Sex Reversal in Birds

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Key Words
Avian · Chicken gonad · Sex determination · Sex reversal

Abstract
Sexual differentiation in birds is controlled genetically as in mammals, although the sex chromosomes are different. Males have a ZZ sex chromosome constitution, while females are ZW. Gene(s) on the sex chromosomes must initiate gonadal sex differentiation during embryonic life, inducing paired testes in ZZ individuals and unilateral ovaries in ZW individuals. The traditional view of avian sexual differentiation aligns with that expounded for other vertebrates; upon sexual differentiation, the gonads secrete sex steroid hormones that masculinise or feminise the rest of the body. However, recent studies on naturally occurring or experimentally induced avian sex reversal suggest a significant role for direct genetic factors, in addition to sex hormones, in regulating sexual differentiation of the soma in birds. This review will provide an overview of sex determination in birds and both naturally and experimentally induced sex reversal, with emphasis on the key role of oestrogen. We then consider how recent studies on sex reversal and gynandromorphic birds (half male:half female) are shaping our understanding of sexual differentiation in avians and in vertebrates more broadly. Current evidence shows that sexual differentiation in birds is a mix of direct genetic and hormonal mechanisms. Perturbation of either of these components may lead to sex reversal.

Birds show some of the most striking sexually dimorphisms seen in vertebrates. Males frequently have gaudy plumage and can exhibit elaborate courtship dances, while females are often more cryptically coloured. Sexual dimorphisms in anatomy, physiology, and behaviour all stem from the process of sex determination, the earliest genetic event during embryonic life that sets the sex of an individual. While many studies, primarily in mammals, refer to sex determination as differentiation of the embryonic gonads into ovaries or testes, sex is actually determined at fertilization by the inheritance of sex chromosomes. Gonadal sex differentiation is one of many manifestations of sexual differentiation, often referred to as primary sex differentiation. The traditional view of sexual differentiation is that hormones secreted from the gonads direct sexual differentiation of the rest of the body. However, the appearance of sexual dimorphisms that pre-date gonadal sex differentiation, in birds and in other
vertebrates, point to a role for direct genetic factors in the process of sexual differentiation [reviewed in Arnold et al., 2013]. In this review, we will firstly summarise our current understanding of avian sex determination and gonadal sex differentiation, which occurs during embryogenesis. We then provide an overview of sex reversal in birds, both natural and experimentally induced, with emphasis on the key role of oestrogens. Finally, we will summarise the most recent observations pointing to direct genetic contributions to avian sexual development in addition to established hormonal mechanisms. In light of these observations, the term ‘sex reversal’ becomes blurred. ‘Sex reversal’ has traditionally been used to describe individuals showing gonadal and extra-gonadal sexual phenotypes that are opposite to their sex chromosome constitution. In this review, ‘sex reversal’ will be used to describe birds in which the gonads or other sexually dimorphic features are discordant with genetic sex. Increasingly, it is being recognised that sex reversal is not a clear cut phenomenon but can reflect a partial decoupling of sexual phenotypes at various levels (chromosomal sex, gonadal sex, brain sex, plumage, or other sexually dimorphic aspects of anatomy).

**Avian Sex Determination and Gonadal Sex Differentiation**

As in mammals, sex in birds is strictly genetic, governed by the sex chromosome constitution established at fertilization. However, the sex chromosomes of birds are unrelated to those of mammals. Birds have a ZZ male:ZW female sex chromosome system, and a homologue to the mammalian testis-determining gene, *SRY*, is lacking. Currently, the exact mechanism of avian sex determination has yet to be definitively resolved. Sex may be controlled by a dominant acting female (or ovary) determinant carried on the W sex chromosome, or it may depend upon Z chromosome dosage (2 for male, 1 for female), or a combination of both mechanisms. However, in light of the absence of global Z chromosome inactivation in birds [Ellegren et al., 2007; Arnold et al., 2013; Uebbing et al., 2013] the widely accepted mechanism controlling avian sex determination is Z gene dosage [Chue and Smith, 2011; Ayers et al., 2013a]. Under this mechanism, the Z to autosome ratio is important, whereby males (ZZ) have a higher dose of male-determining factors compared to females (ZW). These factors may operate in the gonads and in extra-gonadal sites, such as the brain. Such a mechanism may involve one master Z-linked gene expressed in all tissues, or, more likely in avians, different combinations of Z-linked factors in different tissues that are not dosage compensated or are only partly compensated [Clinton et al., 2012].

