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Factors causing sex differences in birds

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Abstract

In recent years, increasing evidence suggests that sex differences in the phenotype of all tissues is influenced by the inequality of effects of sex chromosome genes in the two sexes. In birds, genes on the Z chromosome are not well dosage compensated, so that most Z genes are expressed higher in ZZ male cells than in ZW female cells. The sex difference in expression of Z and W genes is likely to cause sex differences within cells, in addition to the sex differences caused by different levels of testicular and ovarian hormones. The sexual imbalance in cell physiology has implications for aviculture and novel developments in the poultry industry.

Keywords

sex difference; sex determination; Z chromosome; W chromosome; gonads

The conceptual framework for understanding sex differences in birds and mammals has undergone a dramatic shift in the last 15 years. Sex differences in any tissue are caused by factors that are present in one sex but absent or reduced in the other. The goal, therefore, is to identify the factors that cause sex differences in each relevant tissue, and understand the downstream pathways that are influenced by the sex-biased signals. A primary question is whether the sex-biased signals originate from within the female or male cell, or come from outside the cell. For example, the sex chromosomes inherently produce different levels of gene products in all male and female cells, which act within the cell. Sex-biased signals also act on the cell from the outside, for example hormonal signals coming from the ovaries or testes. As is often the case, the conceptual framework for approaching the problem of sexual differentiation has been developed primarily based on studies of mammals, which led to a dominant theory that was tested in a much more limited fashion in birds. Nevertheless, studies in birds have been crucial in shaping the conceptual framework.

The 20th Century Model: Hormonally Induced Sexual Differentiation

The 20th century model began its development by the earliest years of the century. By 1916, Lillie (Lillie, 1916) found that presumed genetically female freemartin calves were masculinized by hormonal secretions coming from their genetically male twins. He suggested that hormones from the testes normally masculinize sexually dimorphic tissues in males, and that they artificially masculinize those tissues in females exposed to the male hormones during fetal life. The experimental work of Jost (1947)(Jost et al., 1973) substantiated that view, but also underscored the asymmetry of hormone actions. Secretions of the testes were the primary hormones causing one sex to diverge developmentally from the other, and ovaries were thought not to be required for differentiation of the feminine structures. Jost gonadectomized fetal rabbits and found that in the absence of gonads, both

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sexes developed a female-like phenotype, at least early in development. Phoenix et al. (Phoenix et al., 1959) first tested Jost's conclusions in the brain (using behavior as a marker for brain sexual phenotype), and convincingly demonstrated that testosterone treatment of fetal guinea pigs caused them to be permanently more masculine in behavior. Subsequent studies on behavior, and on neural structures that have different structures in males and females, showed again that the permanent divergence of male and female development was caused by testicular secretions in males (Arnold & Gorski, 1984). During the same period (by 1959), the Y chromosome of mammals was found to be required for differentiation of testes (Ford et al., 1959; Jacobs & Strong, 1959; Welshons & Russell, 1959) indicating that one or more Y genes initiates a cascade causing commitment of the gonadal ridge to a testicular fate in males. The critical gene, *Sry*, was discovered by the early 1990s (Goodfellow & Lovell-Badge, 1993). Thus, the 20th Century model stated that the sex of the gonads was determined by genes acting within the gonadal primoridum (in mammals, the presence or absence of *Sry*), but that all subsequent sexual differentiation was caused by sex differences in the level of gonadal hormones. An major exception to this theory was first found in marsupials, where significant sex differences in phenotype develop prior to gonadal differentiation (Renfree & Short, 1988).

An important issue is the timing and nature of gonadal hormone effects that cause sexual differentiation. In some cases sexual differentiation is caused by hormonal actions during early stages of development, when the hormones can have a permanent effect, often called an "organizational" effect (Phoenix et al., 1959; Arnold, 2009b; Arnold, 2009b). At various times of life, however, sex differences are caused acutely and reversibly by the different effects of testicular and ovarian secretions. These are often called "activational" effects, and such sex differences require the ongoing divergent actions of the two types of secretions (Arnold, 2009b).

