

# Sex and death in birds: A model of dosage compensation that predicts lethality of sex chromosome aneuploids

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**Abstract.** Birds show female heterogamety, with ZZ males and ZW females. It is still not clear whether the W is female-determining, or whether two doses of the Z chromosomes are male-determining, or both. This question could easily be settled by the sexual phenotypes of ZZW and ZO birds, in the same way that the sexual phenotypes of XXY and XO showed that the Y is male determining in humans, but that the dosage of an X-borne gene determines sex in *Drosophila*. However, despite extensive searches, no ZZW or ZO diploid birds have been satisfactorily documented, so we must assume that these genotypes are embryonic lethals. Given that ZW and ZZ are viable

and the W contains few genes it is not clear why this should be so. Here I propose that sex chromosome aneuploids are lethal in chicken because, to achieve dosage compensation, a locus on the W chromosome controls the upregulation of genes on the Z in ZW females. ZO birds would therefore have only half the normal dose of Z-linked gene product and ZZW would have twice the amount, both of which would undoubtedly be incompatible with life. Reports of other aneuploids and triploids are also consistent with this hypothesis.

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## Bird sex chromosomes

Unlike mammals, sex in birds is determined by a ZZ male/ZW female sex chromosome system. The chicken Z chromosome is a relatively large chromosome, comprising about 7% of the haploid genome and therefore containing some thousands of genes. The W, on the other hand, is small and heterochromatic, containing few genes (four so far). The Z chromosome of other birds, even the distantly related ratites, shares at least several genes with the chicken Z (Nanda et al., 1998; Ogawa et al., 1998) and is completely homologous by chromosome painting (Shetty et al., 1999), although the W chromosome differs greatly in size, morphology and gene content between species (Solari, 1993; Ogawa et al., 1998; de Kloet, 2001; Shetty et al.,

2003). Thus the bird ZW pair shows striking parallels to the mammalian XY pair.

Like the mammal XY and the snake ZW sex chromosome pairs, bird sex chromosomes clearly evolved from a homologous pair (Fridolfsson et al., 1998), as originally proposed for snakes by Ohno (1967). This hypothesis is supported by the observation that all the genes that have so far been isolated on the W have homologs on the Z chromosome, from which they clearly evolved (Hori et al., 2000; O'Neill et al., 2000; Itoh et al., 2001). Comparative gene mapping (Graves and Shetty, 2000; Nanda et al., 2000) shows that the gene contents of the bird ZW pair and the mammal XY pair do not overlap, implying that the two systems evolved independently from different autosome pairs. The ground state, in which the ZW chromosomes were undifferentiated, is represented by the turtle chromosome 5, which was demonstrated by chromosome painting to be completely homologous to the chicken Z (Graves and Shetty, 2001), but which remains an autosome pair in these species that have temperature sex determination.

Two questions are immediately apparent. How does the Z and W chromosome constitution determine sex in the bird embryo? And how do males and females cope with the two-fold dosage difference of all the thousands of genes on the chicken Z chromosome?

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## Bird sex determination

Two possible ways in which the Z and W chromosome constitution could determine sex are by a female-determining gene(s) on the W, or by the differential dosage of gene(s) on the Z.

A dominant female-determining gene on the W chromosome would be analogous to the male-dominant testis determining gene SRY on the mammalian Y. There is no male-specific SRY homolog in chickens (or, indeed, in any vertebrate other than therian mammals; Griffiths, 1991; Graves, 2002). The search for a female-determining gene on the W has been frustrating. Whereas it was possible (though not easy) to positionally clone the SRY gene in mammals using males with small parts of the Y chromosome (Sinclair et al., 1990), these rare clinical cases are not available in birds. Four genes have been isolated from the W chromosome using strategies including exon trapping from BACs and various subtractive methods (Dvorak et al., 1992; Ellegren, 1996; Hori et al., 2000; O'Neill et al., 2000; Itoh et al., 2001), but the presence of similar homologs on the Z, as well as their expression patterns render most of these unconvincing candidates for a female determining role. However, one of these genes (*ASW/PKCI*) remains of interest and others may yet be discovered, so the existence of a female-determining gene on the W chromosome obviously cannot be ruled out.

A dosage system, in which two copies of a gene on the Z chromosome determines male differentiation in ZZ embryos, and a single copy female differentiation of ZW embryos, is formally analogous to the system in *Drosophila*, in which sex is determined by dosage of a gene on the X, rather than the presence or absence of the Y (Cline and Meyer, 1996). A candidate dosage-sensitive sex determining gene was discovered in 1998. *DMRT1* was first isolated from a region of human chromosome 9, deletions of which were associated with male to female sex reversal (Raymond et al., 1998). It was subsequently found to be present on the Z and not the W in chickens (Nanda et al., 2000). Two doses of this gene could be critical to testis determination in chickens as well as humans. Such a role for *DMRT1* is compatible with the finding that the *DMRT1* gene is present on the Z chromosome but not the W in the emu (Shetty et al., 2003), although the emu W is otherwise highly homologous to the Z.

