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Brief Report

Experimental manipulation of yolk testosterone affects digit length ratios in the ring-necked pheasant (*Phasianus colchicus*)

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Abstract

In humans, most of the mammals and one bird species studied so far, the relative length of individual digits is sexually dimorphic. Most studies of humans have been concerned with the ratio between second (2D) and fourth digits (4D), whereas some studies of humans and other mammals have also investigated other digit ratios. Inter- and intra-sexual variation in 2D:4D may depend on differential exposure to androgens during embryonic life, and the genetic mechanisms linking 2D:4D to androgens may be mediated by *Hox* genes. Because *Hox* genes are conserved in vertebrates, similar patterns of variation in digit ratios might be expected across vertebrate classes. The observation of correlations between digit ratios and physiological, psychological and performance traits in humans has generated interest in exploring the possibility that digit ratios are a marker of embryonic exposure to androgens, which have diverse consequences on several phenotypic traits. However, the hypothesis that digit ratios depend on androgen effects during development has never been tested experimentally. In this study, we increased testosterone concentration in ring-necked pheasant eggs and measured length ratios between the second, third and fourth digits of both feet in fully grown offspring. Females from testosterone-injected eggs had larger 2D:3D in the left foot, whereas this was not the case in males. The other digit ratios were unaffected by hormone treatment in both sexes. However, digit ratios showed no sexual dimorphism among controls. Thus, present results are consistent with the hypothesis that variation in testosterone levels during development affects digit ratios.

Keywords: Development; Digit ratios; Directional asymmetry; *Phasianus colchicus*; Sexual dimorphism; Testosterone

Introduction

In humans, the relative length of fingers has been known to be sexually dimorphic for decades (Phelps, 1952). In particular, the length ratio between the distal extent of the second (index) and the fourth (ring) fingers (2D:4D) has been investigated in several studies, where males have been found to have smaller values than females (Manning, 2002; Manning et al., 1998; McFadden and Shubel, 2002). It has been hypothesized that inter- and intrasexual variation in 2D:4D depends on exposure to different levels of androgens during embryonic development of males and females and among individuals of the same sex (Brown et al., 2002a; Lutchmaya et al., 2004; Manning, 2002; Manning et al., 1998; Peters et al., 2002).

A link between digit development and exposure to androgens during fetal life may be enforced by the simultaneous effect that some homeobox genes have in controlling the development of fingers, toes, and the urogenital system (Manning et al., 1998). Because these genes are conserved in vertebrates (e.g. Herault et al., 1997; Kondo et al., 1997; Mortlock et al., 1996; Peichel et al., 1997), similar relationships between exposure to sex hormones and relative digit length should exist also in non-human vertebrates.

Besides 2D:4D, sexual dimorphism in humans exists also for length ratios between other pairs of forelimb digits, men showing smaller 2D:3D, 2D:5D, 3D:4D and 3D:5D (McFadden and Shubel, 2002), or 2D:3D and 2D:5D

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(Manning et al., 2003) than women. However, sex-related variation in length ratios between digits or metapodials has been documented also for two species of baboons, gorillas, and chimpanzees (McFadden and Bracht, 2003, 2005; Roney et al., 2004). In addition, some evidence has been presented that men have larger hindlimb 2D:4D and 3D:4D than women (McFadden and Shubel, 2002). In mice, Brown et al. (2002b) reported smaller hindlimb 2D:4D, and Manning et al. (2003) found smaller 2D:3D, 2D:4D, and 2D:5D among males compared to females. In the only bird species studied so far, the zebra finch (*Taeniopygia guttata*), right foot 2D:4D (data for other digit ratios were not reported) were larger in males than females and in both sexes increased with laying order of the egg of origin (Burley and Foster, 2004). In the zebra finch, yolk testosterone concentration declines with laying order (Gil et al., 1999), which is consistent with the idea that 2D:4D is negatively influenced by high testosterone levels.

