Gonadal and Endocrine Analysis of a Gynandromorphic Chicken

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Birds have a ZZ male and ZW female sex chromosome system. The relative roles of genetics and hormones in regulating avian sexual development have been revealed by studies on gynandromorphs. Gynandromorphs are rare bilateral sex chimeras, male on one side of the body and female on the other. We examined a naturally occurring gynandromorphic chicken that was externally male on the right side of the body and female on the left. The bird was diploid but with a mix of ZZ and ZW cells that correlated with the asymmetric sexual phenotype. The male side was 96% ZZ, and the female side was 77% ZZ and 23% ZW. The gonads of this bird at sexual maturity were largely testicular. The right gonad was a testis, with SOX9$^+$ Sertoli cells, DMRT1$^+$ germ cells, and active spermatogenesis. The left gonad was primarily testicular, but with some peripheral aromatase-expressing follicles. The bird had low levels of serum estradiol and high levels of testosterone, as expected for a male. Despite the low percentage of ZW cells on that side, the left side had female sex-linked feathering, smaller muscle mass, smaller leg and spur, and smaller wattle than the male side. This indicates that these sexually dimorphic structures must be at least partly independent of sex steroid effects. Even a small percentage of ZW cells appears sufficient to support female sexual differentiation. Given the lack of chromosome-wide dosage compensation in birds, various sexually dimorphic features may arise due to Z-gene dosage differences between the sexes. **(*Endocrinology* 159: 3492–3502, 2018)**

The long-held paradigm of vertebrate sexual differentiation invokes embryonic gonadal development (ovary or testis) directing somatic sex differences via the release of sex steroid hormones. This concept was expounded by Alfred Jost following his classic castration experiments on rabbit embryos. In these studies, removal of the embryonic gonads resulted in feminization of the body, in both XX and XY embryos (1, 2). Many subsequent studies have generally supported the Jost model of sexual differentiation in mammals, in which the gonads have a central role in directing somatic sexual differentiation (3–7). In therian mammals, the Y chromosome-linked *SRY* gene initiates testicular development during embryonic life (8–10). Testosterone released from the testes then acts to masculinize the reproductive tract in male embryos (XY), and, in the absence of testosterone, a female phenotype prevails (XX embryos). Under this scenario, the ovaries are not required for feminization of the reproductive tract. This general model has been extended to other vertebrates over the years, although *SRY* is absent beyond mammals and other genetic triggers of gonadal sex differentiation exist (11–15). Although testosterone has a masculinizing effect in mammals, in avians, it is estrogen that has a more pervasive role in sexual differentiation (13, 16–19). Gonadectomy of avian embryos results in masculinization of the internal and external reproductive

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Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

structures, due to the loss of estrogen (20, 21). In birds, estrogen has a key role in both gonadal and extragonadal tissues during embryonic development (19, 22–24). The rate-limiting enzyme in estrogen synthesis is aromatase (*CYP19A1*), which is expressed in female but not male embryonic gonads, in chicken and other avian species (25–28). Injection of an aromatase inhibitor into day 3 or 4 fertile chicken eggs can induce robust masculinization of genetically female embryos. Birds develop as apparent males, with testes and masculine features that last through to adult stages (18, 29). In both mammals and birds, the hormonal theory of sexual differentiation has found widespread acceptance over many years. Jost considered that defeminization is produced by the testes in male mammals, whereas demasculinization was produced by the ovary in female birds (30).

Several lines of evidence point to direct genetic factors having a major contribution to sexual differentiation, operating in conjunction with endocrine signals. Firstly, sexually dimorphic gene expression has been detected in the gonads and, notably, in extragonadal tissues prior to gonadal sex differentiation. This has been reported for both mammal and bird embryos (31–35) [reviewed by Arnold (36)]. Secondly, a number of somatic sexual dimorphisms have been reported that predate gonadal sex differentiation and sex steroid synthesis. These include, for example, size differences between early XX and XY mouse embryos (37), structural differences in the brain (38), and the development of scrotum and mammary anlagen in wallabies (39, 40). Thirdly, naturally occurring gynandromorphs support direct genetic effects upon sexual differentiation. Gynandromorphs are bilateral sex chimeras, with female features on one side of the body and male features on the other (41–43). Agate *et al.* (44) described a gynandromorphic zebra finch that had male feathering and masculinized brain on the right side and female feathering and brain features on the left. More recently, Zhao *et al.* (45) reported three adult gynandromorphic chickens that were sexually asymmetric. The male side had a large leg with spur, large muscle mass, and large wattle, whereas the female side had smaller spur, smaller muscle mass, smaller wattle, and female plumage (45). Such birds cannot be explained by hormones, which are expected to flow equally to both sides of the body, suggesting direct genetic effects. Taken together, all of these data support the notion that vertebrate sexual differentiation is at least partially cell autonomous, involving direct or indirect effects of the sex chromosomes in individual cells.

Birds have a ZZ male; ZW female sex chromosome system (46). In the case of all documented gynandromorphic birds, the male side is predominantly ZZ and the female predominantly ZW (41). The gonads of gynandromorphic chickens are reportedly testis (composed of mainly ZZ cells), ovarian (mainly ZW cells), or ovotesticular (a mix of ZZ and ZW cells) (45). The genetic basis for gynandromorphic development is unclear, but it has been postulated to involve failure of extrusion of the polar body during female meiosis and subsequent fertilization by two Z-bearing sperm, leading to ZZ-ZW chimeric embryos (42, 45). The exact genes involved in sexually dimorphic development are unknown. Birds lack a system of global Z chromosome dosage compensation, so that males (ZZ) have on average higher levels of Z-linked gene expression compared with females (47–50). The ~80-megabase chicken Z chromosome has ~1000 genes (51), so any one or more of these genes could contribute to male vs female cellular fate independently of hormones. The Z-linked gene, *DMRT1*, plays a central role in testis development in chicken, and probably all birds (52, 53). However, it is not expressed beyond the gonads, suggesting that other Z-linked genes, or W-linked genes, may underlie gynandromorphic development.