Although sex is determined at fertilization, genes involved in sexual differentiation become active later in development. The vast bulk of research on vertebrate sex determination has previously focused on gonadal sex differentiation, that is, the morphogenesis of testes or ovaries during embryonic life. However, evidence from bird embryos (and other vertebrates) now suggest that genes governing sexual differentiation may also become active in other tissues, such as the brain, in some cases prior to gonadal sex differentiation [Agate et al., 2003; Nef et al., 2005; Lin et al., 2010; Zhang et al., 2010; Ayers et al., 2013b]. For example, sexually dimorphic gene expression exists in chicken blastoderms, early embryos, and in the early brain, prior to formation of the genital ridge, the undifferentiated or so-called bipotential gonad [Zhang et al., 2010; Ayers et al., 2013b]. This indicates that sexual differentiation may at least partly depend upon ‘sexual differentiation genes’ active in various parts of the embryo and not just in the gonads [Arnold and Itoh, 2011]. These genes may be different to those expressed in the gonads. Hence, sexual differentiation may depend upon both direct genetic (cell autonomous) and hormonal mechanisms. The direct effects of gene expression on sexual differentiation, independent of hormones, has been termed cell autonomous sex identity (CASI) and will be discussed further below [Zhao et al., 2010; Clinton et al., 2012].

The chicken embryo has been a widely studied model of avian gonadal sex differentiation [reviewed in Chue and Smith, 2011; Guioli et al., 2014]. The embryonic gonads develop on the ventromedial surface of the embryonic kidneys (mesonephros). In both sexes, the undifferentiated gonads initially comprise an outer layer of coelomic epithelium and underlying cords of somatic cells, the medulla. Primordial germ cells, having migrated from the extraembryonic germinal crescent via the bloodstream, are scattered primarily in the medulla. Gonadal sex differentiation in the chicken is first visible histologically from day 6.0, equivalent to Hamilton and Hamburger (HH) stage 29 [Hamburger and Hamilton, 1951, 1992]. Differentiation commences at approximately the same stage in both sexes. In males (ZZ), the cells of the medullary cords condense and coalesce into Sertoli cells, the first cell type to emerge in the testis, while the outer epithelial layer progressively flattens. Germ cells held in the cords subsequently undergo mitotic arrest. The outer ep-
ithelial layer does not hyper-proliferate. In the female (ZW), the outer cortical layer of the left gonad thickens and accumulates germs cells, which later enter meiotic arrest, while the inner medulla develops characteristic lacunae or fluid-filled cavities [Smith et al., 2008; de Melo Bernardo et al., 2015]. The right gonad of the female grows somewhat but fails to elaborate a thickened cortex, and germ cells do not enter meiosis, consistent with a loss of local Raldh2 enzyme expression (which synthesises the meiosis inducer, retinoic acid). While some modest asymmetry is initially also observed in males, marked asymmetry of gonadal sex differentiation is diagnostic of female chicken embryos and in most, but not all, other avians that have been examined [Guioli et al., 2014] (fig. 1).

Although the right female gonad does not become a functional ovary and ultimately regresses, it nevertheless expresses key female marker genes during embryogenesis [Andrews et al., 1997; reviewed in Lambeth and Smith, 2012], indicating that the ovary-determining pathway is initially engaged in the right as well as the left ZW gonad. In females, both the left and right medulla synthesise oestrogen from the onset of gonadal sex differentiation, and oestrogen is required for proper ovarian differentiation in birds, having a paracrine or autocrine role in the developing ovary [Scheib, 1983]. Hence, disturbances in oestrogen synthesis or action can induce quite robust sex reversal in birds [Vaillant et al., 2001; Ayers et al., 2013c]. In females only, the cortically-located germ cells undergo meiotic arrest from day 13, with the expression of the Stra8 meiotic initiator and Symp3 [Smith et al., 2008].