A repetitive region (MHM for male hypermethylated) on the chicken Z chromosome close to the *DMRT1* locus has recently been found to be undermethylated and transcribed on the single Z of ZW females, but highly methylated and repressed on both Z chromosomes of ZZ males (Teranashi et al., 2001). Undermethylation and expression of MHM appears to be under the control of a factor on the W chromosome rather than the number of Z chromosomes, since MHM is methylated and silent in ZZZ triploids, but undermethylated and expressed in ZZW triploids. The non-coding female-specific MHM transcript was found to accumulate at the MHM locus on the Z chromosome, suggesting that it represses the nearby *DMRT1* gene to reinforce the dosage difference that triggers female or male sex determination.

Distinguishing between the hypotheses of a female-determining gene on the W and a dosage-sensitive male determining

**Table 1.** Phenotypes of sex chromosome aneuploids predicted on the alternative hypotheses of a female dominant gene on the W chromosome, and a dosage-sensitive gene on the Z, two copies of which are required for male development

Sex chromosomes	Female dominant on W	Dosage sensitive on Z
ZZ	M	M
ZW	F	F
ZO	M	F
ZZW	F	M

gene on the Z would be straightforward if sex chromosome aneuploids were available in birds. The observation that in mammals XXY individuals are male with Klinefelters Syndrome (Jacobs and Strong, 1959) and XO are females with Turners Syndrome (Ford et al., 1959) implied that X dosage is irrelevant to sex and the Y chromosome bore a testis determining gene. SRY was subsequently positionally cloned and characterized (Sinclair et al., 1990). In *Drosophila*, on the other hand, the observation that XXY diploids are female and XO diploids are male immediately implied that dosage of X-borne genes determined sex. These genes have been cloned and characterized (Cline and Meyer, 1996). In birds, the W-borne female determinant hypothesis predicts that ZO would be male and ZZW female, whereas the Z-borne dosage sensitive gene hypothesis predicts that ZO would be female and ZZW male (Table 1).

Over decades, several intensive searches have been conducted for chickens with aberrant sex chromosome constitutions. No ZO chickens have ever been reported. The single report in 1933 of a ZZW chicken is considered dubious because of the poor resolution of cytogenetic techniques of the time (Clinton, 1999). A very extensive search of about 12,000 parrots produced five birds with aberrant bands at Z and W-linked loci that were interpreted as recombination events between the X and Y chromosomes (Halverson and Dvorak, 1993). Two of these birds had intersexual phenotypes.

In the absence of ZZW and ZO diploid birds, studies of triploid birds have provided tantalizing clues. Triploids are relatively common in some selected lines (Thorne et al., 1993). Of the three possible sex chromosomes constitutions, ZZZ is male, as expected. ZZW triploid birds are more interesting, being hatched as females, but taking on male characteristics on maturation, with masculinization of the gonads. This suggests that the W does have some feminizing effect, but that this is ultimately overpowered by the dosage of Z chromosomes. There is a report of a female ZWW chicken embryo with abnormal ovary development (Bonaminio and Fechheimer, 1993).

Why are there no ZZW or ZO diploid chickens among the many thousands that have been screened? It must be assumed that they are early embryonic lethals. This raises a conundrum: if ZZ and ZW embryos are viable, and the W contains virtually no genes, why should ZO and ZZW embryos die?

## Dosage compensation in birds?

The second question raised by the bird ZZ/ZW system in which the W contains few genes concerns the need for some form of dosage compensation to equalize the amount of product from genes on the Z. Many genes are not sensitive to dosage and may be present in one or two active copies without detrimental effect (as demonstrated by the true recessive alleles at many loci on sex chromosomes and autosomes in mammals and birds). However, the Z is a large chromosome, containing thousands of genes, and it would be remarkable if none of them were dosage-sensitive. An extra copy of most human autosomes is lethal early in embryonic or fetal life.

The question of dosage compensation in birds was initially approached by comparing the amount of a Z-linked enzyme in males and females of several birds. A 2:1 ratio of aconitase between males and females implied that this locus is not dosage compensated (Baverstock, 1982). An absence of Z chromosome inactivation was implied by the observation that the two Z chromosomes replicate synchronously (Schmid et al., 1989), and there is no sex chromatin.