Previous studies of gynandromorphic birds have not closely examined the gonads and sex steroid output. In this study, we describe the gonads and endocrine profile of a gynandromorphic chicken that had male features on the right and female features on the left. The gonads of this bird at sexual maturity were largely testicular. The right gonad was a testis, with $SOX9^+$ Sertoli cells, $DMRT1^+$ germ cells, and active spermatogenesis. Histologically, the left gonad was primarily testicular, but with a small number of peripheral $aromatase^+$ follicles. The bird had very low levels of serum estradiol and high levels of testosterone, as expected for a male, although the testosterone level was very high. Despite the elevated testosterone, the bird was female on one side of the body. It had a low percentage of ZW cells on the female side, but still had female sex-linked feathering, smaller muscle mass, smaller leg and spur, and smaller wattle than the male side. This indicates that sexually dimorphic structures such as the wattle, spur, and feathering must be at least partly independent of sex steroid effects. Even a small percentage of ZW cells appears sufficient to support female-type sexual differentiation.

Materials and Methods

Birds

A naturally occurring gynandromorph was identified in a population of commercial brown egg–laying hybrid chickens in Victoria, Australia. In this hybrid line, males are white, and females are brown in color. The bird was held and later euthanized at the Werribee Animal Health Facility at the Commonwealth Scientific and Industrial Research Organisation (Werribee, Victoria, Australia; approved by the Commonwealth Scientific and Industrial Research Organisation Animal Ethics Committee, protocol no. 1834). The bird was identified as potentially gynandromorphic based on marked asymmetry in plumage color, which is sex-linked in this hybrid line. Males

have white feathering, and females have brown feathering. The bird was white on the right side of the body and brown on the left side. It showed male-type behavior, such as crowing. It was raised to sexual maturity (30 weeks) and compared with age-matched male and female chickens.

Molecular sexing and chromosomal analysis

Molecular sexing by PCR was carried out on feather pulp from both sides of the body of the gynandromorph at 17 weeks of age and again at postmortem, from different tissues of the body. Tissues were digested in PCR-compatible proteinase K buffer, and the genomic DNA was used for rapid PCR sexing. By this method, ZW cells can be detected by the presence of the W-linked *Xho*I repeat sequence (54). In control birds, only females (ZW) have the *Xho*I PCR product. Amplification of *18S rRNA* gene in both sexes served as an internal control. As chickens have nucleated red blood cells, we anticipated some ZW cells flowing from the female side to the male side. To definitely identify ZZ-bearing and ZW-bearing cells, karyotyping was performed on fibroblasts isolated from left- and right-side connective tissues. Abdominal connective tissue was taken from each side, minced, and plated into flasks in culture medium (DMEM plus 10% fetal calf serum plus antibiotics). After 5 days, fibroblast cultures were established. Fibroblasts were also derived from control female and male samples.

Metaphase chromosome preparation

Metaphase chromosome spreads were prepared from short-term culture of whole blood and also from fibroblast cell lines following protocol described in Ezaz *et al.* (55) with slight modification (mitogen and culture temperature). Briefly, ~100 μL heparinized (sodium heparin) blood was used to set up 2 mL culture in DMEM (GIBCO) supplemented with 10% fetal bovine serum (JRH Biosciences), 1 mg/mL L-glutamine (Sigma), 10 mg/mL gentamycin (Multicell), 100 units/mL penicillin (Multicell), 100 mg/mL streptomycin (Multicell), and 3% pokeweed mitogen (Sigma). Cultures were incubated at 35°C for 96 hours in 5% CO_2 incubator. Six hours prior to harvesting, 75 ng/mL colcemid (Roche) was added to the culture. Metaphase chromosomes were harvested and fixed in 3:1 methanol/acetic acid following the standard air-drying protocol. Cell suspension was dropped onto glass slides and air-dried. For 4′,6-diamidino-2-phenylindole (DAPI) staining, slides were mounted with antifade medium Vectashield (Vector Laboratories) containing 1.5 mg/mL DAPI. Images were captured and analyzed using a Zeiss Axioplan epifluorescence microscope equipped with a charge-coupled device camera (Carl Zeiss) and Isis florescence imaging platform (MetaSystems).

Plasma sex steroid measurements

At the time of euthanasia (30 weeks), blood was collected from the gynandromorphic bird and from control male and female chickens. Blood was centrifuged to separate out red cells, and plasma was stored at −20°C prior to hormone assay. Testosterone and 17β-estradiol were measured by chemiluminescent immunoassay (Biochemistry Department, Royal Children's Hospital, Melbourne, Victoria, Australia).

