Transcriptome profiling reveals male- and female-specific gene expression pattern and novel gene candidates for the control of sex determination and gonad development in *Xenopus laevis*

Rafal P. Piprek1 · Milena Damulewicz2 · Jean-Pierre Tassan3 · Malgorzata Kloc4,5,6 · Jacek Z. Kubiak3,7

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Abstract

*Xenopus laevis* is an amphibian (frog) species widely used in developmental biology and genetics. To unravel the molecular machinery regulating sex differentiation of *Xenopus* gonads, we analyzed for the first time the transcriptome of developing amphibian gonads covering sex determination period. We applied microarray at four developmental stages: (i) NF50 (undifferentiated gonad during sex determination), (ii) NF53 (the onset of sexual differentiation of the gonads), (iii) NF56 (sexual differentiation of the gonads), and (iv) NF62 (developmental progression of differentiated gonads). Our analysis showed that during the NF50, the genetic female (ZW) gonads expressed more sex-specific genes than genetic male (ZZ) gonads, which suggests that a robust genetic program is realized during female sex determination in *Xenopus*. However, a contrasting expression pattern was observed at later stages (NF56 and NF62), when the ZW gonads expressed less sex-specific genes than ZZ gonads, i.e., more genes may be involved in further development of the male gonads (ZZ). We identified sexual dimorphism in the expression of several functional groups of genes, including signaling factors, proteases, protease inhibitors, transcription factors, extracellular matrix components, extracellular matrix enzymes, cell adhesion molecules, and epithelium-specific intermediate filaments. In addition, our analysis detected a sexually dimorphic expression of many uncharacterized genes of unknown function, which should be studied further to reveal their identity and if/how they regulate gonad development, sex determination, and sexual differentiation. Comparison between genes sex-specifically expressed in developing gonads of *Xenopus* and available transcriptome data from zebrafish, two reptile species, chicken, and mouse revealed significant differences in the genetic control of sex determination and gonad development. This shows that the genetic control of gonad development is evolutionarily malleable.

Keywords Testis · Ovary · Sex determination · Gonad development · *Xenopus* · Transcriptome

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Introduction

*Xenopus laevis* is a good model to study molecular mechanisms of gonad development because the structural changes in developing gonads and the master gene determining sex, the W-linked DM-domain gene (*dm-w*), are well known. The *dm-w* is located on W chromosome and thus is present only in the genetic females (ZW) (Yoshimoto et al. 2008). At the earliest stage of gonad development, the gonads are undifferentiated and bipotential. The expression of *dm-w* triggers ovary development, while its absence promotes testis development. It is believed that the DM-W protein blocks the DMRT1 (doublesex and mab-3-related transcription factor 1) involved in male sex determination (Yoshimoto et al. 2010). In addition to the *dm-w*, many other genes, which act independently or downstream of *dm-w*, are involved in the development of bipotential gonads into the ovaries or the testes (Piprek et al. 2016). However, the expression and role of many genes involved in gonadal development is still vague. At the initial stage of gonadogenesis (NF50, Nieuwkoop-Faber stage 50), the gonads consist of the gonadal cortex and the medulla. The gonadal cortex contains coelomic epithelium and the germ cells, which adhere to the interior face of the epithelium. The medulla is sterile and contains medullar cells only (Piprek et al. 2016, 2017). At this stage, the sex-determining genes (*dm-w* and *dmrt1*) are expressed in the somatic cells of the gonads. In the absence of *dm-w*, i.e., in the differentiating testis (ZZ), around stage NF53, the cortex and medulla fuse. Subsequently, around stage NF56, the germ cells become enclosed by the somatic cells, which results in the formation of testis cords (Piprek et al. 2017). The typical structure of the testis, i.e., fully differentiated testis cords separated by the interstitium, is established at stage NF62. In contrast, in differentiating ovaries, which express *dm-w*, the germ cells remain in the cortical position, and at stage NF56, the ovarian cavity forms inside the gonad. Around NF62, the ovaries are fully differentiated, with the oocytes located in the cortex (Piprek et al. 2017; Yoshimoto et al. 2008). This divergent development of the female and male gonads has to be controlled by differential gene expression. A global analysis of *Xenopus* gonad transcriptome, which we performed in this study, is the step in obtaining a broad database of gene expression pattern in developing male and female *Xenopus* gonads.

