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# Specific Features of Sex Determination in Birds on the Example of *Gallus gallus* domesticus

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*and Antonina Vladimirovna Trukhina*

## Abstract

The chapter is devoted to the consideration of sex determination in birds. The appearance of heteromorphic sex chromosomes (birds) has led to the emergence of some specific W chromosomal signal, which provides estrogen control of the development of a heterogametic sex. At present, two hypotheses about sex determination in birds compete. One of these hypotheses considers the number of Z chromosomes as a key sex-determining factor, while the other hypothesis supposes the presence in W chromosome of the key gene controlling ovarian development or suppressing the appearance of testes. Into the modern scheme of the genetic control of sex determination in birds (practically within the hypothesis of dose compensation), an epigenetic mechanism was added. The appearance of gonads in birds is most likely determined by sex hormones and to the greatest extent by estrogen under the control of W chromosome. It is desirable to pay attention to noncoding RNAs, their connection with the W chromosome and their role in bird sex determination.

Keywords: sex determination, sex hormones, sex chromosomes, sex-determining genes, bird, Gallus gallus domesticus

## 1. Introduction

Sex is characterized by a set of features that ensure sexual reproduction. We distinguish the primary definition of sex—the emergence of one of two types of gonads (organs somatic of nature), their sexual differentiation into final system, and the development of two types germ cells. In different groups of vertebrates, different mechanisms of sex determination are realized. We consider hypothetical schemes of such a process in birds using the example of *Gallus gallus domesticus* [1]. On the one hand, chicken is an important model object of fundamental genetics, especially embryogenetics [2], and on the other hand, it has significant practical importance for humans: 210 million tons of meat and 1482 billion eggs per year [2, 3]. Both males and females are fattened in broiler production. There is currently no economically worthwhile use of the male of egg breeds. Therefore, the 1-day cockerels are destroyed, and this applies to 330 million chickens annually in

the European Union alone [1, 4, 5]. This raises ethical issues, and understanding the principles of gender genetics, as well as gender selection algorithms in early embryos, is extremely important from a fundamental and applied point of view.

## 2. Sex determination in bird

In birds, females are heterogametic. Embryos with two Z chromosomes in birds develop as males, and those with ZW chromosomes develop as females (**Figure 1**).

At present, two hypotheses about sex determination in birds compete. One of these hypotheses considers the number of Z chromosomes as a key sex-determining factor, while the other hypothesis supposes the presence in W chromosome of the key gene controlling ovarian development or suppressing the appearance of testes. A decrease in the expression of doublesex and mab-3-related transcription factor 1 (DMRT1) protein in ovo as a result of RNA interference of this gene leads to the feminization of embryonic gonads in genetic males. In the feminized left gonad, a decrease in the expression of the SRY-box transcription factor 9 (SOX9) gene and an increase in the expression of the cytochrome P450 aromatase (CYP19A1) gene were observed. This observation confirms the hypothesis of the presence of dose compensation in the DMRT1 gene. In feminized right gonad, the expression of the corresponding genes is very different, indicating differential sensitivity to DMRT1 between the left and right gonads. Germ cells in feminized gonads are distributed as in the ovaries. All this indicates that the DMRT1 gene is necessary for the development of testes [6]. But there is no clear evidence that this gene controls the primary sex determination (the appearance of testis or ovary).
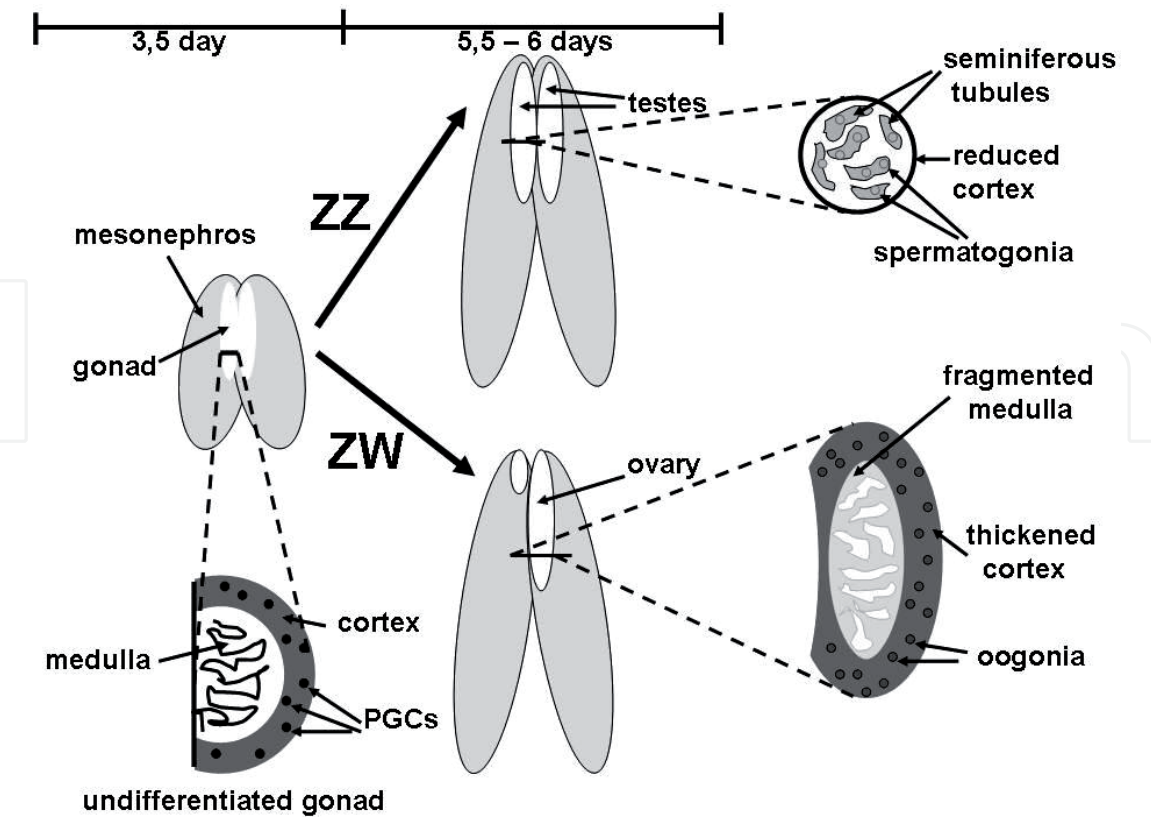