As in other vertebrates, the reproductive ducts of birds arise from 2 paired embryonic structures, the Wolffian and Müllerian ducts, and the fate of these ducts is influenced by hormones [Gasc and Stumpf, 1981]. Both ducts are initially present in both sexes. In the chicken embryo, the paired Müllerian ducts regress in male (ZZ) embryos from around day 9 of incubation under the influence of gonadal anti-Müllerian hormone (AMH), a glycoprotein hormone secreted by developing Sertoli cells. In contrast, the Wolffian ducts develop into male reproductive structures, the vas deferens and epididymis, under the influence of gonadal androgens. In females (ZW), the right Müllerian duct also regresses in line with atrophy of the right gonad [Romanoff, 1960]. This is consistent with the fact that female gonads also synthesise AMH, although at lower levels than in males. The left female Müllerian duct develops into a functional oviduct, serving the left ovary. During embryonic life, the left female duct is thought to be protected from AMH by the action of local oestrogens [Tran and Josso, 1977; Hutson et al., 1982]. In females, due to a lack of gonadal androgens, the Wolffian ducts regress along with the mesonephric kidney [reviewed in Lambeth and Smith, 2012].

With respect to gonadal sex differentiation, the best candidate ‘sex determinant’ under the Z-dosage hypothesis is DMRT1, a gene that encodes a zinc-finger like transcription factor. This gene is present in 2 doses in male birds (ZZ) and in one dose in females (ZW). It is posited that DMRT1 controls the direction of gonadal sex differentiation, with 2 doses leading to testis development and 1 dose being compatible with ovarian development in birds [reviewed in Chue and Smith, 2011; Graves, 2014]. Homologues of DMRT1 with male-associated functions have been reported in other vertebrates, from fishes to mammals [reviewed in Matson and Zarkower, 2012]. In the fly, Drosophila melanogaster, and the worm, Caenorhabditis elegans, DMRT1-related genes (doublesex and mab-3, respectively) also have male-associated functions [reviewed in Zarkower, 2001]. In the chicken embryo, the most widely used avian model system, DMRT1 is expressed exclusively in the urogenital system (gonads, germ cells, and Müllerian ducts), and always more highly

**Fig. 1. A** Schematic of gonadal sex differentiation in the chicken embryo showing the likely involvement of key sexual differentiation genes, reproduced from Schmid et al. [2015] with permission from S. Karger AG, Basel. Genes implicated in avian sexual differentiation outcomes are shown with male-expressed genes in blue, female-expressed genes in pink, and circle sizes representative of relative expression levels. Functional validation has confirmed the involvement of DMRT1, HEMGN, and Aromatase in the chicken sexual differentiation pathways. DMRT1 is expressed at higher abundance in the male and is central to the initiation of the testis development pathway, including AMH, HEMGN, Prostaglandin D2 (PDG2) and SOX9. DMRT1 is present at lower levels in the female with MHM (male hyper-methylated) potentially antagonising the effects of DMRT1 in female (ZW) gonads. R-SPO1 and WNT4 lead to stabilized β-catenin and active Wnt signalling, which are important for female gonad development in mammals. This pathway is expected to be analogous in chicken sex determination but has yet to be proven experimentally. GATA4 is present in both sexes and may be activating DMRT1. The undifferentiated gonad consists of a medulla (blue), which in the male proliferates and differentiates to form the seminiferous cords, and the outer epithelial layer (pink), which in the female proliferates to form the cortex of the ovary. Germ cells are represented by black dots/circles. **B** DMRT1 mRNA expression in embryonic chicken gonads, showing the gonads (G), mesonephric kidney (Ms), and Müllerian duct (Md).

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Genes involved in chicken gonadal sex differentiation

DMRT1 mRNA expression in embryonic chicken gonads

Female (ZW)  Male (ZZ)
in male (ZZ) gonads (fig. 1B) [Smith et al., 1999, 2003]. In the gonads, this gene is expressed in the medullary cords, which in males give rise to the critical Sertoli cell lineage. Knockdown of gonadal \( DMRT1 \) expression in early male chicken embryos results in feminisation of the gonads, disruption of male markers, such as SOX9, and ectopic activation of female markers, such as CYP19A1 (P450 Aromatase) and FOXL2 [Smith et al., 2009a]. Conversely, overexpression of \( DMRT1 \) in female gonads (ZW) results in activation of testis pathway proteins, such as SOX9 and AMH [Lambeth et al., 2014]. Taken together, these observations suggest that gonadal sex differentiation in the chicken is regulated by \( DMRT1 \) gene dosage, which antagonizes ovary-promoting genes (fig. 1). An initial 2-fold \( DMRT1 \) expression difference between male (ZZ) and female (ZW) gonads appears to be amplified during chicken gonadal development, presumably due to a positive feedback loop [reviewed in Lambeth et al., 2014].