Histology and immunofluorescence

Left and right gonads were taken from the gynandromorphic bird. Following macroscopic analysis, gonads were sliced into smaller pieces, fixed overnight in Bouin fixative, processed into paraffin blocks, and then processed for either hematoxylin and eosin staining or immunofluorescence. For the latter, 6-μm sections were dewaxed, washed in PBS, and then subjected to antigen retrieval (0.1 mol citrate buffer at a temperature of 98°C for 30 minutes using a Dako PT Link antigen retrieval instrument). Sections were blocked for 1 hour in PBS plus 2% BSA and then incubated overnight at 4°C with primary antibodies. The following antibodies were used: rabbit anti-DMRT1 (1:2000; in-house; RRID: AB_2665399) (56); rabbit anti-SOX9, a male marker (1:6000; Millipore; RRID: AB_2239761) (57); and rabbit anti-P_{450}aromatase, a female marker (1:5000; in house; RRID: AB_2734780) (58). Sections were washed three times in PBS and then incubated with fluorophore-conjugated secondary antibodies (Alexa Fluor 488; Invitrogen). Sections were counterstained with DAPI, mounted, and imaged under an epifluorescent microscope.

Results

The gynandromorphic chicken at sexual maturity (30 weeks) had female features on the left side of the body and male features on the right side (Fig. 1A). The

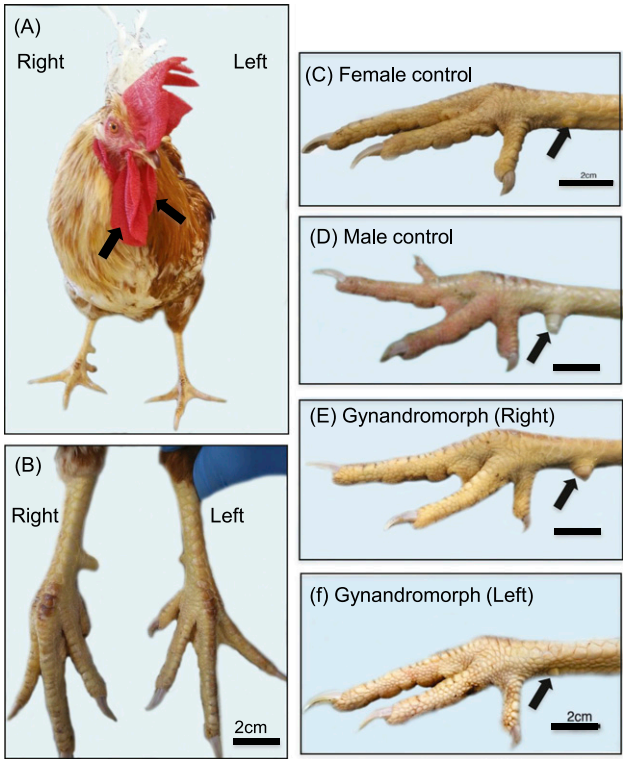


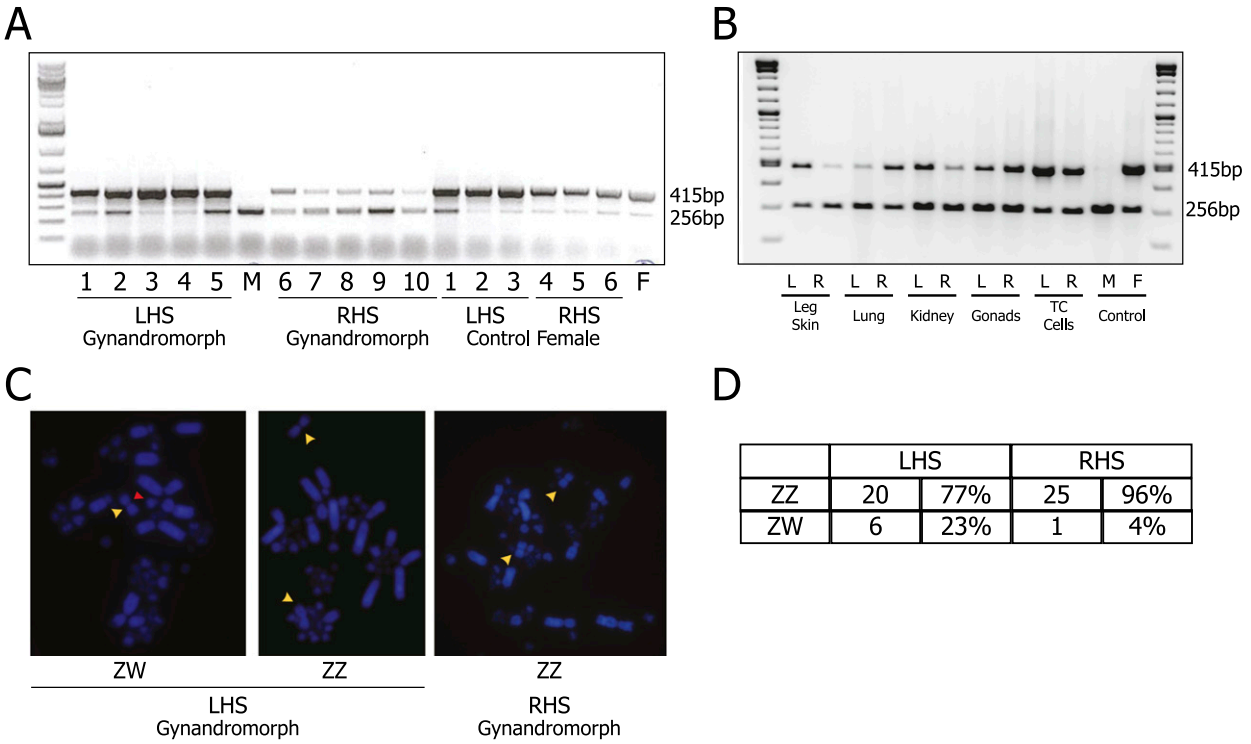
Figure 1. Gynandromorphic chicken, showing asymmetric sexually dimorphic traits. (A) Male phenotype is present on the right (white feathering, with larger wattle, arrow), whereas the female phenotype is present on the left (brown feathering and smaller wattle, arrow). (B) Larger leg on the right male side compared with the left female side. (C) Small nodular spur on the leg of a control (30-wk-old) female chicken. (D) Large well-developed spur on a control (30-wk-old) male chicken. (E) Spur on the right leg of the gynandromorph at 30 wk is prominent and male-like in size. (F) Spur on the left leg of the gynandromorph at 30 wk is very small and female-like. Scale bars, 2 cm.