Research on birds confirmed the potent roles of gonadal hormones to cause sexual differentiation. For example, Wolff and Wolff (Wolff, 1959; Wolff, 1951) removed the gonads of chick and duck embryos, and found that in the absence of gonadal hormones, the Müllerian ducts persisted in both sexes, but that a masculine syrinx and phallus would develop in both sexes. (These studies mirrored those of Jost in rabbits; (Jost, 1947). Thus, it was concluded that the testes normally cause sexual differentiation of Müllerian derivatives by inhibiting Müllerian development in males. In this regard the bird and mammalian pattern were deemed to be similar. However, differentiation of the masculine form of the syrinx and phallus of ducks was thought to be inhibited by ovarian secretions, so that the factors causing non-gonadal sexual differentiation were ovarian hormones. Thus, by the 1960s, sexual differentiation of some tissues was thought to be caused by permanent effects of gonadal hormones in one sex, but it was testicular secretions that appeared to cause the sexes to diverge in mammals, and ovarian secretions in birds.

Later extensive studies of sexual differentiation of brain and behavior confirmed an important, if not dominant, role for gonadal hormones in sexual differentiation. For example, increases or decreases in levels of estrogens in quail alter the sexual differentiation of copulatory behavior. Estrogens inhibit normal masculine development in males and females (reviewed by (Balthazart & Adkins-Regan, 2002; Balthazart et al., 2009).. The results again suggested that ovarian estrogens play an important role to suppress the development of the brain's capacity to produce masculine patterns of behavior.

The Passerine song circuit as a model sexually dimorphic system

In Passerine birds, males sing a courtship song that is usually much more elaborate than female song. In some species, such as the zebra finch, only males sing. The telencephalic

brain regions controlling song are 5–6 times larger in volume in males than females, and virtually every cellular parameter is sexually dimorphic (Nottebohm & Arnold, 1976; Arnold & Schlinger, 1993) This dramatic sexual dimorphism offers advantages for studying the factors that cause sexual differentiation.

Initial experiments on zebra finches in the 1980s and 1990s tested the dominant hormonal model, and manipulated the levels of estrogens and androgens using treatments of hatchlings or embryos with hormonal agonists or antagonists (Gurney & Konishi, 1980; Wade & Arnold, 2004; Arnold, 1997). Estrogen treatment of females caused dramatic masculinization, and such females sang, unlike normal females. This result suggested that sexual differentiation of the song system was similar to that of mammalian brain regions, in that a sex steroid (estradiol), a metabolite of testosterone, presumed to be secreted from the testes of males, cause a masculine pattern of brain development. The masculinization caused by estradiol, however, was incomplete, and in some traits, the estradiol-treated females were only half as masculine as untreated males. Eventually, various attempts to deprive males of estrogens or androgens failed to cause sex reversal of the song system. Males treated with drugs that block sex hormone action were fully masculine in the song circuit and behavior (Arnold & Schlinger, 1993). Masculine development in males therefore appeared not to be sensitive to gonadal hormones.

In our own lab, a turning point came based on experiments in which testes were induced to develop in genetic females (Wade & Arnold, 1996). Because ovarian development requires the action of estrogens in birds, treatment of female embryos with inhibitors of aromatase (estrogen synthase), prior to ovarian differentiation, causes the differentiation of a testis on the right side, and usually an ovotestis on the left. Genetic females treated with the aromatase inhibitor, which had considerable amounts of testicular tissue that were endocrinologically active, nevertheless were nearly completely feminine in their neural song circuit, and they did not sing (Springer & Wade, 1997). That result suggested to us that sexual differentiation might be primarily not controlled by gonadal secretions. The primary alternative to hormonal sexual differentiation was brain autonomous, direct effects of sex chromosome genes acting within brain cells themselves (Arnold, 1996).