However, the question of dosage compensation in birds has been re-examined recently using real-time quantitative RT-PCR to compare the amounts of mRNA from nine Z-borne loci in male and female chickens. Six of these loci, including the gene coding for aconitase (now named *IREBP*), showed dosage equivalence in the two sexes, implying that most Z-borne loci are dosage compensated (McQueen et al., 2001). Only one gene showed a significant sex difference, and, intriguingly, this gene (*ScII*) has an ortholog in *Caenorhabditis elegans* that is involved in dosage compensation. As expected on the hypothesis that *DMRT1* dosage differences are involved in sexual differentiation in birds, the *DMRT1* gene also appears to escape dosage compensation, being expressed in the genital ridge at twice the level in ZZ male embryos as in ZW female embryos (Raymond et al., 1998).

This dosage compensation does not seem to be accomplished by inactivation of one Z chromosome, as might be expected by analogy to mammalian X chromosome inactivation, since RNA FISH for five Z-borne genes showed expression from both Z chromosomes (Kuroda et al., 2001). This implies either that the single Z of females is upregulated to match the ZZ dosage in males (parallel to the *Drosophila* system of upregulating the single X in XY flies), or that the two Z chromosomes in male are downregulated to match the single dosage in ZW females (as in the downregulation of the two X chromosomes in XX *C. elegans* hermaphrodites) (Marin et al., 2000).

### A model for W-control of Z activity

Thus dosage compensation does occur in birds, but does not appear to involve Z inactivation. It is likely, therefore, that dosage compensation operates either by the upregulation of the single Z in females or the downregulation of both Z chromosomes in males.

How is the bird dosage compensation system regulated? The repetitive sequence (MHM) on the Z chromosome that is undermethylated and transcribed in ZW females, but hypermethylated and repressed in ZZ males (Teranishi et al., 2001), is suspiciously similar to the locus that controls X chromosome inactivation in mammals. XIST is regulated by DNA methylation, and transcribed into an untranslatable RNA that does not leave the nucleus, but accumulates on the X. Likewise, dosage compensation in *Drosophila* depends on two redundant X-borne genes *roX1* and *roX2* that produce non-coding RNAs that associate with the X (Marin et al., 2000). MHM transcript may therefore be involved in dosage compensation, as well as having a role in controlling *DMRT1* activity and thence sex determination.

Does the W chromosome influence Z chromosome activity in female birds? In mammals, the presence or absence of the Y chromosome is irrelevant for inactivation, since one X is inactivated in XX females and XXY Klinefelter males. The Y is also irrelevant for dosage compensation in *Drosophila*, since the single X is upregulated in XO as well as XY flies.

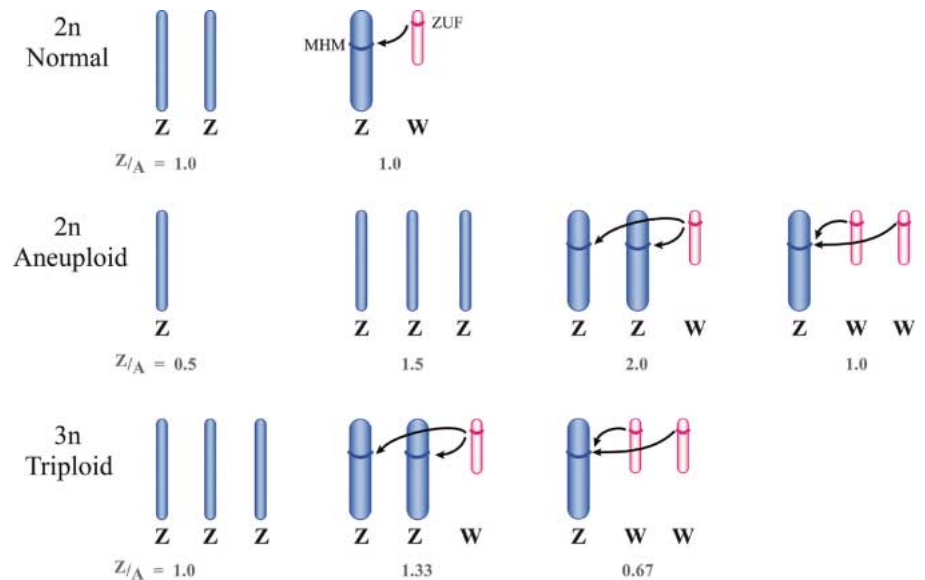
However, the presence in chickens of the MHM locus on the Z whose methylation depends on the presence of the W (Teranishi et al., 2001), suggests that the W chromosome may play a part in upregulating Z chromosome activity. Methylation of the MHM sequence is evidently under the control of a factor on the W chromosome, called F by the authors but here named *ZUF* for Z Upregulating Factor. If MHM is involved in dosage compensation, this W-borne factor could control upregulation of the single Z in ZW birds.

This model for dosage compensation in birds in which *ZUF* on the W chromosome is responsible for two-fold upregulation of genes on the Z chromosome, predicts that the Z/autosome ratio will be 1.00 in both sexes. In ZZ males, there is no upregulation, so the dosage of Z-borne genes is 2Z, and the Z/autosome ratio is 1.00 (Fig. 1; Table 2). In ZW females, the *ZUF* locus on the W upregulates the single Z by a factor of 2, producing a dosage of 2Z, and again a Z/autosome ratio of 1.00.