**Figure 1.**
*A hypothetical scheme of primary sex differentiation in Gallus gallus. Initially gonads are undifferentiated. They consist of an outer cortex and underlying medullary layer. Primordial germ cells (PGCs) are visible on 3.5 day and are located mainly in the cortex. On the 5.5–6th day (stage 28–30), bilateral testes appear in ZZ embryos, and in ZW embryos, the left gonad gives ovary and the right one regresses (adapted from [1]).*
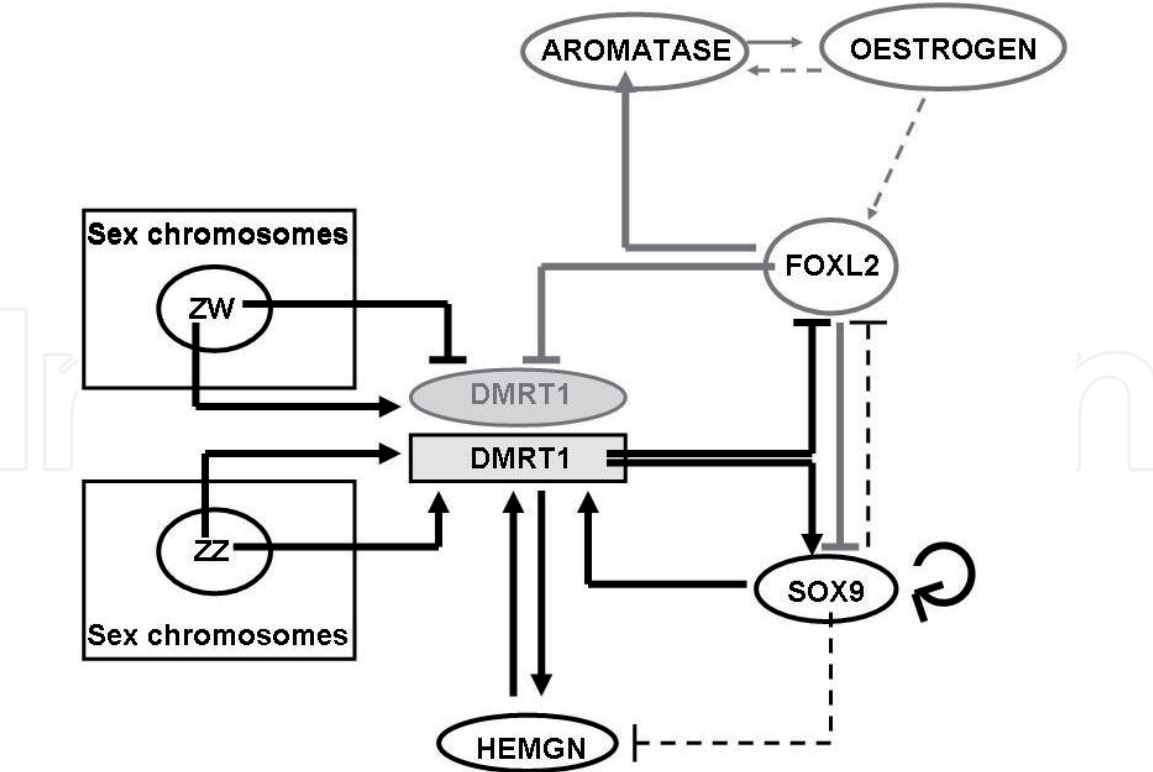
**Figure 2.**
*The simplified gene regulatory network controlling primary sex determination of chicken. Black (from male genes) and gray (from female genes) arrows represent positive (→) and negative (⊣) interactions, and dashed arrows indicate indirect or proposed interactions between Z and Z chromosomes and between Z and W chromosomes (adapted from [9]).*

**Figure 2** presents a hypothetical scheme of genetic control of primary sex differentiation in Gallus gallus. The gonad appears on the 3.5th day (stage 22) as thickening on the surface of mesonephros. It consists of the epithelial layer of somatic and germ cells and medullary cordate layer (epithelial cords), which is mixed with mesenchymal cells. On the 6.5th day (stage 30), the first genes that determine sex are activated.

Into the modern scheme of the genetic control of sex determination in birds (practically within the hypothesis of dose compensation), an epigenetic mechanism was added ([7, 8], **Figure 2**).