The study of a rare gynandromorphic zebra finch advanced the conclusion that sexual differentiation may be primarily controlled by the sex chromosome constitution of brain cells, rather than the gonadal hormonal milieu of the brain (Agate et al., 2003). Lateral avian gynandromorphs, long a target of interest by investigators of sexual differentiation (Witschi, 1939), possess masculine traits on one side of the body, and feminine traits on the other side. The lateralization of sexual phenotype is striking, especially when the plumage is sexually dimorphic, and often the dividing line between the male plumage and female plumage is sharp and at the midline. Until the advent of molecular reagents to genotype the two sides, the sex chromosomal constitution was not understood. Various possibilities included ZZ/ZO, or ZO/ZW, or ZZ/ZW. Even in the absence of such information, however, investigators in the early 20th century concluded that sex differences in phenotype were not always controlled by gonadal hormones in these animals, but were controlled by “intercellular” factors (Witschi, 1939; Allen et al., 1938). Even though a testis was often found on the male side, and an ovary on the female side, the levels of circulating gonadal hormones on the two sides were likely to be the same, so that sexual differentiation seemed not to be caused by gonadal hormones (Burns, 1961). We were fortunate to be able to study a gynandromorphic zebra finch for the first time with newly developed molecular probes (Agate et al., 2003). The study established that W genes were represented more in the genome on the phenotypically female half (thus excluding the ZZ/ZO genotype). Expression of W genes (normally constitutively expressed in females) was dramatically higher on the female side of the brain, with a sharp dividing line of W gene expression at the midline, similar to the

boundary seen in the plumage. However, a small population of cells expressing W genes was found on the male side, indicating that the division of genetically male and female cells was not absolute and that each side likely contained cells of the other genetic sex. Furthermore, Z genes that are normally constitutively expressed in both sexes were expressed higher throughout the male side relative to the female side, suggesting a ZZ/ZW genotype rather than ZO/ZW. Analysis of DNA content established that the cells were diploid, excluding other possibilities such as ZZZ/ZZW. In the brain, the sexual phenotype of the song circuit of the gynandromorphic finch was more masculine on the genetically male side of the bird than on the female side. Thus, the sexual phenotype of brain cells was correlated with the genetic sex of brain cells under conditions when the levels of gonadal hormones could be assumed to be equivalent on the two sides of the brain. That finding suggested that the imbalance of expression of Z or W genes, within brain cells themselves, was a primary determinant of the sexual phenotype of brain cells. Nevertheless, the song circuit on the female side of the brain was considerably more masculine than that of normal females, so the presence of male tissue (e.g., in the testes or in the brain itself) caused some masculinization of presumed genetically female cells on the female side.

Analysis of gynandromorphic chickens, using improved molecular reagents (e.g., BAC clones for interphase DNA fluorescent in situ hybridization) to establish the number of Z and W chromosomes on a cell-by-cell basis, shows that gynandromorphs are mixtures of ZZ and ZW cells (Zhao et al., 2010), and that ZZ and ZW cells are both present on each side. On the side with ZZ cells predominating, the phenotype of various tissues (muscle, bone, leg, plumage, etc.) is more masculine than on the side on which ZW cells predominate. Thus, the sexual phenotype of many different types of cells correlates with their sex chromosome complement of cells, suggesting wide-spread influence of sex chromosome complement on sex-biased phenotype. Although studies of gynandromorphs and other naturally-occurring intersex birds yield critically important information on the factors that could cause sex differences, these studies nevertheless are correlational and do not directly manipulate the putative factors to examine their sex-specific effects on phenotype.