The \( Z \) and \( W \) sex chromosomes are highly heteromorphic in the chicken and in many other birds [Nanda et al., 2008; Zhou et al., 2014]. The chicken \( Z \) chromosome has some 1,000 genes, most of which are unrelated to sex, but there is a bias of sex-related genes on the \( Z \) [Bel-lott et al., 2010]. The chicken \( W \) chromosome is a degraded version of the \( Z \), with few bona fide genes, probably around 25–45 [Ayers et al., 2013b; Moghadam et al., 2012; Smeds et al., 2015]. It is noteworthy that \( DMRT1 \) is also \( Z \)-linked in all other birds, including the ratites (flightless emu, ostrich, etc.), suggesting that it might be a universal sex determinant among avians [Shetty et al., 2002]. In the emu, \textit{Dromaius novaehollandiae}, the \( Z \) and \( W \) are homomorphic and pair over most of their length during female meiosis, exchanging genetic material. Significantly, however, there is a terminal chromosomal region in the emu (and ostrich) that does not pair and this harbours \( DMRT1 \). Through this mechanism, \( DMRT1 \) is retained as \( Z \)-linked, and the dosage mechanism of \( DMRT1 \) is thereby preserved even among ratites. Zhou et al. [2014] identified various evolutionary strata on the avian \( Z \) sex chromosome. \( DMRT1 \) is located on what is probably the most ancient evolutionary strata shared amongst all major lineages, supporting the notion that it is a universal testis determinant in birds [Chue and Smith, 2011; Zhou et al., 2014]. Recently, another \( Z \)-linked gene, \textit{HEMGN}, has been implicated in testicular morphogenesis in the chicken [Nakata et al., 2013] (fig. 1A). This gene encodes a nuclear protein, Hemogen, which is involved in haematopoiesis in mammals (but not gonadal sex differentiation). It is expressed in male but not female embryonic chicken gonads, and forced overexpression in ZW females can induce upregulation of male marker genes, including \( DMRT1 \) [Nakata et al., 2013]. It is possible that \textit{HEMGN} lies upstream of \( DMRT1 \) in chicken gonadal sex differentiation. However, if this is the case, then another sex-linked molecular mechanism is required to trigger its expression in male but not female gonads. This mechanism could involve a female determinant carried on the \( W \) sex chromosome. Under this scenario, the so-called dominant \( W \) hypothesis for avian sex determination would apply.

According to the dominant \( W \) hypothesis, the \( W \) chromosome would carry a female-determining gene (fig. 1A), analogous (but not homologous) to the mammalian \( SRY \) gene [Smith, 2007]. The avian \( W \) chromosome has until very recently been poorly characterised. Advances in chicken transcriptomics and whole chromosome assembly in chicken and other avians have revealed a largely heterochromatic \( W \) that harbours perhaps 25–46 genes in the region that does not recombine with the \( Z \) and is hence \( W \)-specific [Chen et al., 2012; Ayers et al., 2013b; Smeds et al., 2015]. Most of these are highly homologous to copies on the \( Z \) (gametologues) with no obvious link to sex [Ayers et al., 2013b; Smeds et al., 2015]. These \( W \) genes may be sensitive to dosage and hence are retained as functional partners of their \( Z \) gametologues. However, one gene, \textit{HINTW}, is somewhat divergent from its \( Z \) homologue. It is present in multiple copies on the avian \( W \), it appears to have undergone positive selection over evolution, and it has been presented as a possible female (or ovary) determinant [Smith, 2007]. The \( Z \)-linked copy, \textit{HINTZ}, encodes a histidine triad nucleotide binding protein, an enzyme that can hydrolyse AMP from lysine residues. The \( W \)-linked copy, \textit{HINTW}, lacks the key catalytic domain of \textit{HINTZ} and could act as a dominant negative in avian sex determination, blocking a possible testis-function of \textit{HINTZ}. However, mis-expression of \textit{HINTW} does not perturb male development in chicken embryos [Smith et al., 2009b]. Another gene on the chicken \( W \) that is absent from the \( Z \) is \textit{FET1} (female-expressed transcript 1), a retroviral-related sequence that is expressed in early ZW embryonic chicken gonads. However, its expression is asymmetric, while key ovary genes such as aromatase are bilaterally expressed, undermining a role in bird ovarian differentiation. In summary, a convincing female or ovary determinant has yet to emerge from the avian \( W \) sex chromosome [Smith, 2007].
Sexual differentiation of the soma beyond the gonads in birds has long been considered to conform to the usual vertebrate paradigm. That is, androgens and oestrogens synthesized by the embryonic and post-hatching gonads masculinise or feminise the brain and certain other tissues, respectively (fig. 2). As in other vertebrates, the embryonic gonads synthesis and secrete gonadal sex steroids according to the genetic sex [Tanabe et al., 1979, 1983; reviewed in Clinton and Haines, 2001]. These hormones influence the development of sexually dimorphic traits. In the chicken, these traits include enlarged spurs, wattles, and comb in males, compared to smaller features in females. Masculinisation of the comb and wattle in males is attributed to testosterone [Zeller, 1971; Shanbhag and Sharp, 1996; Yoshioka et al., 2010; Lambeth et al., 2016a]. The mechanism regulating leg spur development seems to vary among species or even among strains [Lambeth et al., 2016a]. In one strain of chickens, removal of the left ovary after hatching (ovariectomy) induces the small female spur to enlarge and lengthen so that it resembles that in a male, while in another strain, it does not [Valdez et al., 2010]. Differences in body mass between the sexes may be under direct genetic control, as body mass conforms to genetic sex even when sex hormone levels are experimentally manipulated, at least in chicken [Valdez et al., 2010; Lambeth et al., 2016a].