Among anurans, a transcriptome analysis was performed only in *Silurana* (*Xenopus* tropicalis) and only on already sexually differentiated gonads (from stage NF58) (Haselman et al. 2015). Thus, the genes expressed before and during the sexual differentiation of amphibian gonads are still unknown. The aim of our study was to examine the transcriptome of developing *Xenopus* gonads from the earliest stage of gonad development. We studied the gene expression pattern in four different stages of gonad development: the undifferentiated gonad during the period of sex determination (NF50), gonads at the onset of sexual differentiation (NF53), the differentiating gonads (NF56), and during the developmental progression of differentiated gonads (NF62) (Fig. 1).

Results and discussion

Sex-specific changes in the level of gene expression

In developing *Xenopus laevis* gonads (stages NF50, NF53, NF56, and NF62 combined), we detected the expression of 63,084 transcripts in total. We found that while the expression level of the majority of genes was similar between stages and between male and female gonads, a subpopulation of genes showed distinct changes in the expression level between stages and sexes, which suggested that they may play a role in sex determination and/or sexual differentiation (Figs. 2A, B and 3, Tables 1 and 2).

Analysis of gene expression level in the gonads showed that in the genetic females (ZW), the gonads at the onset of sexual differentiation (NF53) had 376 genes with upregulated expression and 1078 genes with downregulated expression in comparison to the undifferentiated gonad during sex determination period (NF50) (Fig. 2, Table 1). In the differentiating ovaries (NF56), only 143 genes were upregulated and 128 genes were downregulated in comparison to NF53 (Table 1). In differentiated ovaries (NF62), there were 918 genes with upregulated expression and 1834 genes with downregulated expression in comparison to NF56 (Table 1).

The genetic male (ZZ) gonads at the onset of sexual differentiation (NF53) had 659 genes with upregulated expression and 436 genes with downregulated expression in comparison to NF50 stage (Fig. 2, Table 1). In differentiating testes (NF56), 340 genes were up- and 340 downregulated in comparison to NF53 stage. The differentiated testes at stage NF62 had 334 genes with upregulated expression and 831 genes with downregulated expression in comparison to NF56 stage.

Altogether, these data indicate that in both sexes, the transcriptional regulation is more robust during early gonadal development, i.e., at the onset of sexual differentiation of the gonad (NF50-NF53) and in the already differentiated gonads NF56-NF62 than in the differentiating gonads (NF53-NF56).
The comparison of gene expression level in between ZW and ZZ gonads showed significant differences between the sexes and revealed sexually dimorphic pattern of gene expression. At the initial phase of gonad development, i.e., in the undifferentiated gonads (NF50), there were 1192 genes (i.e., 3.4%) with sexually dimorphic expression (≥2-fold change). Eight hundred twenty genes showed higher expression in ZW (genetic females), and only 372 showed higher expression in ZZ (genetic males) gonads (Fig. 3, Table 2). This indicates that female sex determination in *Xenopus* involves a robust transcriptional regulation. In contrast, in mice, during the sex determination period (between embryonic day E10.5 and E12.5), a higher number of genes were upregulated in the XY (genetic males) than in the XX (genetic females) gonads (Nef et al. 2005), which suggested that programs of sex determination may be diverse among vertebrates.

Our analysis showed that at NF53, i.e., at the beginning of sexual differentiation of *Xenopus* gonads, 1083 genes (i.e., 3%) showed sexually dimorphic expression (≥2-fold change), which was slightly lower number than at NF50 (during sex determination). One hundred ninety-three genes showed higher expression in ZW gonads, and 890 in ZZ gonads (Fig. 3, Table 2). Thus, at the onset of sexual differentiation, more genes were specifically expressed in ZZ (male) gonads than in ZW (female) gonads in *Xenopus*, which was opposite
to the mouse, where more genes were specifically expressed in XX (female) than XY (male) gonads at the beginning of sexual differentiation (E13.5) (Nef et al. 2005). This again indicates differences in the molecular programs of gonad development among vertebrates.