Studies involving intersexual transplantation of tissue are therefore a significant advance, because they directly manipulate the sex of cells placed into a male or female cellular and hormonal milieu. Using chicks expressing a GFP transgene to label the sex of the transplanted tissues, mesodermal cells harvested before gonadal differentiation were transplanted into embryos prior to gonadal differentiation (Zhao et al., 2010). In general, transplanted cells that adopt a somatic cell fate in the gonads later show differentiated gene expression patterns that were typical of the sex of the donor tissue, not the host gonad, confirming that the genetic sex of somatic cells of the gonads plays a significant role in determining sexual phenotype

This research in birds has impacted research on differences between XX and XY cells in eutherian mammals. Our own research in zebra finches motivated us to test the “direct genetic” hypothesis extensively in mice, a genetically tractable species that allows one to manipulate the sex chromosome complement of cells that develop in either a testicular or ovarian environment. These studies show that in specific cases the sexual phenotype of mice differs in XX and XY mice irrespective of their gonadal sex. (De Vries et al., 2002; Arnold & Chen, 2009; Arnold, 2009a; Arnold, 2009b). The results indicate that the endogenous inequality of expression of X and Y genes, which characterizes every XX and XY cell, causes cells to be different. Thus, sexual phenotype is caused both by factors encoded on the sex chromosomes, and by factors impinging on the cells from the outside, for example by gonadal hormones.

A unified model of sexual differentiation

Whereas the 20th century model of sexual differentiation placed the gonads in a primary position in the causal hierarchy of factors causing sex differences in nongonadal tissues, a more accurate model starts emphatically with the sex chromosomes (Figure 1; (Arnold, 2009b). To our knowledge, all sex differences in phenotype are caused ultimately by the difference in sex chromosome complement in the zygote. On balance, averaging across individuals, all other factors are thought to be equal in the male and female zygote. The imbalance of expression of Z and W genes in birds causes ZZ and ZW cells to be different, just as the imbalance of expression of X and Y genes in mammals causes sexual differentiation. The Z and W genes act differentially in male and female tissue, both gonadal and non-gonadal tissues, to cause sex differences in phenotype. One of the most important events of sexual differentiation is the differentiation of the gonads, not only because of the primary position of the gameteproducing organs among tissues that have an important functional sex difference, but also because the differentiation of the gonads establishes the life-long sex difference in secretion of gonadal hormones, which have profound effects to cause sex differences in phenotype of various organs. Although some authors (Zhao et al., 2010) have suggested that direct genetic (cell autonomous) sex-biased effects of sex chromosome complement cause the majority of sex differences in somatic tissues in birds, that conclusion is premature and likely false. Certainly gonadal hormones have dramatic effects on sexual phenotype, as decades of research has demonstrated (Burns, 1961; Balthazart et al., 2009; Witschi, 1961). The relative importance of gonadal hormones and sex chromosome complement is actually difficult to gauge because few if any studies manipulate the two factors to weigh their relative effects.

Sex chromosome evolution

Heteromorphic sex chromosomes, such as XX/XY chromosomes in mammals and ZZ/ZW chromosomes of birds or snakes, are thought to evolve when a dominant gonad-determining mutation occurs on an autosome (Graves, 2006). The XY and ZW sex chromosomes evolved from different ancient autosomes. Although birds and snakes both possesses ZZ/ZW systems, the origin of sex chromosomes is different (Matsubara et al., 2006). This suggests that the occurrence of dominant gonad-determining mutation is random, and these mutations become the driving force of sex chromosome evolution regardless their locations. As reviewed previously (Graves & Shetty, 2001; Stiglec et al., 2007), the small W chromosome of carinate birds is the ancient autosomal partner of the Z chromosome, which has lost most genes once shared with the Z and accumulated repeat sequences. For example, the chicken W chromosome mainly consists of three repetitive sequences (EcoRI -, XhoI -, and SspI - repetitive sequences), and only 10Mb is the non-repetitive sequence which possibly contains genes (Mizuno et al., 2002).

The mammalian Y and avian W chromosomes, each of which is passed clonally in one sex from generation to generation, have accumulated more changes than the other sex chromosome common to both sexes, X and Z. Recently, comparative analysis of human X and chicken Z chromosomes demonstrated that these two different chromosomes followed convergent evolutionary pattern: a low gene density, acquisition/amplification of testis genes (Bellott et al., 2010). Thus, despite their opposite evolutionary history, X and Z share specific features that separate them from autosomes. Yet, the gene expression patterns of X and Z chromosome are quite different.