In chicken and in other birds, the sexes also show sexually dimorphic feathering, with males often displaying more colourful or elaborate feather patterning. Sexually dimorphic feathering is generally attributed to sex steroid hormones. In the Galliformes (chickens, turkeys, etc.) female feathering is induced by oestrogens, and male plumage is the default pattern in the absence of significant oestrogen (fig. 2). For example, female peafowls develop the long and colourful tail plumage of males after ovariectomy and hence loss of oestrogen [reviewed in Owens and Short, 1995]. In a line of bantam chicken with a genetic mutation leading to constitutively active aromatase enzyme activity and hence ectopic oestrogen synthesis, both sexes show female-type (‘heny’) feathering [George and Wilson, 1980; Matsumine et al., 1991]. In seasonally breeding passerines (perching birds) testosterone is important for male plumage [Lindsay et al., 2011]. Sex reversal of the plumage can therefore occur when sex steroid levels are disturbed, through gonadal disease, for example. Sex steroid hormones also play well established roles in sexual differentiation of the avian brain, although

**Avian Sexual Differentiation beyond the Gonads: Role of Hormones and Direct Genetic Effects**

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Sex Dev 2016:10:288–300
DOI: 10.1159/000448365

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**Fig. 2.** Sexual dimorphisms in the adult chicken, reproduced from Mayer et al. [2004] with permission from Elsevier. Photographs of a male (A) and female (B) adult chickens demonstrating sexual dimorphisms (head ornaments, spurs, breast musculature, and plumage). Distinct differences can be seen in tail feathering (C) between the male (left) and the female (right).
there is evidence for direct genetic control of the song centre, in at least some birds [Arnold, 1996; Agate et al., 2003].