## 3. Sex reversal

Sex reversal is the phenomenon whereby organisms developing at sex-specific conditions hatch the opposite sex. This can be caused by factors acting as estrogen promoters or inhibitors, increasing or decreasing the number of female offspring, through controlling aromatase [10]. Synthetic aromatase inhibitors (an enzyme catalyzing the synthesis of estrogens) can cause steady inversion of sex from female to male. In this case, the left gonad becomes an ovotestis, or a testis, and the right gonad becomes a testis. In most experiments injection of aromatase inhibitors in ovo is carried out on the 3rd or 4th day of incubation [11, 12]. The proportion of individuals with sex inversion and two testes is increased with an earlier introduction of the inhibitor at the beginning of incubation [13]. However, females with inversed sex have got testes with an abnormal development and with an abundance of abnormal spermatozoa in the seminiferous tubules. The experimental results suggest that the abnormal development of the testes in the sex-reversed

female chicken is jointly regulated by sex-related genes and long noncoding RNAs (lncRNA); Wnt (the term **wnt** is an amalgam of **wingless** (Wg) and **int**) and transforming growth factor beta/the bone morphogenetic protein signaling pathways (TGFβ/BMP signaling pathways) play an essential role in regulating developmental pathways during embryogenesis, including a very important role in the differentiation of gonads and in maintaining their function in chickens [14]. Unfortunately at the moment there is no clear concrete data on these issues. It was previously noted that the earliest expression of aromatase in birds is detected only on the 5th day of embryonic development in the medullary layer of the ovary. It is also worth noting that the appearance of aromatase has recently been demonstrated already in the mother's body, after oogenesis in the theca of early follicles [15]. The effect of estrogens on female gonadogenesis follows from the classical scheme of primary sex determination in Gallus gallus. At the same time, in experimental males injection of estradiol results in reversible feminization [16].

In recent study Morris et al. described the gonads and endocrine profile of a gynandromorphic chicken. Its right side had male features, and the left side had female features. Almost all cells (96%) on the right side had a ZZ karyotype, and the left side had a mixture of cells with ZZ and ZW karyotypes. Moreover, the number of cells with the ZW karyotype was much smaller than with the ZZ karyotype. A reduced percentage of cells with the ZW karyotype (23%) did not affect the manifestation of female traits. Based on these observations, Morris et al. concluded that even a small percentage of ZW cells is sufficient for female differentiation. They also confirm the hypothesis of the existence of cell autonomy, on which sexual differentiation in birds depends [17].

Thus, comparing the results of experiments on the inversion of sex and the search for a sex-determining gene in chickens, it can be assumed that the primary determination of the sex of birds is determined by the estrogens content in the early stages of embryogenesis. The role of DMRT1 is associated with the correct regular inclusion of spermatogenesis genes.

## 4. Female heterogametic system

A heterogametic sex is a genetically determined sex that corresponds to the presence in the cells of the body of two different sex chromosomes or one, in a double dose leading to the formation of an alternative sex. Heterogametic individuals give two groups of gametes (according to the content of different sex chromosomes). In animals with a heterogametic male sex, the letters X and Y are used to designate sex chromosomes. Individuals, normally carrying a pair of sex chromosomes X and Y or one chromosome X, are males, and two chromosomes X are females. This group includes mammals, most species of insects, and many other groups. If the heterogamous sex is female, then other designations for sex chromosomes are used—Z and W. Individuals with the genotype ZZ are males; ZW are females. Heterogamous female sex is characteristic of Lepidoptera insects, reptiles, and birds. In these species, sex is not determined during fertilization, but during meiosis. There is no full dose compensation. Female heterogametic systems are very different from the male heterogametic systems. For example, dosage compensation is incomplete in birds. Z-linked genes are higher expressed in males (with two Z chromosomes) than in females (with one Z chromosome), and only a subgroup of genes have the same dose of expression between the sexes. Two male hypermethylated (MHM) regions (MHM1 and MHM2) were found on the Z chromosome with extreme differences in DNA

|  | Female heterogametic system | Male heterogametic system |
|---|---|---|
| System of sex chromosomes | ZW/ZZ | XX/XY |
| Gender carrying with two types of gametes | Female | Male |
| Type of gametes that determines gender | Eggs | Spermatozoids |
| An event that leads to sex determination | Meiosis (first division) | Fertilization |
| Moment of sex determination regarding fertilization | Long before fertilization | In the moment of fertilization |
| Type of gametes in which material is stored for the development of a heterogametic sex | Eggs | Eggs |
| Role of eggs before fertilization | Storage of substances necessary for the development of zygotes, including various types of RNA, during egg maturation | Storage of substances necessary for the development of zygotes, including various types of RNA, during egg maturation |
| Role of sperm in egg fertilization | Start of development, introducing genetic material associated with the Z chromosome | Start of development, introducing genetic material associated with the Y chromosome |
| Possible involvement of sex chromosomes in the storage of substances | Possible involvement of Z or W chromosomes | Only the X chromosome is involved |
| Dose compensation | Incomplete for Z (male) | Complete for X (female) |
| Feature of the alternative sex chromosome (W or Y) | Almost completely consists of repeats, completely heterochromatic | Almost completely consists of repeats, completely heterochromatic |
| Origin of alternative sex chromosome (W or Y) | Maternal | Paternal |

**Table 1.**
*The similarity and difference between the female and male heterogametic system.*

methylation between male and female chicken [18, 19]. Most of the samples analyzed showed a limited effect of MHM1 on transcription of DMRT1. The role of DMRT1 in determining sex in chickens, which may depend on developmental stage and tissue, needs further evaluation [19]. The similarity and difference between the female and male heterogametic system are shown in **Table 1**.