At NF56, i.e., in the differentiating gonads, only 421 genes (i.e., 1.2%) showed sexually dimorphic expression (≥2-fold change). This stage showed the lowest percentage of genes with sexually dimorphic expression among all stages. Seventy-five genes had higher expression in ZW, and 346 in ZZ gonads (Fig. 3, Table 2). Thus, more genes were highly expressed in ZZ gonads (differentiating testes) than in ZW (differentiating ovaries). We previously showed that the testis differentiation in *Xenopus* is a complex process during which the basement membranes between gonadal cortex and medulla disintegrate, the cortex and medulla fuse, and the germ cells and somatic cells gather to form the testis cords (Piprek et al. 2017). This sequence of profound structural changes certainly requires an involvement of a number of different genes, which is reflected in the high number of genes expressed in ZZ gonads at this stage.

At stage NF62, the sexual dimorphism of gene expression is the most pronounced. At this stage, 3224 genes (i.e., 5%) showed sexually dimorphic expression (≥2-fold change). However, only 594 genes showed higher expression in ZW (ovaries), and as many as 2630 in ZZ (testes) gonads. This is the stage when the gonads of both sexes are already differentiated and fully prepared to perform their sex-specific functions, and therefore the sexual dimorphism is evident not only at structural but also at molecular level.

### The expression of genes during different stages of ovary development

We found that in ZW gonads at stage NF53, in comparison to stage NF50, 376 genes had upregulated expression. The list of genes is presented in Suppl. Table 1, and chosen genes are presented in Table 3. Functional analysis grouped these genes in several distinct categories shown in Table 4. Among the upregulated genes, monoacylglycerol O-acyltransferase 2 gene 1 (*mogat2.1*) is involved in synthesis of diacylglycerol (DAG) that acts as a messenger lipid in cell signaling (Toker 2005); retinol-binding protein 2 (*rbp2*) is involved in retinoic acid regulation; extracellular proteins: collagen 2 and collagen 9, cysteine protease cathepsin K, epithelium-specific intermediate filaments: keratin 14 and keratin 19, estrogen receptor 1 (*esr1*), and synuclein gamma. At this early stage, the germ and somatic cells proliferate, and somatic cells start gathering in the gonad center forming medulla (Fig. 1A, C). Collagens accumulate between the gonad cortex and medulla (Piprek et al. 2017). Importantly, around stage NF50, a sex determination period takes place and gene expression analysis suggest
that DAG, retinol, and estradiol may be involved in *Xenopus* sex determination.

We also found that in ZW gonads at stage NF53, there were 1078 genes with a downregulated expression in comparison to stage NF50. All these genes are listed in Suppl. Table 2, and chosen genes are presented in Table 3. Functional analysis grouped these genes in three categories shown in Table 4. Among these downregulated genes, there were signaling protein chordin (*chrd*), retinol-binding protein (*rbp4*), several protease inhibitors serpins, signaling proteins *wnt11b* and *igf3* (insulin-like growth factor 3), transcription factors *dmrt2*, and *mafb* (Table 3).

In developing ZW gonad at stage NF56, in comparison to stage NF53, there were 143 genes with upregulated expression (Suppl. Table 3, and chosen genes are presented in Table 5). Functional analysis grouped these genes in three categories shown in Table 4. One of important genes upregulated in this period is a neurotrophin receptor a-1 (*p75NTRa*) (Table 2); its role in gonad development has never been studied; however, its upregulation suggests that neurotrophins (ligands of this receptor) can play a role in ovarian differentiation. We also found that in ZW gonad at stage NF56, in comparison to stage NF53, there were 128 genes with downregulated expression (Suppl. Table 4, and chosen genes are presented in Table 5). Functional analysis grouped these genes in several categories shown in Table 4. At NF56 stage, more genes responsible for reorganization of extracellular matrix and epithelial differentiation in ZW gonads are expressed than at stage NF53. Between stages NF53 and NF56, the medulla cells disperse, which results in the formation of the cavity in the oovary center (Fig. 1E). The mechanism of this event is not known and would be interesting to study how the neurotrophins, extracellular matrix, and epithelial differentiation are involved in this process.

