^2 = 8.9$, $p = 0.0029$) followed by ESR1_12 (QTDT, d.f. = 1, $\chi^2 = 5.3$, $p = 0.021$). For female hopping activity, neither the ESR1 locus (QTDT, d.f. = 6, $\chi^2 = 4.8$, $p = 0.57$) nor the AR locus (QTDT, d.f. = 5, $\chi^2 = 7.1$, $p = 0.21$) showed significant overall effects.

Table S2 (electronic supplementary material) lists for each haplotype, the estimated effect (i.e. the slope in a mixed model of linear regression) that a single copy of that haplotype has on digit ratio (33 separate analyses, each limited to the respective informative full-sibling families, assuming no dominance deviation from additivity). Similar to the QTDT analysis none of the AR, but two of the ER haplotypes (ESR1_7 and ESR1_10) showed strong and highly significant effects on digit ratio in the mixed-effects model analyses. To examine dominance effects, these two most significant models were repeated with haplotype copy number as a three-level predictor (d.f. = 2). The parameter estimates from these models (figure 2) revealed little dominance deviation from linearity in the effects of the two haplotypes, i.e. two copies had approximately twice the effect of a single copy. The negative effect of ESR1_7 on digit ratio is not just an artefact of two ESR1_7 copies meaning zero ESR1_10 copies, because the effect of ESR1_7 is still significant after accounting for the effect of ESR1_10 (lmer, d.f. = 1, $\chi^2 = 6.3$, $p = 0.012$). However, the estimated effect sizes (see electronic supplementary material, table S2) are of course estimated against a shifting reference of the pooled effect of all other alleles.

To test how much of the total phenotypic variance in digit ratio can be explained by the 13 ESR1 haplotypes together, we ran a multiple regression with the allele counts of each haplotype (except the most common ESR1_7) as the 12 predictors. The overall effect was highly significant when compared with a null model (lmer, d.f. = 12, $\chi^2 = 86$, $p < 10^{-12}$). Predicted values (by the parameter estimates for the 12 fixed effects from this model) are plotted against the observed phenotypic values in figure 3 ($n = 1,156$, $r^2 = 0.113$). Hence, the 13 ESR1 haplotypes explain 11.3 per cent of the phenotypic variation in digit ratio, which corresponds to 16.1 per cent of the estimated additive genetic variance in digit ratio (see heritability estimate in figure 4).

The *x*-axis of figure 3 represents a vector of estimated effects of ESR1 genotype on digit ratio. To see whether this vector of genetic variation has pleiotropic effects on male and female mating behaviour, we correlated this genotypic prediction with the behavioural data. In line with the expectations from genetic correlations (Forstmeier 2005, and below), male song rate was positively correlated with 'predicted digit ratio' ($r = 0.147$, $n = 585$, lmer accounting for family ID, $z = 2.89$, $p = 0.004$), and female hopping activity was negatively correlated with 'predicted digit ratio' ($r = -0.088$, $n = 551$, lmer accounting for family ID, $z = -2.03$, $p = 0.043$).
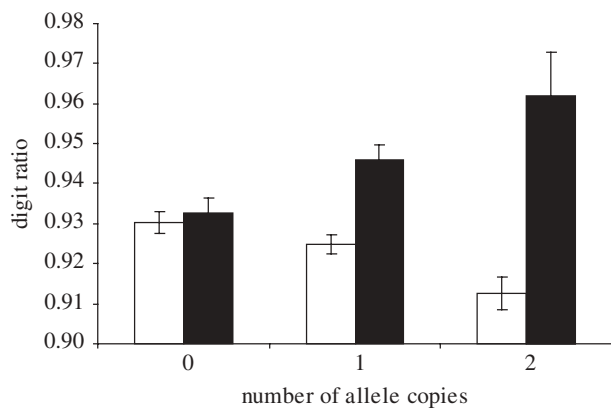
Figure 2. Mean $\pm$ s.e. digit ratio of individuals carrying 0, 1 or 2 copies of the oestrogen receptor (ER) haplotypes ESR1_7 and ESR1_10. Parameter estimates from mixed-effects models are shown. Open bars, ESR1_7; filled bars, ESR1_10.
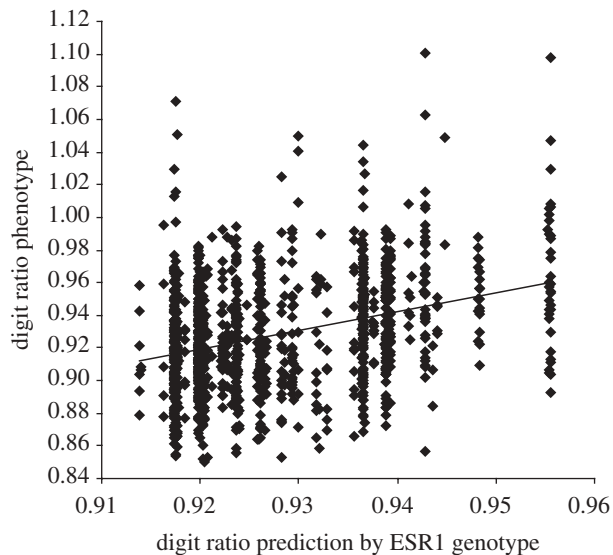