The genome can differentiate tissue-specific. An excellent example of this is the presence of the germline-restricted chromosome (GRC) in the genome of some songbirds. It is absent in somatic cells. GRC is inherited through the female germline and eliminated from the somatic cells during early embryogenesis. Also it was found that GRC contains genes that are paralogous to genes on autosomes and the Z chromosome. More than 38 GRC-linked genes were found in a large number of copies. There are no mobile elements on GRC. The GRC is enriched in genes that are highly expressed in gonads and are involved in the development of female gonads. Germline-restricted chromosomes are probably widespread in the highly dynamic evolutionary history of songbirds, which leads to significant differences between the genome of germline cells and the genome of somatic

cells. This is a new mechanism for minimizing the genetic conflict between germ line cells and somatic cells [20, 21].

## 5. W chromosome and its role

Chicken W chromosome has a length of about 7.08 Mb and contains 28 genes. It was shown that the decay of genes specific for the W chromosome is not random, and therefore it was suggested that the content of the surviving genes associated with the W chromosome was under high evolutionary pressure due to absence of recombination. There are no genes on the W chromosome that determine sex. In the evolution of bird karyotypes, the W chromosome has undergone extensive degradation and accumulation of repetitive DNA [22, 23]. Notably, the most common W chromosome-specific repeats *XhoI*, *EcoRI*, *SspI*, and newly described (GGAAA)$_n$ are compacted in densely packed chromomeres and do not transcribe at the lampbrush stage. *XhoI*, *EcoRI*, and *SspI* were shown to lack any significant homology between Galliformes, Ciconiiformes, and Passeriformes. Komissarov et al.
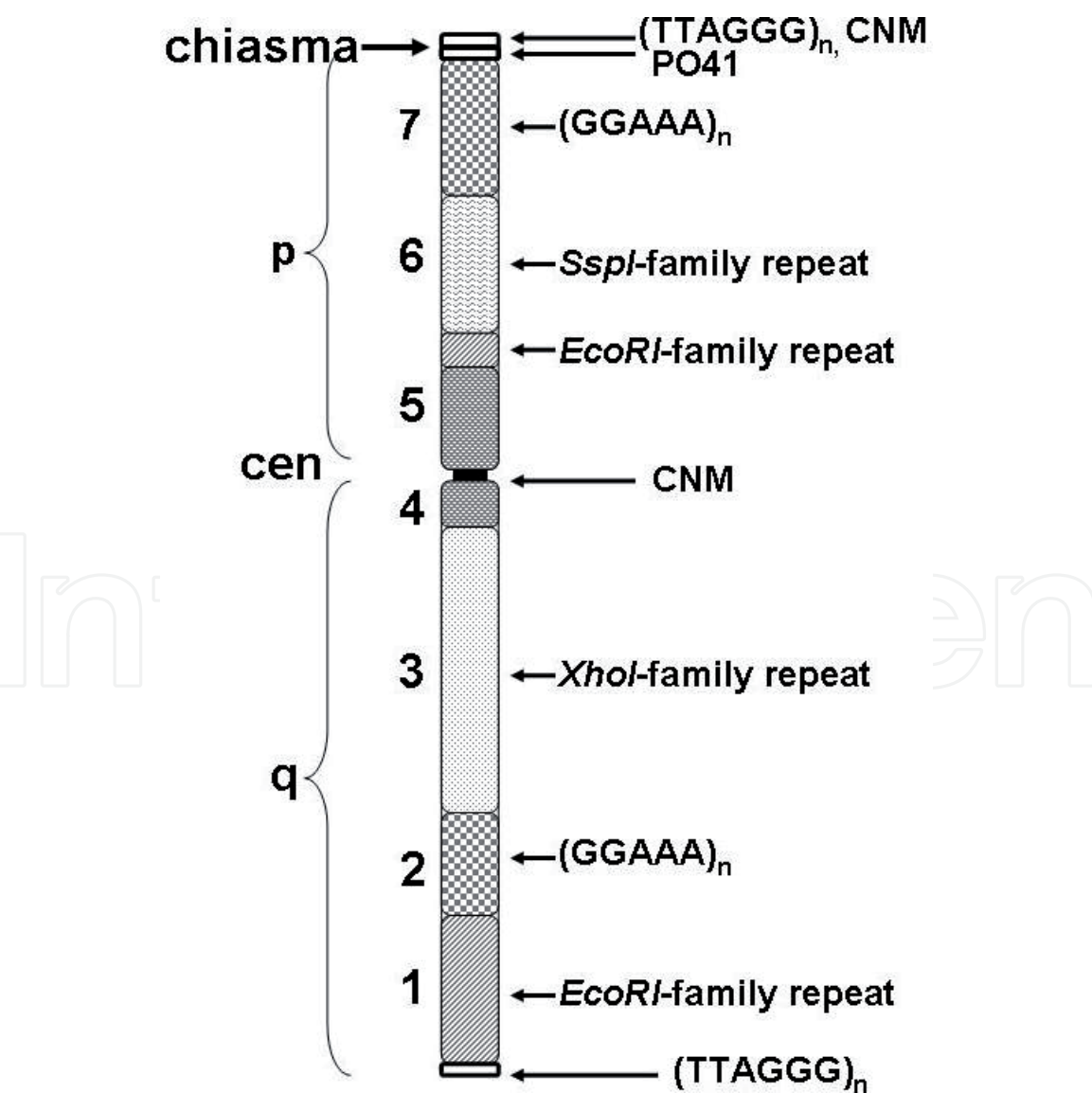