Gonadal Hormone Synthesis and Sex Reversal in Birds

Most instances of sex reversal reported in birds are related to alterations in sex steroid hormone action, most notably oestrogen, which plays a central role in ovarian differentiation (fig. 1A). In the chicken embryo, oestrogen synthesis by the gonad is female-specific. Expression of the rate-limiting enzyme in oestrogen synthesis, P450 aromatase, occurs only in female gonads at the onset of embryonic gonadal sex differentiation (day 6.0; HH 29) (fig. 1A) [Andrews et al., 1997]. Oestrogen-receptor alpha is expressed in the outer cortex, and to a lesser extent in the underlying medulla, mediating the effects of local oestrogen production. Injection of aromatase blockers such as fadrozole into chicken eggs prior to the onset of gonadal sex differentiation induces development of testes in ZW embryos [Elbrecht and Smith, 1992; Wartenberg et al., 1992; Smith et al., 2003]. In most studies, these birds can develop as phenotypic males, with male sexual dimorphisms that in some cases persist beyond hatching, characterised by well-developed testes, spurs, comb, and wattle [Elbrecht and Smith, 1992; Burke and Henry, 1999; Vaillant et al., 2001, 2003]. In some cases, gonads can revert to ovotestis or ovarian structures, which may reflect different degrees of aromatase suppression in different instances [reviewed in Vaillant et al., 2001]. The ZW male chickens produced by aromatase enzyme inhibition at embryonic stages can exhibit spermatogenesis as adults [Elbrecht and Smith, 1992]. Experiments with induced sex reversal and generation of chimeric animals containing ZZ and ZW germline cells both suggest that when ZW cells are present in a testis, meiosis I proceeds normally and ultimately Z-bearing sperm are derived that can function to produce offspring, while the W, however, is compromised in its ability to give rise to functional spermatozoa and thus far has only been shown capable of fertilizing an oocyte through assisted reproductive technologies [Kagami et al., 1995; Shimada et al., 2007; Takagi et al., 2007]. Conversely, oestrogens injected into eggs prior to gonadal sex differentiation can feminise male gonads, characterized by a thickened gonadal cortex and fragmented medulla [Scheib, 1983]. However, this effect is transient, and ZZ birds revert to a male gonadal pheno-
type after hatching, suggesting that the presence of 2 Z chromosomes might ultimately override the feminizing effects of oestrogen.

While a central role for oestrogen in avian gonadal sex differentiation and sex reversal is well established, a decisive role for testosterone in testis formation has not been defined for birds. Indeed, in chicken embryos, the developing female gonad is reported to express a higher level of androgen receptor than its male counterpart, where it may have a role [Katoh et al., 2006], while testosterone is virtually undetectable in embryos of either sex at embryonic stages [Tanabe et al., 1986]. Interestingly, the regressive right gonad of the female bird has an innate tendency to form a testicular-like structure. During masculinisation due to aromatase inhibition, it is always the right gonad that becomes more testicular in appearance. Furthermore, if the left ovary is removed or becomes dysfunctional due to diseases, the right gonad can differentiate into a testis [Groenendijk-Huijbers, 1965, 1967]. It is hypothesized that oestrogen activity suppresses development of the right gonad, and its loss following removal of the ovary allows the right gonad to become masculinised. Loss of oestrogen through a diseased ovary may explain rare instances of sudden female-to-male sex reversal among adult birds [Owens and Short, 1995].

Another role for hormones in influencing avian gonadal sex differentiation had been revealed through grafting experiments. Embryonic ZW gonads can be masculinised when a day 13 embryonic testes is grafted onto the extraembryonic membranes, whereby the host gonads develop as testes [Maraud et al., 1990]. Given the dubious role of testosterone in avian embryonic gonadal sex differentiation, the masculinising hormone is thought to be AMH. This provokes the idea that AMH may have a central role in avian testis development, and hence sexual differentiation. However most recently, we knocked down AMH gene expression in embryonic chicken gonads using RNA interference and found no effects upon gonadal development, apart from growth reduction in both sexes [Lambeth et al., 2015]. This suggests that AMH is not required for testis development in birds, in keeping with the data for eutherian mammals. Meanwhile, we have detected partial sex reversal (partial feminisation) of embryonic male chicken gonads following RNAi-mediated knockdown of gonadal DMRT1 expression. The left gonad shows a female-type histology, although the right can be either undeveloped or testicular. These effects involved ectopic activation of aromatase in the feminised male gonads, again pointing to the importance of oestrogen synthesis for ovary development. DMRT1 may nor-
mally antagonise aromatase expression, probably via the transcription factor FOXL2 (fig. 1A) [Hudson et al., 2005; Pannetier et al., 2006; Wang et al., 2007].