Figure 3. Digit ratio of 1156 adult zebra finches plotted against predicted values from a multiple regression with ER genotype (mixed-effects model, d.f. = 12, $r^2 = 0.113$, $p < 10^{-12}$). Predictions are based on parameter estimates for each of the 13 ER haplotypes. The 13 haplotypes allow for 91 possible haplotype combinations (13 homozygotes and 78 different heterozygote combinations), 69 of which were represented in our pedigree sample (explaining the categorization along the *x*-axis).

Figure 4*a* shows the heritabilities of and the genetic correlations between the three traits of interest. Environmental correlations between the traits were weak but in the same direction as the estimated genetic correlations (digit ratio versus song rate $r_e = 0.103 \pm 0.080$; digit ratio versus hops $r_e = -0.090 \pm 0.086$). After accounting for the effect of ESR1 genotype ('predicted digit ratio') on each of the three traits, the residual genetic variances in these traits were still slightly correlated with each other, though to a lesser extent (figure 3*b*). By accounting for 'predicted digit ratio', the genetic covariance between digit ratio and song rate was reduced by 63 per cent, the genetic covariance between digit ratio and female hops by 31 per cent.
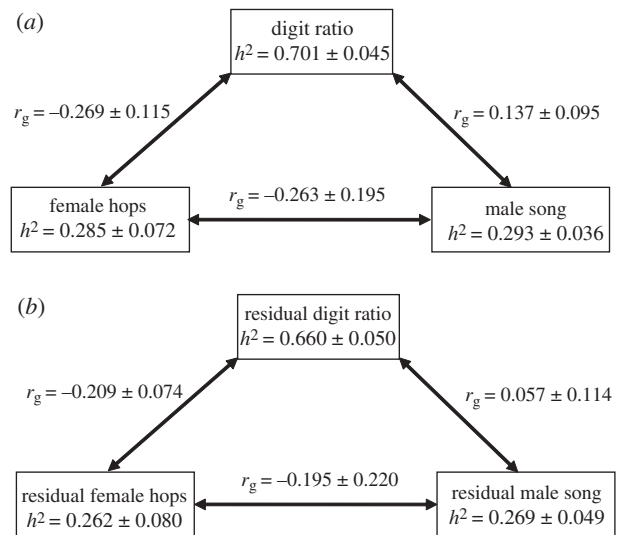
Figure 4. (*a*) Heritability of and genetic correlations between digit ratio, amount of female hopping in a four-way choice chamber and male courtship song directed at females in pairwise encounters. (*b*) The same relationships after removing the suspected effect of ESR1-genotype on these genetic correlations. This is done by accounting for the effect of genotype-predicted digit ratio (*x*-axis of figure 3) on each of the three traits as a continuous fixed effect (d.f. = 1). The corrected traits 'residual digit ratio', 'residual female hops' and 'residual song rate' tend to show weaker genetic correlations than in (*a*). The reductions in the respective heritability estimates from (*a*) to (*b*) closely follow the expectations (e.g. digit ratio additive genetic to environmental variance: $V_a/V_e = 0.701/0.299$; residual digit ratio expected $V_a/V_e = (0.701 - 0.113)/0.299 = 0.663/0.337$, observed $V_a/V_e = 0.660/0.340$).