Ineffective sex chromosome dosage compensation and the male-biased expression of most Z genes

The imbalance in the number of copies of Z genes is thought to generate sex-specific selection pressures (Charlesworth, 1996). As genes were gradually lost from the evolving W chromosome, the former allelic partner Z genes would be present in the male (ZZ) at twice the genomic dose of the female (ZW). Because Z genes often function in similar gene networks in both sexes, the difference in genomic dose might mean that one sex has a non-optimal expressed dose of the gene. In several XX/XY systems, a chromosome-wide sex-specific dosage compensation mechanism has evolved, which adjusts the expressed dose of one sex to be closer to that of the other sex. For example in mammals, one X chromosome is transcriptionally silenced (inactivated) in XX cells so that cells in both sexes have one active X chromosome. The inactivation of the X chromosome does not abolish sex differences in the expression of X genes, but rather reduces sex differences in expression of genes so that it is not much greater than the sex differences found in autosomal genes (Itoh et al., 2007). To assess the effectiveness of X-inactivation globally, as well as the degree of sexual differentiation of gene expression, one can plot the distribution of sex differences (male to female ratios of expression) found in all genes in any tissue (Itoh et al., 2007). The distribution of sex differences in mammals shows that the majority of genes, in somatic tissues such as brain, adipose, or liver, have sex differences in expression near 1 (sexual equivalence), but that some genes show moderately greater expression in one of the two sexes (Figure 2) (Itoh et al., 2007). Very few genes have a level of expression that is two-fold or greater in one sex than the other. Genes on the X chromosome are not as a group expressed higher in females, but rather are expressed in M/F ratios that are quite comparable globally to M/F ratios of autosomal genes. Thus, X-inactivation is quite effective, not because it eliminates sex differences in X genes, but because it avoids strong female-bias in X gene expression and makes the distribution of sex differences imilar to that of autosomal genes.

In birds, however, the mechanisms of dosage compensation are ineffective. Z genes as a group are expressed higher in males than in females in various tissues (Figure 2; (Itoh et al., 2007)), in different species (chicken: (Itoh et al., 2007; Ellegren et al., 2007), zebra finch: (Itoh et al., 2007; Itoh et al., 2010), European crow (*Corvus corone*): Wolf & Bryk, 2011); common whitethroat (Naurin et al., 2011) and at various stages of development including long before the gonads have differentiated (Ellegren et al., 2007; Scholz et al., 2006; Zhao et al., 2010). The distributions of M/F ratios of expression indicate that the majority of Z genes are expressed higher in males than females, with a mean in the range 1.4 – 1.8 fold higher across the entire chromosome. This constitutive bias in Z gene expression suggests that male and female cells are likely sexually differentiated by inherent differences in the gene pathways that involve Z genes. Indeed, differentiation of the gonads may occur because of the inherent difference in expression of the Z gene *DMRT1*. Knock down of DMRT1 causes ovarian development in ZZ male chick embryos (Smith et al., 2009), although the influence of an upstream W gene cannot be ruled out. Because avian cells differ in their degree of dosage compensation from mammalian cells, it is likely that the role of Z genes as the originators of sexual dimorphisms in tissue function is greater than the role of X genes in mammals. Quantitatively, we predict greater numbers of sex differences caused by the imbalance of expression of Z genes within bird cells than by X genes within mammalian cells (Itoh et al., 2007).

Another perspective on this issue comes from considering the evolution of sex differences in physiology. Sex differences evolve when selection pressures on one sex differ from those on the other, when it is adaptive for specific cell types to be different in the two sexes. When

such selection pressures exist, a specific physiological process (gene network) evolves sensitivity to a sex-specific regulatory signal, such as a sex hormone or genes expressed in a sexually dimorphic manner, so that the physiological process differs in the two sexes. In avian cells, many more sex-biased signals are directly expressed from the sex chromosomes than in mammalian cells (Itoh et al., 2007; Arnold et al., 2008). Natural selection may find it easier to drive sex differences from Z signals than from X signals.