**Figure 3.**
*Chicken W lampbrush chromosome diagram with localized repeat blocks. Chromomeres are numbered from the free end of the W chromosome to the chiasma region. Loop-related repeats are indicated by thin arrows. Non-transcribed repeat locations are marked with thick arrows (adapted from [23]).*

| Ploidy | Sex chromosomes | *ZUF* | Z dosage | Z/A ratio | Phenotype |
|---|---|---|---|---|---|
| Normal | | | | | |
| 2A | ZZ | — | 1*2Z = 2 | 1,00 | M |
| 2A | ZW | + | 2*1Z = 2 | 1,00 | F |
| Aneuploid | | | | | |
| 2A | ZO | — | 1*1Z = 1 | 0,50 | M (dead) |
| 2A | ZZZ | — | 1*3Z = 3 | 1,50 | M (dead) |
| 2A | ZZW | + | 2*2Z = 4 | 2,00 | F (dead) |
| 2A | ZWW | + | 2*1Z = 2 | 1,00 | F |
| Triploid | | | | | |
| 3A | ZZZ | — | 1*3Z = 3 | 1,00 | M |
| 3A | ZZW | + | 2*2Z = 4 | 1,33 | F/M |
| 3A | ZWW | + | 2*1Z = 2 | 0,67 | F (dead) |
| *ZUF = Z upregulated factor.* | | | | | |

**Table 2.**
*The number of Z chromosomes and phenotype of aneuploid chickens [27].*

suggested independent accumulation of specific DNA repeats, which occurred after initial divergence of Z and W chromosomes (**Figure 3**, [23]).

The determination of the function of repeating sequences on Y or W chromosomes is in its infancy, but it is clear that these sequences play a functional role in gene regulation and chromatin structure [23]. Banded krait minor (Bkm) satellite repeat in the W chromosome consists of tandem arrays of GATA nucleotides. Tissue-specific protein that binds specifically to Bkm repeats, known as Bkm-binding protein (BBP), is involved in the coordinated decondensation of the heterogametic sex chromosomes in germ cells. It is known that GATA repeats play a conservative role of insulators [24, 25].

The transcriptome of sex-inversed chickens has a wide variety of lncRNA classes compared to the transcriptome of ordinary males. lncRNAs are classified as a separate class of non-protein-coding genes. These genes do not encode proteins and do not have open reading frames; their functional constraints differ from those for protein-encoding genes or genes where most of the nucleotide sequence is necessary for a function similar with tRNA. Despite this, relatively few lncRNAs have been studied in detail. They present high sequence divergence between species. Nonetheless, similar or equivalent lncRNAs perform the same functions in different organisms [26]. The W chromosome is necessary for the appearance of the ovary. Individuals containing it are always females (**Table 2**) [27, 28]. A specific factor, called F or ZUF (Z upregulated factor), is involved in gene dose compensation in determining sex. This W-specific factor can control gene expression from the single Z chromosome of the heterogametic sex [27]. Unlike mammals, we never see birds with differences in the number of Z and W chromosomes; it seems that there are no bird equivalent to women with XO with one X chromosome and men with XXY chromosomes. Perhaps such changes are lethal in birds.

## 6. The ability to choose the sex of the descendants of female birds

There is some experimental evidence that sexual cleavage in birds may not be random [29, 30]. This corresponds to the previously reported effects of hormonal

manipulations on the offspring. It appears that there is a critical level of corticosterone needed to manipulate the sex of the offspring and that this level must be achieved within a relatively short time (**Figure 4**, [31–34]).

Proposed preovulatory mechanisms for a primary sex ratio bias in birds include (1) asynchronous sex-specific follicular development, (2) segregation distortion or meiotic drive, and (3) selective resorption of postmeiotic and preovulatory follicle (**Figure 5**).