4. DISCUSSION

We found that genetic variation at the oestrogen but not the androgen receptor locus was strongly associated with digit ratio and also with male and female mating behaviour, thereby explaining—at least in part—the genetic covariance between the two and hence the indicator function of digit ratio. After accounting for the effect of the ESR1 gene polymorphism, a weak genetic and environmental covariance remains between digit ratio and mating behaviour. This is consistent with the idea that both genetic and environmentally induced variation in oestradiol levels (and potentially also testosterone levels) will also contribute to the covariance between digit ratio and mating behaviour (figure 1). The lack of an effect from the AR gene could either mean that testosterone and the receptor do not play a role or that the receptor is involved but is functionally monomorphic in our population.

The estimated effect of the ESR1 locus (explaining 11.3% of the phenotypic variation in digit ratio) most probably represents a minimum estimate. Regarding the potentially high number of untyped polymorphisms in the gene region it seems unlikely that the genotyped microsatellite loci themselves are the functional genetic variants causing those effects. It seems more likely that the markers are in linkage disequilibrium with the real causal genetic polymorphism, be it a coding sequence polymorphism affecting protein function or a difference in the promoter region affecting transcription rate. If linkage between functional polymorphisms and marker polymorphisms was perfect, the true effect of the locus

would be close to the estimated 11.3 per cent, while if linkage was incomplete, the true effect would have been underestimated. Such underestimation could be an alternative explanation for the residual genetic covariance between digit ratio and mating behaviour.

Strong linkage disequilibrium between microsatellite markers and functional variants is expected owing to genetic bottlenecks in the breeding of our captive population. In the most extreme case, there would be only 13 different haplotypes of the locus, and all copies of one haplotype would be identical by descent. This scenario would lead to perfect linkage between markers and functional variants. The infrequent occurrence of crossovers in the central parts of chromosome 3 where the ER gene is located also means that linkage disequilibrium blocks extend over large distances (Backström *et al.* 2010). This implies that the functional variants which cause variation in digit ratio and covariance with mating behaviour could potentially also lie in one of the neighbouring genes. While this cannot be excluded until the functional variants are identified, we consider this possibility rather unlikely, given the very strong *a priori* expectations (based on previous work) pointing towards the major sex hormone receptors (figure 1).

(a) What does digit ratio reflect to what extent?

Probably, the most common statement in the human digit ratio literature is that digit ratio is thought to be an indicator of foetal testosterone levels. In the following, we examine the evidence for this statement, by reviewing the relative importance of the four factors (testosterone, AR, oestradiol and ER) proposed in figure 1. Measurements of foetal testosterone in humans during gestational weeks 11–18 showed that the sexes differed from each other by at least three within-sex standard deviations (Abramovich 1974; Reyes *et al.* 1974; Rodeck *et al.* 1985; for discussion see electronic supplementary material, appendix). We would expect to see the same effect size of three standard deviations difference between the sexes in their digit ratios if digit ratio depended solely on foetal testosterone during that period (for a discussion see electronic supplementary material, appendix). However, the observed sexual dimorphism in digit ratio is only about 0.48 standard deviations (Hönekopp & Watson 2010), suggesting that the within-sex correlation between foetal testosterone and digit ratio is only about $r = 0.157$ (for explanations see electronic supplementary material, figure S2). In other words, the within-sex variation in foetal testosterone explains only about 2.5 per cent of the within-sex variation in digit ratio. This rough calculation rests on the assumption that between-sex differences can be regarded as a linear continuation of within-sex variation, which need not be true. However, the view that foetal testosterone explains only little of the variation in digit ratio is further supported by the recent finding that individuals with a male karyotype that are completely insensitive to androgens also show only slightly increased digit ratios (by up to 0.6 male standard deviations, $p = 0.04$, one-tailed; Berenbaum *et al.* 2009), hardly exceeding the average digit ratio for women. Moreover, digit ratios in androgen-insensitive males varied just as much between individuals (s.d. = 0.032) as in the control population (s.d. = 0.033).