**Sex Chromosome Aneuploidy and Avian Sex Reversal**

The sexual phenotype of sex chromosome aneuploids would readily address the question of Z dosage versus a dominant W underlying avian sex determination. 2A:ZZW birds with a female phenotype and 2A:Z0 birds with a male phenotype would support the dominant W hypothesis, while if such birds showed male and female phenotypes, respectively, then the Z dosage model would be favoured. Unfortunately, such sex chromosome aneuploids appear to be very rare in birds and indeed may be embryo-lethal [Graves, 2003; Forstmeier and Ellegren, 2010]. However, triploid ‘intersex’ birds have been described (3A:ZZZ and 3A:ZZW) [Tiersch et al., 1991]. Thorne and Sheldon [1993] described a line of triploid chickens that showed aberrant sexual development. Despite being triploid, these birds survived and could be analysed during embryonic development, hatching, and sexual maturity. Adult 3A:ZZZ birds were relatively normal males (although spermiogenesis was impaired), while 3A:ZZW were described as sterile intersexes [Thorne et al., 1991] that exhibited unilateral ovotestes at hatching. The right gonad was testicular, consistent with previous observations that the right gonad of both sexes has an innate testicular predisposition, while the left had a thickened outer ovarian-type cortex together with seminiferous cords in the medulla. However, the ovarian compartment regressed over time [Lin et al., 1995].

The triploid chicken data suggest that 2 Z chromosomes are associated with testis cord formation in the gonadal medulla, and a W chromosome is associated with ovarian cortex formation, and that the former can override the latter. A presumed lack of oestrogen synthesis by the medullary compartment may have contributed to the failure to maintain an ovarian type cortex, although the birds were phenotypically female at hatching, which indicates either enough oestrogen present for somatic feminisation or direct genetic effects of the sex chromosomes upon non-gonadal tissues with some degree of cell autonomous sexual differentiation (see below). However, interpretation is complicated by the fact that the birds were triploid: the autosomes were present in 3 copies along with the sex chromosomes. Hence 3A:ZZZ birds had a Z:A ratio of 1, as in 2A:ZZ birds, while 2A:ZW birds have a ratio of 0.5, and 3A:ZZW birds have a ratio of 0.66. The data are consistent with the possibility that chicken sex depends not on the absolute dosage of Z sex chromosomes but upon the Z:autosome ratio, analogous to the pattern in *Drosophila*, in which the X:autosome ratio is key. Other apparent ZZW female birds have been described in passerines (perching birds). Arit et al. [2004] described an apparent 2A:ZZW female great reed warbler (*Acrocephalus arundinaceus*), breeding in a natural population, based on the inheritance of Z-linked microsatellites. More recently, also using Z-linked inheritance, Kupper et al. [2012] reported an apparent triploid ZZW Kentish plover that had female plumage and reproduced as a female. However, in these cases of ZZW passerines, the gonads were not assessed, sex chromosome mosaicism could not be excluded, and definitive karyotypes are lacking. Nevertheless, these data do leave open the possibility of a female-determining gene on the avian W sex chromosome. If so, then a gene or genes on the avian W sex chromosome may antagonize a Z-linked factor(s).
Avian Gynandromorphs and the Concept of Cell Autonomous Sex Identity

The phenomenon of gynandromorphy has been reported in birds and provides further insight as gynandromorphic birds are bilateral sex chimeras, male on one side of the body and female on the other (fig. 3). Gynandromorphic chicken and zebra finches have been described [Agate et al., 2003; Zhao et al., 2010], and 3 gynandromorphic chickens have been studied in some detail. The cells on the male side of the body were largely ZZ and cells on the female side were largely ZW. The gonads reflected the sex chromosome composition. Thus, testes were present when most cells were ZZ and ovaries when most cells were ZW [Zhao et al., 2010]. How can gynandromorphs develop in the context of the hormonal paradigm of sexual differentiation? One possibility is that the response of cells to sex steroid hormones may depend upon the sex chromosome constitution (ZW or ZZ). For example, ZZ cells may respond more strongly to serum testosterone levels than ZW cells, generating male structures on one side and female on the other in the case of the ZZ:ZW gynandromorphs. It is interesting in this context that there is a preponderance of genes related to sex and re-
production on the avian Z sex chromosome (as expected due to the evolution of sexually antagonistic alleles), and there is a lack of global dosage compensation, hence setting up an inequality between ZZ and ZW cells [Graves, 2014]. Which genes involved in avian sex hormone function are sex-linked? Sex steroid receptors are autosomal in birds, not sex-linked, although some other aspects of hormonal signalling might be sex-linked. The more likely explanation underlying the phenotype of gynandro-morphic birds is that the sexual identity is at least partly cell autonomous, involving direct genetic factors independent of hormonal signalling [Clinton et al., 2012]. Hence, tissues on the male and female sides develop sexually dimorphic features based on genotype – Z dosage or a dominant W chromosome effect. The lack of a global dosage compensation system in birds might mean that these direct genetic effects are mediated by Z-linked gene dosage or Z:autosome ratio. This idea is supported not only by the occurrence of gynandro-morphic birds but by the observation that sexual dimorphisms have been reported in avians well before gonadal sex differentiation and hence pre-dating gonadal sex hormone synthesis. Differences in gene expression between the sexes have been reported in early avian embryos, for example see Schmid et al. [2015].