There are hypotheses that explain sex ratio bias (SR bias). These are sex-specific fertilization and sex-specific embryo mortality. Now they are not relevant. Hormonal fluctuations caused by external factors that the female encounters alter the normal process of meiosis I (MI, the first meiotic division), blocking the segregation of the first polar body (PB) (**Figure 6**, [32]). But not only maternal hormones and other components of the yolk might affect the result. Temperature is another factor responsible for differential embryonic mortality in birds.
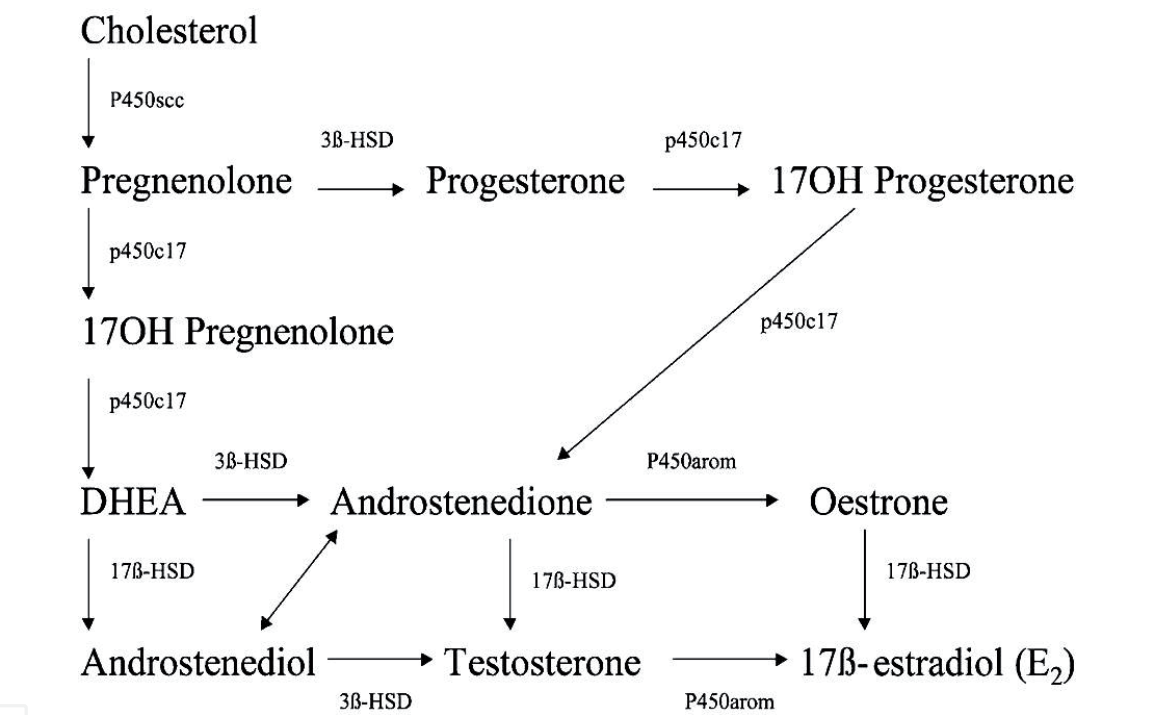


**Figure 4.**
*The proposed scheme of biosynthesis of important steroid hormones (adapted from [34]).*
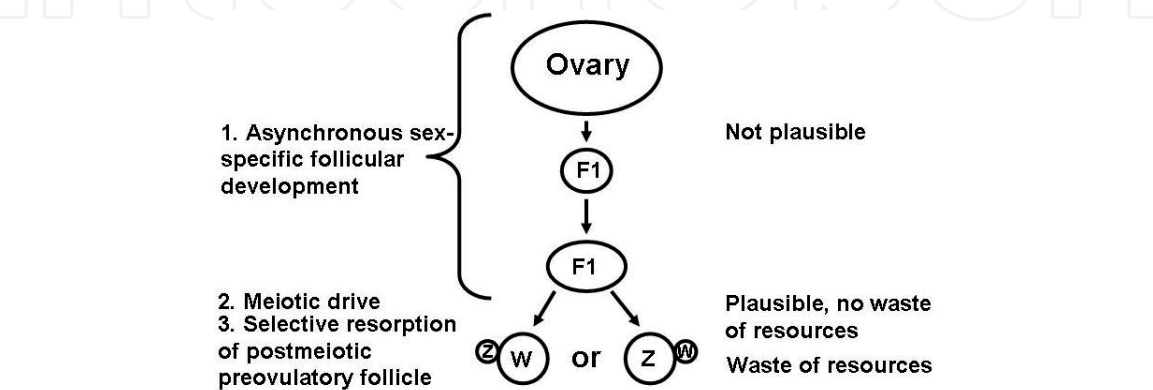


**Figure 5.**
*The suggested mechanisms of bias of the primary sex ratio in birds. (1) The first mechanism implies the existence of factors within the follicles prior to meiosis. (2) Meiotic drive suggests that factors during meiosis I cause nonrandom segregation of sex chromosome. (3) Third mechanism involves sex-specific follicle abortion and subsequent resorption after meiosis and before ovulation (adapted from [35]).*