Specialized regions of the Z chromosome

Against the background of the male-biased expression of Z genes, the MHM (male hypermethylated) region of the chicken Z chromosomes stands out as an area of sexual equivalence and female-biased expression. The MHM non-coding RNA is expressed only in females, and repressed in males probably because the male's DNA is more strongly methylated than that of females (Teranishi et al., 2001). Acetylated lysine 16 of histone 4, a histone mark associated with higher gene expression, is concentrated in the region around MHM of females but not males (Bisoni et al., 2005; Itoh et al., 2010), giving rise to the hypothesis that MHM has evolved a function as a dosage compensation machinery that increases expression of genes near MHM in females to a level comparable to that of males. That hypothesis is supported by the observation that the MHM region is specialized, with more sexually equivalent expression of genes, relative to the rest of the Z chromosome (Melamed & Arnold, 2007; Melamed et al., 2009). The MHM RNA is non-coding, as are two other genes intimately involved in dosage compensation in other species, mammalian *Xist* and Drosophila *roX*. The MHM region, however, appears to be present only in galliform birds, not in Passerines such as the zebra finch (Itoh et al., 2010), indicating that if MHM is an incipient dosage compensation mechanism, that it has evolved only in one branch of the avian tree.

Summary and prospectus

The study of birds has helped catalyze a change in the conceptual framework for the understanding of sexual differentiation of all tissues. The theory of somatic sexual differentiation that emerged from the 20th century placed gonadal differentiation at the center of the universe, so that the main question focused on which gonadal hormones cause the diverse sex differences found in tissues, and which downstream mechanisms were activated by the hormones. That theory is no longer viable. A more appropriate starting point for the study of sexual differentiation is the endogenous inequality of the sex chromosomes themselves (Figure 1). All sex differences stem from these chromosomes, in all tissues, and some sex differences are not the result of the different effects of male and females gonadal hormones (Arnold, 2009b).

The realization that many or all avian cell types show higher expression of Z genes in males than in females, because of the inequality in number of Z chromosomes, has important ramifications for the poultry industry and for attempts to make chimeric birds or engineer avian traits. The inherent differences in the physiology of somatic ZZ and ZW may limit the ability of cells of one sex to show characteristics of the other sex (Zhao et al., 2010). Better study of the sex chromosomes will no doubt lead to an improved understanding of their role in specific gene networks that control traits that are desirable in poultry flocks. For example, the greater muscle mass of males than females is quite likely caused by the male-female differences in expression of sex chromosome genes within muscle cells themselves (Zhao et al., 2010), possibly due to the Z-linked *growth hormone receptor* (*GHR*) gene (Rubin et al., 2010). Thus, sex differences in effects of Z and W genes may be harnessed to improve commercially valuable traits in poultry flocks. Study of the chicken Z chromosome will no

doubt require better understanding of the fascinating MHM region, which is poorly sequenced and poorly understood at present.