CASI is also supported by our recent studies showing that male (ZZ) chicken embryos, chronically overexpressing aromatase and elevated serum oestrogen levels, nevertheless develop male-type sexual dimorphisms as adults [Lambeth et al., 2016a]. Meanwhile, chronic overexpression of AMH at embryo stages in chicken can block gonadal sex differentiation and sex steroid synthesis, yielding adult birds that have a mixture of male and female traits [Lambeth et al., 2016b]. In these birds, body weight conformed to genetic sex, for example, despite a blockage in gonadal sex differentiation and sex steroid hormone synthesis [Lambeth et al., 2016b]. These most recent studies support the proposal that sex in birds may be at least partially cell autonomous [Clinton et al., 2012]. Hence, DMRT1 dosage may underlie the direction of gonadal sex differentiation in embryos, while differentiation of other sexually dimorphic structures may involve other Z-linked genes as DMRT1 is not expressed outside the gonads. However, how can this cell autonomy data be reconciled with the fact that a blocking oestrogen action in chicken embryo leads to robust masculinisation of birds? It has been noted that such hormonally sex-reversed birds are not totally masculine, with comb, wattle, and other features that are not fully male, or that sex reversal is often temporary, thus pointing to a role for direct genetic factors [Clinton et al., 2012]. Our own recent data on experimentally sex-reversed gonads (by blocking AMH or chronically expressing aromatase genetically) support this view. Figure 4 summarises the role of direct genetic versus hormonal signalling in avian sex determination and sexual differentiation.

**Summary**

There is an evolutionary trend in the incidence of sex reversal among vertebrates, as defined by a discordance between chromosomal sex and somatic sex. Sex reversal and sex change are widely seen in teleost fishes. Sex change among coral reef fishes is well documented and can be mediated by social cues, such as the death of an alpha male or female. In general, fish are very susceptible to sex reversal triggered by gonadal sex steroid hormones, namely oestrogen or androgens [see Baroiller et al., 2016 in this issue]. Amphibians show a similar plasticity, significantly influenced by both oestrogens and androgens [Nakamura, 2013]. In reptiles, both genotypic and temperature-dependent sex determination have been reported. In many lizard species with defined sex chromosomes and genotypic sex determination, sex reversal can occur when an embryo is exposed to altered egg incubation temperatures [see Georges et al., 2016 in this issue]. In these species, the differentiation of the gonads is discordant with the sexual genotype (which can be XX:XY or ZZ:ZW). Such plasticity has become restricted among the higher vertebrates, birds and mammals. Temperature of the egg does not influence gonadal sex differentiation in birds, while oestrogen but not testosterone has a central role in avian gonadal development. Hence, natural loss of oestrogen through left ovarian disease, or experimental loss through unilateral left gonadectomy, allows the right gonad to become a testis and birds acquire male sexual characteristics. Among therian mammals, plasticity is further restricted. The effects of sex steroid hormones are not as important at embryonic stages, presumably due to the evolution of the placenta, which potentially bathes the embryo in maternal sex steroids. Interestingly, in marsupials, gonadal sex differentiation can be manipulated by oestrogen, and sexual differentiation normally begins after birth in the pouch, separate from the placenta.

Research on avian sexual differentiation has been especially useful recently in helping redefine the concept of sex determination. Evidence from birds, and increasingly from other animals, suggests a key role for direct genetic effects upon sexual differentiation of tissues, in the go-
nads, and elsewhere in the body [Clinton et al., 2012; Arnold et al., 2013]. Hence, several pathways may be operational, involving both genetic and hormonal mechanisms in various tissues. This challenges the old dogma that focused purely upon the sex hormone-secreting gonads as the only source of sexual dimorphisms among animals. In birds and in other vertebrates, natural and experimentally induced sex reversal continues to shed light on the complex interplay of genetics and hormones in shaping sexual differentiation.

**References**


**Disclosure Statement**

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
Sex Reversal in Birds

Sex Dev 2016:10:288–300
DOI: 10.1159/000448365