Gonadal sex determination refers to the decision to differentiate as a testis or an ovary [29], such that sex determination occurs chronologically before sex differentiation and glucocorticoids (GCs) could interfere with both processes. For instance, in avian species, corticosterone may influence sex chromosome segregation at the first meiotic division, thereby acting directly on sex determination.
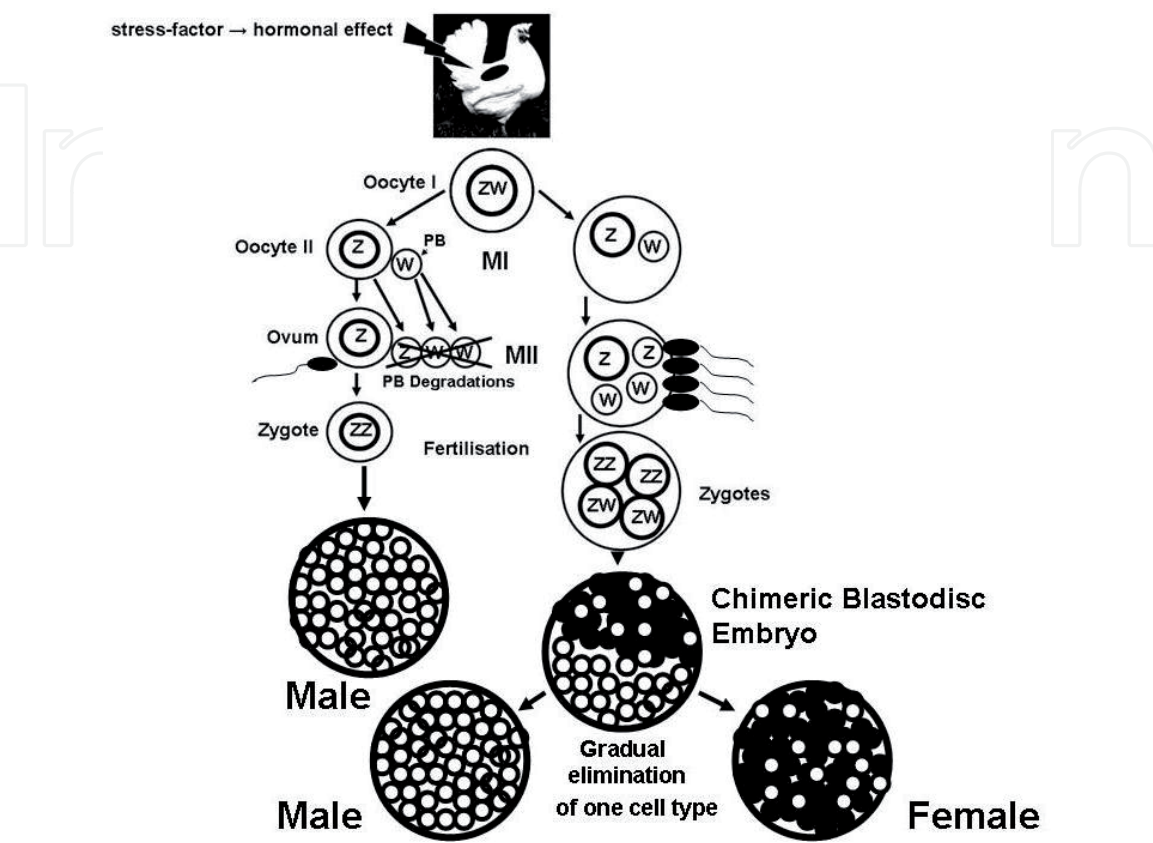


**Figure 6.**
*Exclusion of the first polar body (PB) as a possible mechanism of deviation from the primary SR bias in birds (adapted from [32]).*
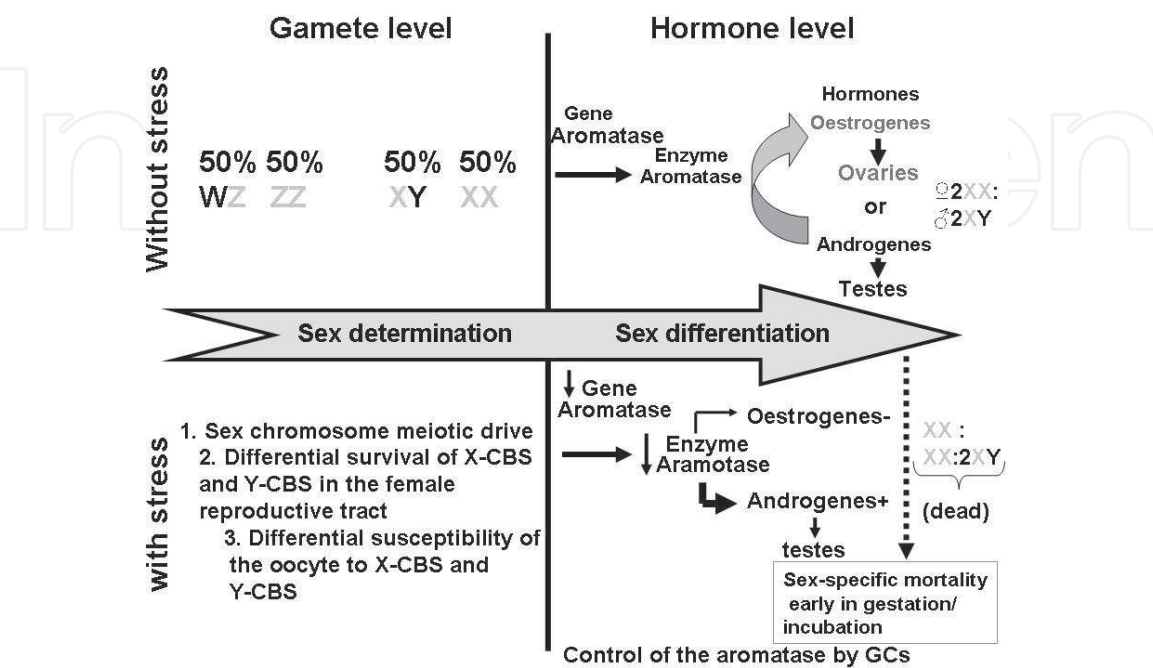


**Figure 7.**
*Mechanism of influence of glucocorticoids (GCs) on determination and differentiation of sex in vertebrates (adapted from [10]).*

This is possible because, in birds, females are the heterogametic sex (ZW chromosomes) and have therefore a high degree of control over the sex ratio of the offspring they produce (**Figure** 7, [10, 34]). Future studies regarding sex ratio bias in birds should focus on the cellular and molecular mechanisms of sex ratio bias by examining the gene and protein expression during meiosis using genomics and proteomics techniques.