gynandromorphy was most apparent in the plumage, which is a sex-linked trait (Z-linked). The *Silver* gene (*S*) yields white feathering and is dominant over the *golden* allele (*s*) that yields brown feathering. The gynandromorph was derived from a commercial hybrid line, produced by crossing white hemizygous females (*S,-*) with brown homozygous males (*s,s*). Hence, all male offspring are predominantly white, and all female offspring are predominantly brown.

The left side of the gynandromorph had brown (female-type) feathering, and the right had white (male-type) feathering. The wattle was significantly larger on the right compared with the left [Fig. 1(A)]. Corresponding with the left female side, the left leg was smaller than the right [Fig. 1(B)]. Spur development was also asymmetric in the gynandromorph at 30 weeks of age. The spur on an age-matched control female at sexual maturity was a small nodule on the lower leg. [Fig. 1(C)], whereas it was a pronounced spur on a control male [Fig. 1(D)]. The gynandromorph exhibited a male-like spur on the right male leg and a smaller nodule resembling that of a control female on the left leg [Fig. 1(E) and 1(F)]. The gynandromorph also exhibited greater breast muscle mass on the right male side [Fig. 1(A)]. Collectively, the bird had external male-type features on the right and female-like phenotype on the left. It crowed like a typical male but failed to mate with other birds.

Molecular sexing and chromosomal analysis

PCR sexing was initially carried out on genomic DNA derived from feather pulp initially at 17 weeks of age. The left female side sexed as female (ZW) due to the presence of the W-linked *XhoI* repeat band, whereas the right male side was largely ZZ (less intense W-linked band; Fig. 2A). PCR sexing was repeated postmortem from tissues representing the derivatives of the three embryonic cell layers—ectoderm (skin), mesoderm (kidney and gonad), and endoderm (lung)—from the left and right side of the gynandromorph. The W-linked band was detected in all tissues isolated from the left female side of the gynandromorph, suggesting ZW cells have contributed to all three germ layers and as such have contributed to the whole left female side of the gynandromorph. The presence of the W-linked band in tissues derived from the



	LHS		RHS	
ZZ	20	77%	25	96%
ZW	6	23%	1	4%

Figure 2. Genotypic analysis of the gynandromorphic chicken. (A) PCR sexing of feather pulp DNA at 17 wk of age, showing strong 415-bp female (W-linked) band on left-hand side (LHS) compared with a weaker band on the right-hand side (RHS) across independent samples. Male and female control genomic DNAs are also shown. (B) PCR sexing of DNA isolated from various tissues postmortem (30 wk). The female-specific (W-linked) band is stronger in samples obtained from the left side and weaker in the right side. In the lung and gonad, the W-linked band is stronger on the right. The presence of the W-linked band on the right could be attributed to mixing ZZ and ZW blood cells on both sides of the body. (C) Karyotype analysis of culture fibroblasts from the left and right sides of the gynandromorph body. The left side was a mix of ZW and ZZ cells, the W being identified as the largest microchromosome (red arrowhead), and the Z is the only submetacentric macro chromosome (yellow arrowheads). (D) Percentage of ZZ and ZW cells from the left and right sides of the body (fibroblasts). ZW cells were primarily detected on the left side of the body. F, female; L, left; M, male; R, right; TC, tissue cultured cells from connective tissue.

right male side is most likely due to the presence of circulating ZW blood cells on this side of the body (Fig. 2B). However, it may also reflect varying levels of ZW cells in different tissues on the male side.

Karyotype analysis of cultured fibroblasts from the left and right sides showed that the gynandromorphic bird was diploid but with both ZZ and ZW cells present. Twenty-six metaphase chromosome spreads were prepared from fibroblasts from both the right and left side of the gynandromorph. The right male side was almost entirely ZZ (96%), whereas those from the left female side were a mixture of male (77% ZZ) and female (23% ZW) cells (Fig. 2C and 2D). The presence of low levels of ZW cells in tissues from the right male side of the gynandromorph also would contribute to the presence of the W-linked band in tissues isolated from the right male side of the gynandromorph.

Gonads and ducts

At 30 weeks of age, control birds had mature gonads. In a control female, a unilateral ovary was present comprising developing follicles and with an associated oviduct. Histologically, maturing and immature follicles were present, comprising oocytes encircled by granulosa cells. Thecal cells were present (Fig. 3A–3C). The control male bird had bilateral testes (Fig. 3D). Within the testis, seminiferous tubules contained Sertoli cells and germ cells in active stages of spermatogenesis (Fig. 3E and 3F). Macroscopically, the gynandromorphic bird at 30 weeks of age had a right testis and a left dysmorphic gonad (Fig. 3G). The right gonad was a typical testis, characterized by seminiferous tubules with Sertoli cells, spermatogonia, and spermatozoa (Fig. 3H and 3I). The left gonad was largely testicular, with a mixture of normal and abnormal seminiferous tubules. The abnormal tubules were less common and characterized by a degenerate structure (loss of Sertoli and germ cells and with considerable cellular debris) (Fig. 3J). Adjacent to the degenerate tubules, some peripheral ovarian follicles were detectable. These follicles were all immature, were encircled by presumed granulosa cells,