Hence, while the shift in mean digit ratio by 0.6 standard deviations is best explained by an androgen effect, the entire remaining individual differences must be owing to other factors. In contrast to these very minor effects, the small-scale study linking digit ratio with genetic variation at the AR locus in men (Manning *et al.* 2003), suggested an effect explaining about 9 per cent of the variation in male digit ratio. Hence, if true, one should rather state that human digit ratio reflects the AR genotype than foetal testosterone levels. Little can be said about the effect of foetal oestrogens in humans (which are only slightly higher in female than male embryos with much overlap between the sexes; Reyes *et al.* 1974), but maybe it is indicative that Lutchmaya *et al.* (2004) found the ratio of amniotic testosterone to oestradiol to be a much stronger predictor of digit ratio than testosterone by itself (r^2 about 27%, $p = 0.004$ versus r^2 about 10%, $p = 0.09$). Note, however, the lack of power ($n = 29$), and the fact that amniotic and foetal sex hormone levels may be only weakly correlated ($r < 0.3$; Rodeck *et al.* 1985).

In birds, Saino *et al.* (2007) found that an injection of a dose corresponding to only one phenotypic standard deviation of yolk oestradiol into pheasant eggs led to feminized digit ratios in male offspring that differed by about 0.9 standard deviations from the male average. If all assumptions hold (as in electronic supplementary material, figure S2), this would reflect a correlation of $r = 0.9$ between yolk oestradiol and digit ratio ($r^2 = 81\%$). This might be an overestimate because injected hormones dissolved in oil may result in much higher concentrations close to the embryo owing to the non-homogeneous distribution of the hormone in the yolk (von Engelhardt *et al.* 2009). With our study, we now can add a first estimate of the effect of the ER locus on variation in digit ratio, with 11 per cent of variance explained.

While it is far from clear which of those four estimates for the effects of testosterone, AR, oestradiol and ER, respectively, will turn out to be the most realistic and representative across species, they might be suggestive enough to redirect some of the attention away from foetal testosterone levels.

It seems worth remarking that in mammals the best evidence involves androgens (Brown *et al.* 2002*b*; Ökten *et al.* 2002; Manning *et al.* 2003; Berenbaum *et al.* 2009; Talarovičová *et al.* 2009), while in birds digit ratio was affected only by oestradiol (Saino *et al.* 2007) but not testosterone (Romano *et al.* 2005) injection into eggs. Future studies could help to clarify whether this is related to the observation that in mammals, as a rule, males have lower digit ratios than females, while reversed sexual dimorphism seems to prevail among amphibians, reptiles and birds (Chang 2008). More data on mammals is clearly needed, given that the association with the AR genotype is based on only 50 individuals (Manning *et al.* 2003) plus 16 individuals with non-functional ARs (Berenbaum *et al.* 2009), and that the experimental treatment with testosterone has only been applied to three pregnant female rats (Talarovičová *et al.* 2009). These sample sizes are too small to reliably estimate effect sizes (see above). Furthermore, future experimental studies should also account for the fact that testosterone can be converted to oestradiol by aromatase, which may happen locally, not only in the

brain, but also in the growth plates of bones (Öz *et al.* 2001) where oestrogens but not testosterone have been found to affect bone growth (Cutler 1997).