In developing ZW gonad at stage NF62, in comparison to stage NF56, there were 918 genes with upregulated expression (Suppl. Table 5, and chosen genes are presented in Table 6). Functional analysis grouped these genes in the many categories (Table 4). Among known genes upregulated in the ovaries at stage NF62 are genes involved in meiosis and oocyte development, such as poly(A)-binding protein, oocyte-specific *pou5f3.3*, zygote arrest 1, zona pellucid proteins (*zp2, zpd, zpy1*), *sycp3* (synaptonemal complex protein 3), and *lhx8* (LIM homeobox 8). This reflects the onset of meiosis at stage NF62 and appearance of first oocytes (Fig. 1G). Also, more genes involved in the regulation of development, such as genes encoding the following: *vegt* protein, growth differentiation factor (*gdf1*), *foxh1, foxr1, wnt11b, ddx25*, and the survivin which prevents apoptosis, were upregulated at stage NF62 than at stage NF56.

In developing ZW gonad at stage NF62, in comparison to stage NF56, there were 1834 genes with downregulated expression (Suppl. Table 6, and chosen genes are presented in Table 6). Functional analysis grouped these genes into several categories (Table 4). Also, many (24) pathways were downregulated, including metabolic pathways, steroid hormone biosynthesis, retinol metabolism, PPAR signaling pathway, and adipocytokine signaling pathway (Table 4). Among known genes downregulated in the ovaries at stage NF62 are the following genes: retinol-binding protein 4 (*rbp4*), *rdh16* (retinol dehydrogenase 16), several serpins, *emx1.2* (empty spiracles homeobox 1), *igf3* (insulin-like growth factor 3), *igfbp1-a* (insulin-like growth factor–binding protein 1), *gata2* (GATA binding protein 2), and chordin. This indicates that retinol pathway and insulin-like growth factor pathway are downregulated at a later stage of ovarian development (NF62), and that these two pathways may be important for earlier stages of ovarian development. The PPAR signaling pathway and adipocytokine signaling pathway are involved in fat tissue differentiation (Ogunyemi et al. 2013) and are probably important for the development of corpora adiposa (fat tissue) at the anterior edges of the developing gonads at stages before NF62. Thus, after the fat tissue had been formed, these pathways are downregulated at stage NF62.

Another interesting gene expressed at the onset of gonadogenesis (NF50), showing upregulation at NF53 and downregulated at NF62 is chordin (*chrd*). Several studies showed that this gene is crucial for early organogenesis (dorsalization, gastrulation, and head development (Pappano et al. 1998; Bachiller et al. 2000), but its role in gonad development is unknown. Overall, our gene expression analysis showed that the later development of the oovary (NF62) is a very transcriptionally active period (many genes become upregulated and downregulated between NF56 and NF62), which may be related to the initialization of meiosis and oocyte formation during this developmental period.

| Compared stages | ZW (females) |  | ZZ (males) |  |
|-----------------|--------------|-----------------|--------------|-----------------|
|                 | Upregulated  | Downregulated   | Upregulated  | Downregulated   |
| NF53 vs. NF50   | 376          | 1078            | 659          | 436             |
| NF56 vs. NF53   | 143          | 128             | 340          | 340             |
| NF62 vs. NF56   | 918          | 1834            | 334          | 831             |

Table 1 Number of genes with up- and downregulated (≥ 2-fold change) expression in ZW and ZZ gonads
The expression of genes during different stages of testis development