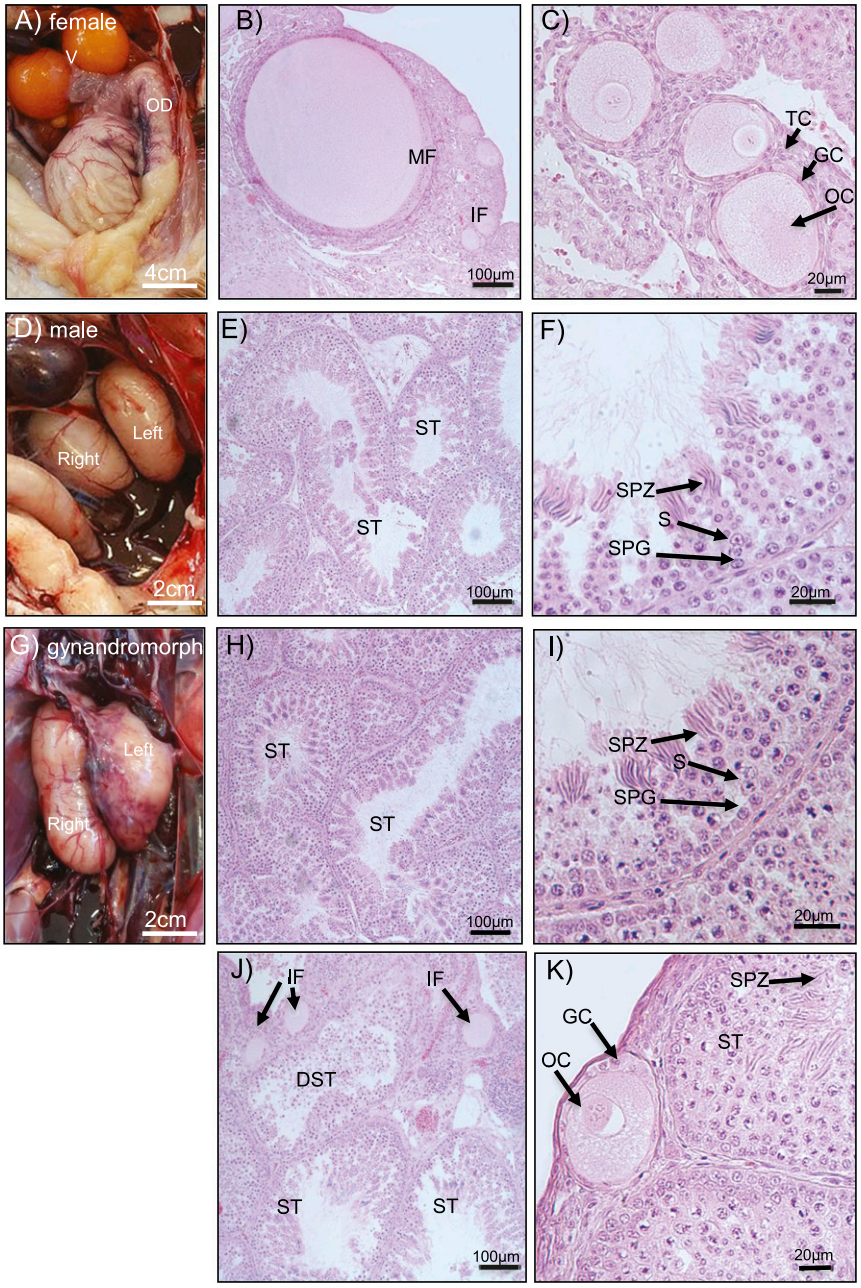


Figure 3. Gonadal morphology and histology of a gynandromorph vs control female and male chickens. (A) Unilateral ovary and oviduct in a control female. (B) Ovary of a female, showing immature and maturing follicles. (C) Higher magnification of a control ovary, showing the oocyte, granulosa cells, and thecal cells. (D) Bilateral right and left testes in a control male. (E) Seminiferous tubules of a control male. (F) High magnification of a control testis, showing Sertoli cells, basal spermatogonia, and maturing spermatozoa. (G) Gonadal morphology in the gynandromorph, showing a right testis and left dysmorphic gonad. (H) Right testis of the gynandromorph, showing well-developed seminiferous tubules. (I) High magnification of right gynandromorph testis, showing Sertoli cells, basal spermatogonia, and maturing spermatozoa. (J) Left gonad of the gynandromorph, showing both seminiferous tubules and immature follicles. A degenerate seminiferous tubule is also apparent. The degenerate seminiferous tubule lacks both somatic and germ cells. (K) High-magnification view of left gonad from the gynandromorph, showing seminiferous tubules and a follicle with an oocyte and apparent granulosa cells. DST, degenerate seminiferous tubule; GC, granulosa cell; IF, immature follicle; MF, maturing follicle; OC, oocyte; OD, oviduct; S, Sertoli cell; SPG, spermatogonia; SPZ, spermatozoa; ST, seminiferous tubule; TC, thecal cell; V, ovary.

and comprised <10% of the total gonadal areas, in representative sections (Fig. 3K). Those seminiferous tubules in the left gynandromorphic gonad, which appeared normal, exhibited Sertoli cells and germ cells in the various stages of spermatogenesis (Fig. 3K). There were no signs of female reproductive ducts in the gynandromorph, implying that anti-Müllerian hormone was present. Sperm ducts were present bilaterally, implying active testosterone action.