## 7. Conclusion

For birds, the DMRT1 gene is considered as the sex-determining gene. The main argument is the change in the sex of male after turning it off with the use of interfering RNA on the 1st day of incubation [6]. There is also the possibility of changing the sex by introducing an aromatase inhibitor, usually on day 5.5 or even at the very beginning of incubation in the direction of the female → male [11–13]. According to recent data, aromatase appears already in the layer of early follicles [14]. The unstable alteration of males into females after injection of estrogen is worth mentioning [16]. The indispensable presence of the W chromosome in females suggests that synthesis of estrogens is induced by genes located on this chromosome. It can be assumed that this process is triggered by the inclusion of a number of factors using lncRNA. The primary sex determination is the onset of testis or ovary before differentiation (**Figure** 8).

*Dmrt1* is unusual in that it is expressed by both Sertoli cells and germ cells. Deletion or inactivation of DMRT1 gene in human resulted in XY male-to-female sex reversal. DMRT1 is required for a stable testis phenotype. It is well known that retinoic acid (RA) signaling between Sertoli cells and germ cells is essential for adult mammalian spermatogenesis. In the absence of DMRT1, RA signaling may also activate genes that can drive male-to-female transdifferentiation. Gonad sex identity may be able to switch at adult stage in some fish species. In mammals the gonadal phenotype remains stable for the rest of life. In birds the function of DMRT1 is not primary sex determination, but maintenance of already formed testis [37]. The
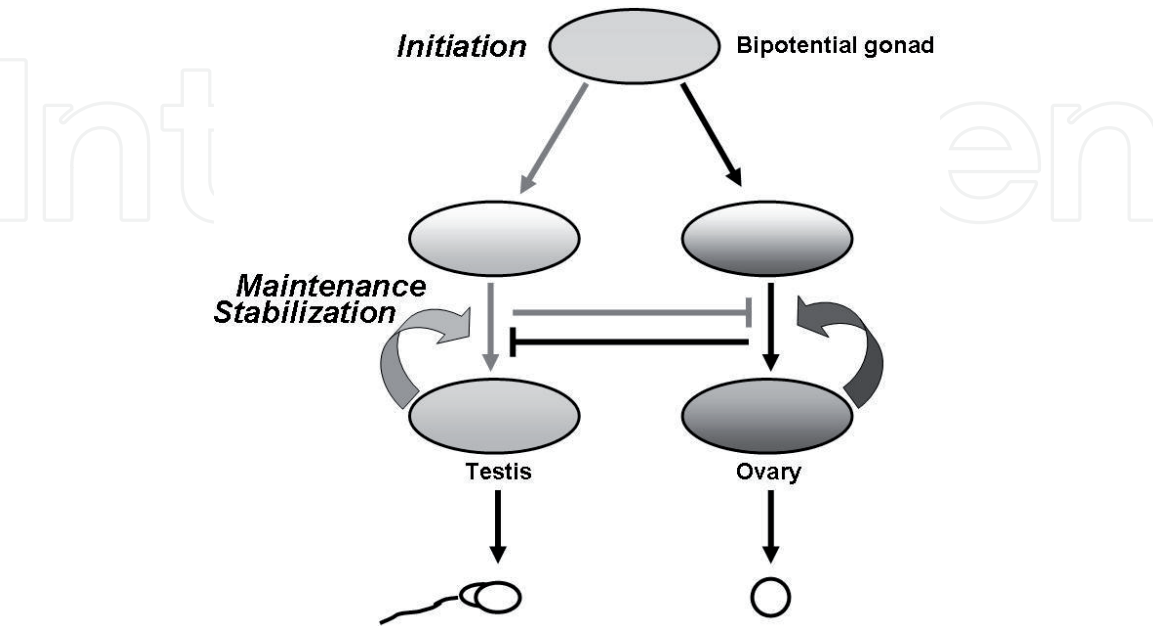


**Figure 8.**
*Stages of gonadal sex determination: the stage of initiation, maintenance, and stabilization. The sex reversal can only occur at stages of initiation and maintenance. The complete sex alteration can only occur at the stabilization stage (adapted from [36]).*

appearance of gonads in birds is most likely determined by sex hormones and to the greatest extent by estrogen under the control of W chromosome. The role of the DMRT1 gene is to maintain and develop testis.

It can be assumed that the W chromosome causes the appearance of ovaries through the control of estrogen synthesis and their interaction with the corresponding receptors. The W chromosome seems to have no specifically female genes. Over the years, it was believed that the main function of RNA is to act as an intermediary in the process of reading a protein sequence from the gene encoding it. Therefore, one of the biggest surprises in modern biology was the discovery that protein-coding sequences comprise less than 2% of the total genome; then it has been found that at least 90% of the human genome is actively transcribed. It should be noted that lncRNA are transcripts that do not encode protein and have a length of more than 200 nucleotides. However, despite debates, the proof that certain lncRNA clearly play first-line roles in development, pluripotency, dosage compensation, establishment of chromatin structure, genome maintenance, and as tumor suppressors or oncogenes is not contested [38, 39]. It assumes that the development of testis in male of chicken is co-regulated by sex-related genes and long noncoding RNA, and Wnt and TGFβ/BMP signaling pathways have a very important role in gonadal differentiation. It is desirable to pay attention to noncoding RNAs, their connection with the W chromosome and their role in bird sex determination.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be considered as a potential conflict of interest.

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