Our analysis showed that in the genetic male (ZZ) gonads at stage NF53, i.e., at the beginning of sexual differentiation, there were 659 genes with upregulated expression in comparison to the stage NF50 gonad (Suppl. Table 7, and chosen genes are presented in Table 7). Functional analysis grouped these genes into several categories (Table 8). There were the following genes with known function: \( \text{igf3} \) (insulin-like growth factor 3), \( \text{rbp4} \) (retinol-binding protein 4), \( \text{vtn} \) (vitronectin), several serpins, \( \text{esr2} \) (estrogen receptor 2), several components of extracellular matrix (collagen 9, matrilin 2), and extracellular matrix (\( \text{timp3} \)) enzymes. A role of these genes in the early phase of ZZ gonad development is not known, and it would be interesting to study if retinol and/or \( \text{igf3} \) are involved in male sex determination in \textit{Xenopus}. Upregulation of PPAR and adipocytokine signaling pathways, characteristic for fat tissue, possibly reflects the onset of the development of the fat bodies at the anterior edge of the gonad.

### Table 3

| Probe name  | Gene symbol | Gene name                  | Log FC |
|-------------|-------------|----------------------------|--------|
| A_10_P009259 | mogat2.1    | Monoacylglycerol O-acyltransferase 2.1 | 6.53907 |
| A_10_P079665 | rbp2        | Retinol-binding protein 2    | 5.67257 |
| A_10_P002950 | col9a1      | Collagen, type IX, alpha 1   | 4.86313 |
| A_10_P005551 | srp2x2      | Sushi repeat–containing protein, X2 | 4.74263 |
| A_10_P000515 | bcan        | Brevican                    | 4.008  |
| A_10_P136703 | krt14       | Keratin 14                  | 3.56258 |
| A_10_P000726 | aldh3b1     | Aldehyde dehydrogenase 3 B1 | 3.5464 |
| A_10_P143593 | csh         | Cathepsin H                 | 3.39144 |
| A_10_P004976 | matn4       | Matrilin 4                  | 3.3843 |
| A_10_P027124 | col2a1b     | Collagen, type II, alpha 1   | 3.12339 |
| A_10_P002931 | matn2       | Matrilin 2                  | 3.10895 |
| A_10_P041821 | sncg-b      | Synuclein, gamma b          | 2.79753 |
| A_10_P032181 | sncg-a      | Synuclein, gamma a          | 2.7756 |
| A_10_P046256 | csk         | Cathepsin K                 | 2.75751 |
| A_10_P165493 | krt19       | Keratin 19                  | 2.48345 |
| A_10_P006607 | col9a3      | Collagen, type IX, alpha 3   | 2.41836 |
| A_10_P033056 | esr1-a      | Estrogen receptor 1         | 2.36005 |
| A_10_P224323 | racgap1     | Rac GTPase activating protein 1 | 2.29739 |
| A_10_P036156 | dcn         | Decorin                     | 2.25563 |
| A_10_P065984 | itga11      | Integrin, alpha 11          | 2.17377 |

### Table 2

| ZW vs. ZZ compared at stages | Upregulated in ZW | Downregulated in ZW |
|-----------------------------|--------------------|----------------------|
| NF50                        | 820                | 372                  |
| NF53                        | 193                | 890                  |
| NF56                        | 75                 | 346                  |
| NF62                        | 594                | 2630                 |

### Table 4

| ZW vs. ZZ compared at stages | Upregulated in ZW | Downregulated in ZW |
|-----------------------------|--------------------|----------------------|
| NF50                        | 820                | 372                  |
| NF53                        | 193                | 890                  |
| NF56                        | 75                 | 346                  |
| NF62                        | 594                | 2630                 |