Immunofluorescence

To further characterize the gonads of the gynandromorph, immunofluorescence was carried out. The expression of marker genes such as *DMRT1* and *SOX9* has not previously been reported in adult chickens. Both of these genes are likely to play essential roles in testicular differentiation at embryos stages in chicken (59–61). In a control hen, strong aromatase expression was observed in somatic (granulosa) compartment of the ovarian follicles. The male markers, SOX9 and DMRT1, were not detected (Fig. 4A–4C). Conversely, in a control male bird, aromatase was not expressed, whereas SOX9 and DMRT1 were detectable in the basal nuclei of Sertoli cells within the seminiferous cords (Fig. 4D–4F). DMRT1 protein was also detected in spermatogonia and in mature (luminal) spermatozoa (Fig. 4F). The right gonad of the gynandromorph had a typical male pattern; aromatase was not expressed, SOX9 and DMRT1 were expressed in somatic (Sertoli) cells of the seminiferous cords (Fig. 4G–4I). The left gonad of the gynandromorph showed areas of peripheral aromatase expression, together with area of SOX9-positive and DMRT1-positive seminiferous tubules (Fig. 4J–4L). The amount of aromatase-positive tissue in the left gynandromorph gonad was estimated to be ~10% based on morphometric analysis of several representative sections.

Sex steroid hormone levels

Blood was taken from the gynandromorph prior to euthanasia, and the serum used for sex steroid hormone measurement. Serum was also derived from age-matched mature male and female chickens of the same strain. The

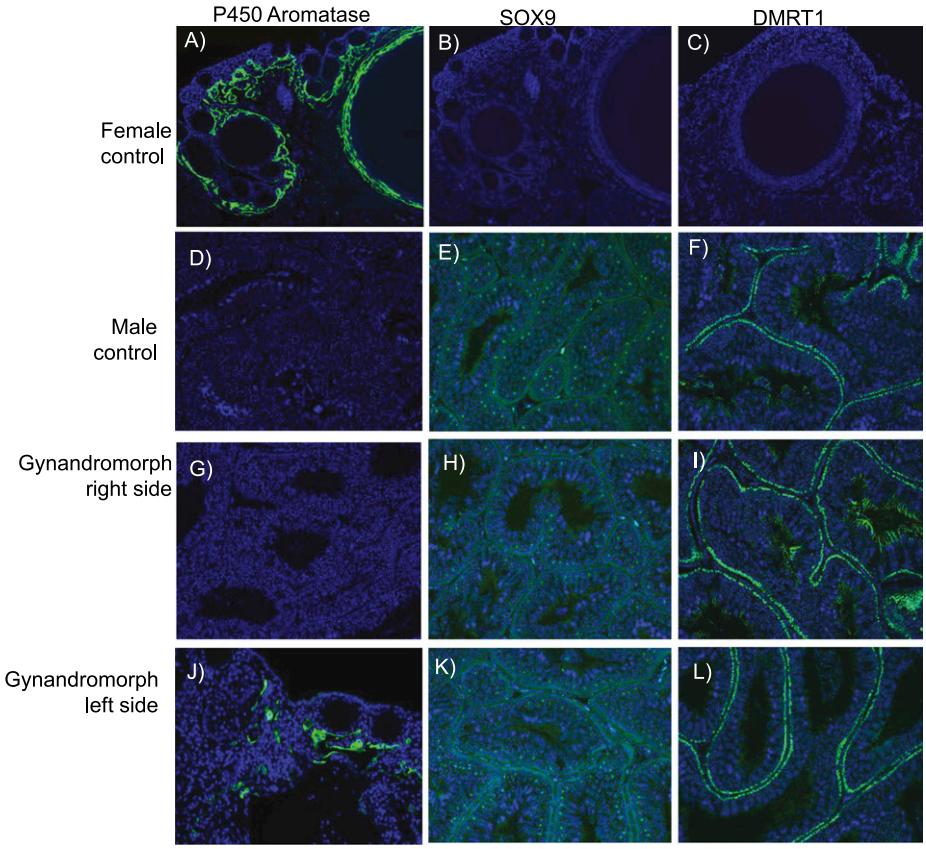


Figure 4. Expression of male and female markers in adult (30-wk-old) chicken gonads and in the gynandromorph. Positive staining is shown in green, counterstained with DAPI. (A) In a control female bird, aromatase enzyme is expressed in the somatic cells of the follicle, whereas (B) SOX9 and (C) DMRT1 were undetectable. (D) In a control male, aromatase is not expressed. (E) SOX9 is expressed in the basal nuclei of Sertoli cells. (F) DMRT1 expression in both basal Sertoli cell nuclei and weakly in mature spermatozoa. (G) The right gonad of the gynandromorph was typically testicular, lacking aromatase. (H) SOX9 and (I) DMRT1. (J) In the left gonad of the gynandromorph, small patches of aromatase-expressing cells were evident. Most of the left gonad comprises seminiferous cords, which stained for (K) SOX9 and (L) DMRT1. No DMRT1 expression was noted in mature spermatozoa.

data are shown in Table 1. The gynandromorph showed a male-type endocrine profile. Testosterone levels were very high; ~10 times the levels seen in a control male. At the same time, the gynandromorph had detectable levels of 17β-estradiol that were higher than those of a typical male but well below those of a normal female chicken. The low but detectable levels of estrogen in the gynandromorph were consistent with the small but detectable areas of aromatase-positive cells in the left gonad.