Interestingly, digit ratios in men and women correlate with several physiological, psychological, performance, and health traits (Bailey and Hurd, 2005; Csathò et al., 2003; Manning, 2002; Manning and Taylor, 2001; McFadden and Shubel, 2002; Neave et al., 2003; Putz et al., 2004; Rahman and Wilson, 2003; Robinson and Manning, 2000; Romano et al., in press; Williams et al., 2000). Several sex-dependent traits have been shown to be expressed in a more 'masculine' form in individuals of either sex with small forelimb 2D:4D (Manning, 2002).

To date, all the studies concerning the relationships between digit length ratios and testosterone levels have adopted a correlational approach. There is therefore no direct experimental evidence that digit ratios are causally linked to embryonic testosterone levels.

In this study, we injected physiological doses of testosterone in the yolk of ring-necked pheasant eggs, while establishing a control group of sham-inoculated eggs, and investigated the effect of increased testosterone levels during development on foot digit ratios of each sex. The ring-necked pheasant is a strongly sexually dimorphic species, with males being larger and having more brightly colored ornamental plumage than females (Cramp, 1980).

Methods

The experiment was done in captivity during spring–summer 2004. A sample of 800 eggs was extracted from a large set of eggs laid by approximately 1000 females kept in 155 separate polygynous groups. Before treatment (testosterone injection or sham injection), the eggs were arranged in random sequence and then split into two groups of 400 eggs. Eggs of each group were then randomly assigned to sub-groups of five eggs (pentads). All five eggs of the first pentad were injected with testosterone (4-androsten-17β-ol-3-one; Sigma, Germany), all five eggs of the second pentad with the solvent of testosterone to serve as controls, and so forth with the following pentads. The two groups of 400 eggs were injected by two different people who were blind to the treatment on a single day. Thus, each injector treated the same number of randomly chosen eggs for each experimental group. Before injection, the eggs were left in a vertical position (acute pole upwards) for approximately 30 min. The eggshell at the acute pole was carefully cleaned and disinfected, and a small hole was opened with a heat-sterilized pin of the same diameter as the needle used for injection. Injection was made using a 250 μl Hamilton syringe mounting a 25 gauge, 16-mm long needle. To seal the hole, a small piece of eggshell was glued over it immediately after injection. Preliminary trials where we injected a red food dye dissolved in sesame oil by this procedure and froze the eggs immediately after injection showed that injection occurred well within the yolk in all of 10 eggs.

The amount of testosterone injected (40 ng in 20 μl sterile sesame oil) corresponded to 2 SD of the average amount estimated for the yolk of 73 pheasant eggs (data kindly provided by F. Dessì Fulgheri, Università di Firenze). However, we checked for testosterone concentration in a random sample of 13 eggs belonging to the same pool of eggs from which the 800 experimental eggs derived (see Testosterone assay). Mean testosterone concentration was 6.95 ng/g (2.28 SD) yielding an estimated mean amount of 73.4 ng (24.0 SD) for an average yolk of 10.6 g. Thus, the amount of testosterone we injected increased testosterone concentration in the egg by 1.67 SD as estimated in the same population of females at the same time of the season. Therefore, the vast majority of the birds in the testosterone injection group originated from eggs which had physiological post-manipulation testosterone concentrations. Control eggs were injected with 20 μl sterile sesame oil using the same procedure as for testosterone injection.

All the eggs were incubated simultaneously in the same incubator on 6 different layers (3 layers for each treatment). Eggs from the two treatments were not mixed in order to distinguish chicks according to the group to which they belonged upon hatching, and treatments were assigned randomly to layers. We are confident that this experimental setting did not bias the results because the eggs were kept in adjacent layers in large (>10,000 eggs) professional incubators which are designed to allow for homogeneous, optimal conditions. In addition, incubation of the two groups of eggs in different layers would have biased the outcome of the analyses only if minute differences in incubation conditions had differential effects on relative digit length and by chance the eggs from particular sex × treatment groups were exposed to different average condition, which is highly unlikely. Chicks were individually marked with numbered plastic bands. Hatching success was 44.5% for testosterone-injected and 42.8% for sham-injected eggs. As egg failures in artificially incubated eggs typically number about 15–20%, injection caused an