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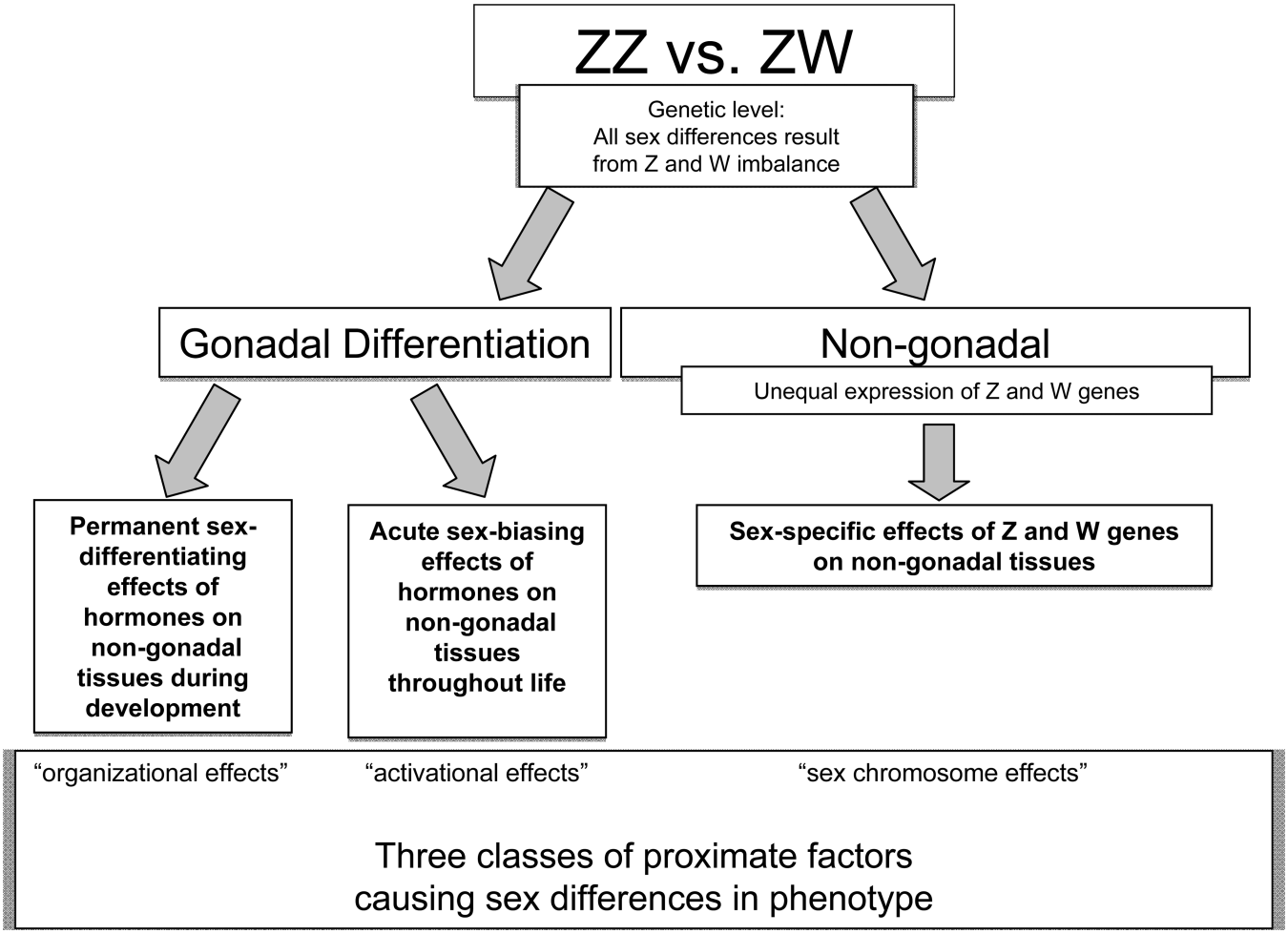

Figure 1.

Modern model of factors causing sex differences in phenotype. All sex differences begin, at the genetic level, with the inequality of expression of sex chromosome genes in every cell. In the gonads, the inequality causes lifelong sex differences in secretions of gonadal hormones, which act throughout the body to cause sex differences in phenotype. The imbalance of expression of Z and W genes also acts within many other (nongonadal) cells types to cause fundamental sex differences in cell development and physiology. Based on Arnold, 2009b.