### Lethality of sex chromosome aneuploids

The involvement of the W in dosage regulation of the Z could also explain why ZO and ZZW diploids are embryo lethals (Fig. 1; Table 2). In ZO embryos, the absence of the W means that there is no upregulation of the single Z, producing a dosage of 1Z and a Z/autosome ratio of 0.5, which would be lethal. In ZZW embryos, the *ZUF* locus on the W upregulates both of the Z chromosomes, producing a dosage of  $2 \times 2Z = 4Z$ , and a Z/autosome ratio of 2.00, which is also lethal.

Other possible sex chromosome aneuploidies, including ZZZ and ZWW diploids, have not been so avidly sought in birds because they are not diagnostic for sex determination. ZZZ diploid birds (whose abnormal karyotype should be obvious) have never been reported, so the ZZZ state is presumably also lethal. The hypothesis readily accounts for this. Lacking a *ZUF* locus, ZZZ would be expected to have a dosage of 3Z and a Z/autosome ratio of 1.50, which is likely to be lethal. The question of ZWW birds is less certain. The hypothesis pre-



**Fig. 1.** Dosage compensation in normal and aneuploid chickens. The Z chromosome is upregulated twofold (indicated by width) by a factor *ZUF* on the W (arrows). Overall dosage of Z-linked product is calculated by the number of Z chromosome multiplied by their 1× or 2× regulation. The Z/autosome ratio is calculated by dividing the Z dosage by the number of chromosome sets.

**Table 2.** Dosage of Z-borne gene product and phenotype in aneuploid chicken

Ploidy	Sex chromosomes	<i>ZUF</i>	Z dosage	Z/A ratio	Phenotype
Normal					
2A	ZZ	–	$1 \times 2Z = 2$	1.00	M
2A	ZW	+	$2 \times 1Z = 2$	1.00	F
Aneuploid					
2A	ZO	–	$1 \times 1Z = 1$	0.5	M (dead)
2A	ZZZ	–	$1 \times 3Z = 3$	1.50	M (dead)
2A	ZZW	+	$2 \times 2Z = 4$	2.00	F (dead)
2A	ZWW	+	$2 \times 1Z = 2$	1.00	F
Triploid					
3A	ZZZ	–	$1 \times 3Z = 3$	1.00	M
3A	ZZW	+	$2 \times 2Z = 4$	1.33	F/M
3A	ZWW	+	$2 \times 1Z = 2$	0.67	F (dead)

dicts that ZWW would be viable with an upregulated Z and a Z/autosome ratio of 1.00, as long as the two *ZUF* loci on the two W chromosomes do not have additive effects on Z upregulation. There are few reports of this karyotype (Clinton, 1999), but ZWW birds might have been missed if they are phenotypically normal females, because the extra W is not easily distinguishable from the numerous small chromosomes of the chicken karyotype.

If, as seems likely, the dosage-regulated testis determining factor (*DMRT1*?) on the Z chromosome escapes upregulation, the sexual phenotypes of ZO birds would be (dead) female, ZZZ and ZZW (dead) male and ZWW (viable) female.

Triploid birds can also be accounted for on this hypothesis. ZZZ triploids lack a W chromosome and have no *ZUF* locus, therefore the three Z chromosomes are not upregulated, the dosage is three-fold, and the Z/autosome ratio is 1.00 (viable male, as observed). ZZW triploid birds, possessing a W chromosome with a *ZUF* locus, would upregulate both Z chromo-

somes, producing  $2 \times 2Z$  (4-fold dosage), and a Z/autosome ratio of 1.33, which is higher than the normal 1.0 but not as high as for ZZZ (1.5) or ZZW (2.0). Conversely, ZWW triploids, having two W chromosome and two *ZUF* loci, would upregulate the single Z, producing a dosage of  $2 \times Z$  and a Z/autosome ratio of 0.67, which is presumably lethal (chromosome deficiency always causing more extreme disruption than an extra copy).

I propose, therefore, that the tolerance for aberrant dosage of Z-borne genes is rather narrow. A normal Z/autosome ratio is always 1.00. Birds with a Z/autosome ratio below 1.00 never survive. Birds with a Z/autosome ratio of 1.33 occasionally survive, but those with 1.50 or 2.00 cannot.

## Conclusion and dire prediction

The hypothesis that a locus (*ZUF*) on the W chromosome induces twofold upregulation of the Z chromosome provides a simple explanation for the absence of ZZW and ZO diploid birds. It predicts that these genotypes will never survive, terminally frustrating all attempts to determine their sexual phenotypes.

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