Discussion

The data presented in this study support the notion that sexual development in chicken is a least partly cell autonomous, because the gynandromorphic bird exhibited both male and female traits despite elevated serum testosterone levels. The data support similar previous reports of gynandromorphic chickens (41, 45) and zebra finches (44). Of the three gynandromorphic chickens described by Zhao *et al.* (45), two were male on the left, female on the right, whereas one was female on the left, male on the right. The bird described in this study was female on the left and male on the right. Hence, either side can be male or female. The presence of both ZZ and ZW in the bird described in this study and those described in previous reports strongly indicate that the gynandromorphs are unlikely to arise from a mutation at the two-cell stage of embryonic development. Previously, it has been proposed that gynandromorphs arise due to failure of extrusion of the second polar body during female meiosis and subsequent fertilization of Z and W pronuclei by two Z-bearing sperm (45). This remains to be formally proven but could be assessed by comparing the paternal Z haplotypes on the left and right sides of the body. Our study adds to the previous findings by documenting serum sex steroid hormone profiles and carrying out detailed analysis of gynandromorphic gonads. The three gynandromorphic chickens described by Zhao *et al.* (45) had gonads that reflected the relative proportions of ZZ- and ZW-bearing cells. Thus, birds had testes on the side that was predominantly ZZ and ovaries on the side that was predominantly ZW. This was also noted in our study. The right side was largely ZZ, and the gonad was a testis, whereas the left side was a mix of ZZ and ZW cells (77%:23%; Fig. 2D) and largely testicular but with some peripheral follicles (Fig. 3J and 3K). These data alone do not prove whether testis formation is driven by Z chromosome dosage or whether the W plays a role in ovarian development. Currently, most evidence points to Z dosage, and the Z-linked *DMRT1* gene, being the molecular trigger for sexual differentiation of avian gonads into testis (2A:ZZ) or ovary (2A:Z) (52, 59, 60).

Table 1. Serum Sex Steroid Hormone Levels at 30 Weeks in Control Female and Male Chickens, Compared With the Gynandromorphic Bird at the Same Age

Bird	Serum Testosterone, nmol/L	Serum 17β-Estradiol, pmol/L
Control female	<0.4	2221
Control male	4.1	<18
Gynandromorph	41.3	39

Studies on chimeric embryos also support the hypothesis that avian sexual differentiation is largely, or partly, cell autonomous, involving direct genetic factors. Zhao *et al.* (45) produced chimeric chicken embryos in which GFP-labeled male (ZZ) pregonadal mesoderm was transplanted into female host embryos and *vice versa*. It was found that the opposite sex tissue did not become incorporated in the host gonadal tissues, instead expressing markers of gonadal sex differentiation typical of the donor tissue. This again supports the view that cells in the chicken embryo have an innate sexual fate, which is independent or at least partly independent of sex steroid hormones. In our study, the left side was genetically female based on PCR of various tissues and confirmed by karyotype analysis of connective tissue fibroblasts. Only a small percentage of ZW cells was detected on the left female side of the body (23%), yet this was enough to induce feminization on that side. In comparison, previously studies have reported higher percentages of ZW cells on the female side [between 40% and 60% in the Zhao *et al.* (45) study)]. This implies that even a small percentage of ZW cells is enough to trigger feminization of chicken tissues. Although the study reported in this paper provides a karyotypic analysis of a gynandromorphic bird, we only karyotyped cells derived from connective tissue. We did not karyotype cells derived from the wattle or other tissues. It is possible that the bird was mosaic for the relative contribution of ZW cells, where, for example, the wattle on the female side may have had a greater percentage of ZW female cells than connective tissue fibroblasts. This is supported by the observation that a W-specific PCR product could be detected on the right (male) side at different intensities in different tissues (refer to Fig. 2B). Although this could reflect different numbers of ZW red blood cells in different tissues of the male side, it could also point to the possibility that different tissues may have had different proportions of ZW cells (on both sides of the body). Nevertheless, it is clear that the left side of the gynandromorph had female traits despite the apparent low percentage of ZW-bearing cells in the bird described in this study.

Furthermore, the chicken described in this study was overtly gynandromorphic despite having a very masculine-biased gonadal and endocrine profile. Firstly, the bird had a typical right testis, featuring Sertoli cells and spermatogenesis, and largely left testis, but with a small number of immature peripheral follicles. (Fig. 3G–3K). Aromatase enzyme expression, a marker of estrogen biosynthesis, was robust in the follicles of an age-matched control female bird, but only a small region of the left gynandromorphic gonad showed aromatase immunoreactivity (Fig. 4A and 4J). This correlated with the peripheral follicles. Accordingly, the gynandromorph had low levels of serum 17β-estradiol, although not as low as a control male. In contrast, the gynandromorph had very elevated levels of serum testosterone (41.3 nmol/L), significantly higher than that measured in an age-matched sexually mature control male (4.1 nmol/L). Serum testosterone levels in sexually mature male chickens have been reported to be in the range of 0.8 nmol/L to 20 nmol/L (62–65). The elevated testosterone levels measured in this study in the gynandromorph could be due to a disturbed hypothalamic-pituitary-gonadal axis if the hypothalamus and/or pituitary were partly feminized and gonadotropins secretion perturbed. Future studies of gynandromorphic chickens should examine this point. Nevertheless, despite the clearly elevated testosterone levels, the wattle and spurs were clearly different on the two sides of the gynandromorph (Fig. 1). It is assumed that the elevated levels of testosterone measured in the adult gynandromorph reflect those during embryogenesis, as implied by the largely testicular gonadal phenotype observed (Figs. 3 and 4).