(b) Sensitive periods and mechanisms

Sexual dimorphism in human digit ratio has been observed already at the earliest stages of digit development (Malas *et al.* 2006; Galis *et al.* 2010), which coincides roughly with the period where male foetuses produce much more testosterone than females (Abramovich 1974; Reyes *et al.* 1974; Rodeck *et al.* 1985). More experimental studies will be needed to pinpoint the developmental time window during which digit ratio is affected by (and hence will be an indicator of) sex hormones. Positive evidence for sex hormone effects on digit ratio points to early (Saino *et al.* 2007) or late (Talarovičová *et al.* 2009) embryo development, while juvenile digit growth seems to be unaffected by oestrogen treatment (Forstmeier *et al.* 2008). One possibility is that the sex hormones affect HOX gene expression in the developing limb bud and thereby shift the anterior–posterior axis, such that the entire morphology of the second digit (or the fourth digit, respectively) would become more 'third-digit like'. Recent arguments about whether the sexual dimorphism lies in skeletal bone lengths or in the fleshy parts of the fingers (Wallen 2009) could be supplemented with more detailed analyses of other aspects of finger morphology, since anterior–posterior shifts in HOX gene expression should affect the entire array of finger features. It might also be rewarding to study digit ratios in zygodactyle birds (like cuckoos, parrots or woodpeckers) where the fourth toe is pointing backwards together with the first toe. Here, shifts in anterior–posterior gradients might lead to parallel sexual dimorphisms in 2D : 3D as well as in 1D : 4D.

(c) Pleiotropic effects on behaviour, psychology and physiology

The study of digit ratio has been particularly appealing because of the suggestion that it might reflect the action of sex hormones during early development, which have well-known organizational effects on brain development and hence adult behaviour and psychology (Wilson 2001; Adkins-Regan 2009). However, correlations between digit ratio and these sex hormone-dependent traits could alternatively come about through receptor polymorphisms (Manning *et al.* 2003; this study) as well as through correlated sex hormone levels across developmental stages (embryo versus adult). Although correlations between adult sex hormone levels and digit ratio have been found to be rather weak (Hönekopp *et al.* 2007; but see Manning *et al.* 1998; Jürimäe *et al.* 2008; Tan 2008), adult sex hormone levels cannot entirely be ruled out as a contributing factor.

Concerning the androgen versus oestrogen debate in relation to behavioural and psychological effects, it is relevant to note that androgens have been found to be the more important sex hormone in mammals (Wilson 2001), while oestrogens play the dominant role in birds (organizational: Adkins-Regan 2009; activational: Balthazart *et al.* 2009). However, there is also growing evidence for some involvement of the oestrogenic pathway

in mammals (Rochira *et al.* 2005). Interestingly, for the male mating behaviour analysed, namely male rate of directed singing towards females, there is already evidence that it is affected by oestradiol acting through the ER (Walters *et al.* 1991). This study suggests that the male sex hormone testosterone gets converted locally in the brain into oestradiol (through the enzyme aromatase), which then binds to the ER and affects the behaviour. Our finding that the ER genotype significantly influences song rate is in line with this earlier study. The effect of the ER genotype on female behaviour (hopping in the choice chamber) remains uncertain, because the effect was only significant when the varying effects of different haplotypes were aggregated into a single predictor ('predicted digit ratio'). This approach maximizes statistical power because it uses up only one degree of freedom, but it assumes that haplotype effects on digit ratio are perfectly correlated with haplotype effects on hopping behaviour. This assumption may not be true. In comparison, 'predicted digit ratio' explained 2.2 per cent of the phenotypic variance in male song rate (d.f. = 1), while a multiple regression involving haplotypes separately (d.f. = 12) explained 5.1 per cent of the phenotypic variance in song rate.

(d) Perspective

For a better understanding of the indicator function of digit ratio we need both a broader and a deeper analysis of the variation in digit ratio and its covariance with other sex hormone-related traits. With broader, we mean that much of the genetic variance in digit ratio is still unaccounted for, and other candidate genes for HOX gene regulation like the ER β, the progesterone receptor, the vitamin D receptor, the retinoic acid receptors (Daftary & Taylor 2006), as well as the HOX gene clusters need to be examined. More depth regarding the ESR1 gene is required in terms of finding the causal polymorphism(s), and studying expression levels in various tissues, sensitivity to oestradiol and effects on HOX gene expression in the phalangeal anlagen.

Furthermore, there is a need for more and better-designed experimental studies. Given the very high heritability of digit ratio it is important to account for genetic relatedness in order to avoid pseudoreplication (e.g. Forstmeier *et al.* (2008) for advice on a more powerful experimental design). Another innovative experimental approach could be to study whether an individual's sensitivity to sex hormone administration can be predicted from its digit ratio. This might be the case (but not necessarily) if digit ratio reflected the sex hormone receptor genotype.

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