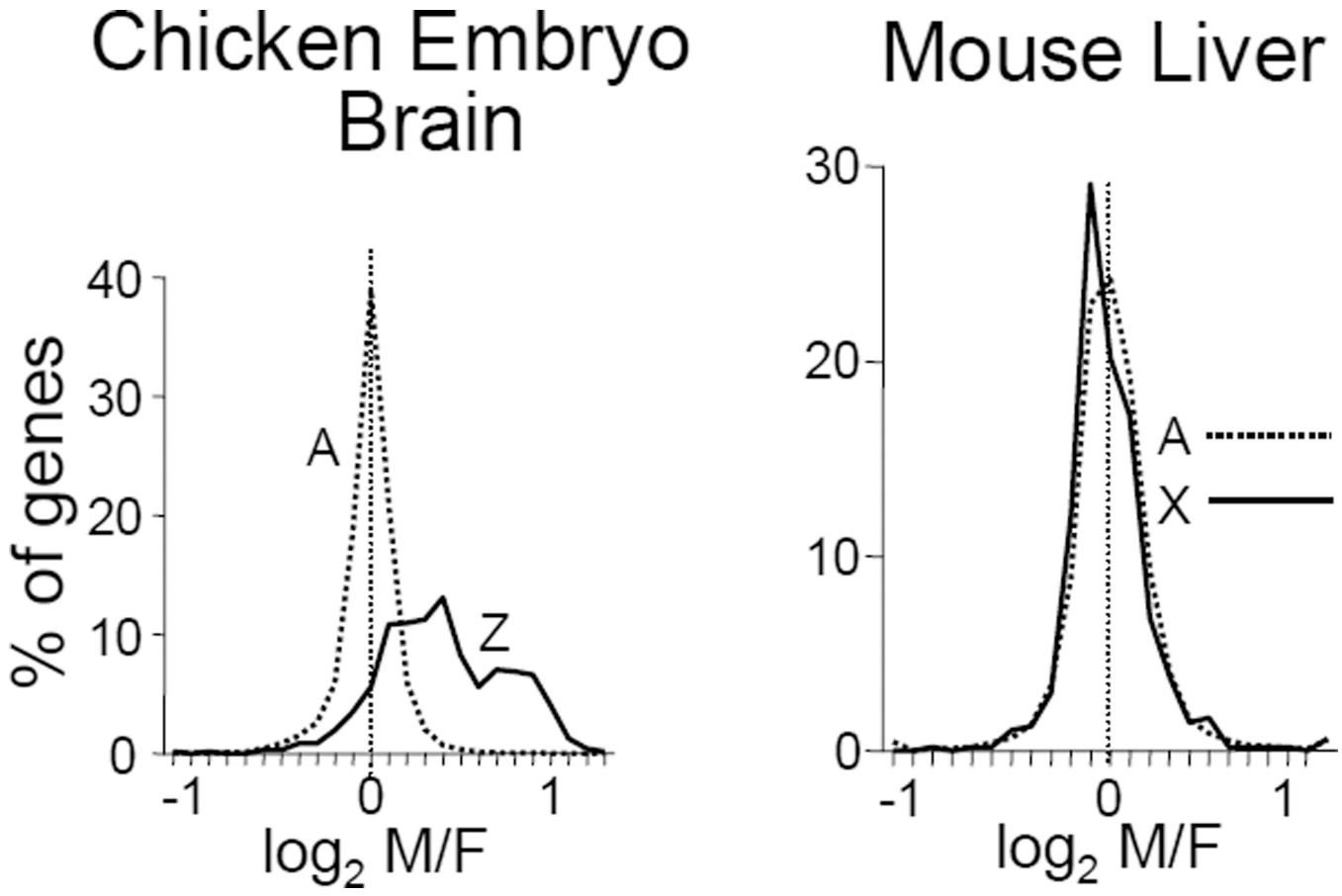

Figure 2.
Global sex differences in gene expression. Histograms show the distribution of sexual dimorphism (male to female ratios) in expression of genes based on microarray expression profiling. Autosomal (A) genes show modal sex ratios near 1 (log M/F = 1). In mammals, X inactivation effectively matches the distribution of sexual dimorphism of X genes to that of autosomal genes. In contrast in birds, Z genes are ineffectively dosage compensated so that the majority of Z genes are expressed higher in males (M/F ratios above 1). From Itoh et al., 2007.