Sexual development in birds appears to be a combination of direct genetic and hormonal factors. For example, enlarged spurs and wattles have long been considered androgen-dependent traits in birds (66). Gonadectomy of male chickens can result in regression of spurs (67), whereas injection of testosterone can cause them to regenerate (66). However, in our recent study of ZZ chickens ectopically overexpressing aromatase and with very high serum estrogens, testosterone was undetectable (presumably metabolized into estrogen by aromatase), but male-type spurs were still present in these birds (63). Together with the current gynandromorph data, this strongly suggests that spurs are partially independent of androgens in Galliformes but are under direct genetic control. Altogether, the data point to a role for both direct genetic and hormonal (androgen) control of spur development, at least in Galliform birds. The wattle may also differentiate according to both genetic and hormonal cues. ZZ chickens ectopically overexpressing aromatase do have reduced wattles (63), which suggests a role for androgens in stimulating wattle development or a role for estrogen in retarding wattle development. However, the gynandromorphic chicken reported in this study showed a patently smaller wattle lobe on the left female side but larger wattle lobe on the right male, suggesting an interplay of genes and gonadal hormones. Another sexually dimorphic trait that was asymmetric in the gynandromorph is feathering, which is also likely to be under both genetic and hormonal control. The dull or cryptic coloring of female birds is linked to estrogen signaling (66, 68–70). However, the gynandromorphic chicken reported in this study, and those described previously, clearly had asymmetric sexually dimorphic feathering (feather color and length). Hence, this trait must also be under genetic and hormonal control. In contrast, the comb is a well-documented target of androgen (71), and the comb on the gynandromorphic bird reported in this study was typically male.

How can a model of cell autonomous sexual development be reconciled with the fact that hormonal manipulation can largely sex-reverse birds? For example, a single injection of the aromatase inhibitor, fadrozole, can induce robust testis formation in genetically female embryos, which can hatch as males and maintain the male phenotype through adulthood (29, 62, 72). Similarly, ovariectomy or loss of the left ovary in adult female chickens can lead to the vestigial right gonad becoming testicular, and the birds become male in appearance (66). Conversely, exogenous estrogen can, at least transiently, feminize genetically male gonads (19). ZW female chicken embryos exposed to testis grafts during embryonic life can become masculinized throughout the body, hatching as males with testes and reaching sexual maturity as cockerels (73, 74). According to Rashedi *et al.* (74), such birds had "large red comb and wattles, spurs, and call." The sexual behavior was also male-like, because they mated with normal females, but eggs obtained were infertile. The masculinization factor in this case was considered to be anti-Müllerian hormone. It is possible that alterations of sexual development induced by hormonal manipulations that feminize or masculinize birds are only partially sex-reversed due to the underlying direct genetic effects (41). Another possibility is that cell-autonomous sexual development set during embryonic stages can be overridden after hatching by hormonal signaling. Estrogen, for example, appears to be required to maintain female features posthatching, and its loss at posthatching stages allows activation of male genetic pathways. According to these models, avian sexual differentiation is a combination of both direct genetic and hormonal effects (41, 45).

The direct genetic effects on sexual development in chickens and other birds may stem from the lack of global Z chromosome dosage compensation (49, 50, 75). Levels

of Z-linked gene expression are, on average, 1.5-fold to 2-fold higher in males (ZZ) compared with females (ZW) (34, 76–78). This applies broadly across tissues and developmental stages. As such, Z-dosage inequality could drive the direct cell-autonomous effects on sexual differentiation. The female-specific W chromosome is another potential source of sexually dimorphic gene expression. However, in the chicken, the 26 or so W-linked genes essentially all have homologs on the Z and are involved in basic cellular processes (79, 80). These genes are likely to be dosage sensitive and have not been lost during W chromosome evolution. The Z chromosome is the more likely source of differential gene expression driving direct genetic effects upon avian sexual differentiation.

An alternative explanation for these observations in the context of cell-autonomous sexual development is that some aspect for sex hormone function is sex-linked. Hence, ZW cells may respond differently to testosterone or estrogen than ZZ cells. ZZ cells may have a greater sensitivity to testosterone, for example, and this could produce a bird that has male-like features on one side (spur and wattle) but less developed female-like features on the other. A logical candidate would be androgen receptor, though this gene is autosomal in chicken (located on chromosome 4). Alternatively, another component of androgen signaling could be sex-linked, such as downstream signaling molecules, cofactors, or androgen targets genes. Interestingly, Lillie (81) in 1931 tried to explain the incidence of gynandromorphic birds by linking the phenomenon to different sensitivity of tissues to estrogen. Feathers on different parts of the body, for example, have different threshold responses to estrogen, and this is linked to growth rates of feather primordia [reviewed in Taber (21)]. Differential growth leading to difference in hormone sensitivity could be influenced by Z-linked genes known to influence growth, such as GH receptor (*GHR*), patched 1 (*PTCH1*), Lim homeobox 1 (*ISL1*), and follistatin (*FST*), among others (82). The sex chromosome complement may have some effect upon growth or some other aspect of the tissue competence to respond to gonadal sex steroids.

Gynandromorphs are naturally occurring, but potentially very informative with respect to the relative roles of genes and hormones in regulating sexual differentiation, in the gonads and in other tissues. With the advent of modern sequencing technologies, future studies should focus on transcriptome analyses of different tissues on the left and right sides of the body. Detailed analysis of hormone-dependent pathways, for example, may be instructive. It would be of value to study the expression of androgen receptor and related hormonal pathways in the left vs right wattle, for example, to determine if a sex-linked component plays a role in gynandromorph development, as discussed above. This could potentially reconcile the observations that hormones can sex-reverse most facets of avian sexual differentiation, whereas the gynandromorphs overtly seem not to support this hypothesis.

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