The expression of genes during different stages of testis development

Our analysis showed that in the genetic male (ZZ) gonads at stage NF53, i.e., at the beginning of sexual differentiation, there were 659 genes with upregulated expression in comparison to the stage NF50 gonad (Suppl. Table 7, and chosen genes are presented in Table 7). Functional analysis grouped these genes into several categories (Table 8). There were the following genes with known function: \( \text{igf3} \) (insulin-like growth factor 3), \( \text{rbp4} \) (retinol-binding protein 4), \( \text{vtn} \) (vitronectin), several serpins, \( \text{esr2} \) (estrogen receptor 2), several components of extracellular matrix (collagen 9, matrilin 2), and extracellular matrix (\( \text{timp3} \)) enzymes. A role of these genes in the early phase of ZZ gonad development is not known, and it would be interesting to study if retinol and/or \( \text{igf3} \) are involved in male sex determination in \textit{Xenopus}. Upregulation of PPAR and adipocytokine signaling pathways, characteristic for fat tissue, possibly reflects the onset of the development of the fat bodies at the anterior edge of the gonad.
Our analysis also showed that in the genetic male (ZZ) gonads at stage NF53, there were 436 genes with downregulated expression (Suppl. Table 8, and chosen genes are presented in Table 7). Functional analysis grouped these genes in the categories shown in Table 8. Some of these upregulated genes are rbp2 (retinol-binding protein 2), receptor of prostaglandin E (ptger3), stromal cell-derived factor 2-like 1 (sdf2l1), and neurotrophin receptor (p75NTRa). Further, studies are necessary to establish what is the exact role of the prostaglandin E, retinol, and neurotrophins in testis differentiation. Importantly, around NF53-NF56, the cortex and medulla fuse in differentiating testes, and the germ cells lose their connection with the superficial coelomic epithelium and disperse in the whole testis (Fig. 1F). There were also 340 genes downregulated at stage NF56 versus NF53.

### Table 4  Number of genes assigned to functional groups up- and downregulated in ZW (genetic female) gonads

| Functional gene groups | ZW (genetic females) |  |
|------------------------|----------------------|---|
|                        | NF53 vs. NF50 | NF56 vs. NF53 | NF62 vs. NF56 |
|                        | Up | Down | Up | Down | Up | Down |
| Signaling factors      | 20 | 61 | 7 | 8 | – | 103 |
| Calcium-binding proteins | 6 | – | 3 | – | – | – |
| Iron-binding proteins  | 4 | – | – | – | – | – |
| Monoxygenases          | 4 | – | – | – | – | 11 |
| Oxidoreductases        | 5 | 11 | – | – | – | 22 |
| Sushi domain–containing proteins | 2 | – | – | – | – | – |
| Metalloproteases       | 3 | – | – | – | – | 8 |
| Intermediate filaments | 3 | – | – | – | – | – |
| EGF-like domain–containing proteins | 3 | – | – | – | – | – |
| ECM-receptor interaction pathway | 3 | – | – | – | – | – |
| Progesterone-mediated oocyte maturation pathway | 4 | – | – | 11 | – | – |
| Proteases              | – | 12 | – | – | – | 18 |
| Hydrolases             | – | 27 | – | – | – | 33 |
| Disulfide bond–containing proteins | – | – | 5 | – | – | 45 |
| Extracellular matrix components | – | – | – | 5 | – | – |
| Markers of epithelial differentiation | – | – | – | 2 | – | – |
| Meiosis regulation factors | – | – | – | – | 8 | – |
| RNA-binding proteins   | – | – | – | – | 15 | – |
| Phosphoproteins        | – | – | – | – | 16 | – |
| Proteins involved in development | – | – | – | 22 | – | – |
| Proteins involved in oogenesis | – | – | – | 3 | – | – |
| Cytoplasmic proteins   | – | – | – | – | 35 | – |
| Cytoskeletal proteins  | – | – | – | – | 12 | – |
| Proteins involved in differentiation | – | – | – | 9 | – | – |
| Nuclear proteins       | – | – | – | – | 45 | – |
| Transcriptional repressors | – | – | – | – | 8 | – |
| DNA-binding proteins   | – | – | – | – | 3 | – |
| Oocyte meiosis         | – | – | – | – | 10 | – |
| p53 signaling          | – | – | – | – | 6 | – |
| Basal transcription factors | – | – | – | – | 4 | – |
| Proteins involved in DNA Replication | – | – | – | 4 | – | – |
| Proteins involved in the formation of dorso-ventral axis | – | – | – | 3 | – | – |
| Secreted proteins      | – | – | – | – | 23 | – |
| Transport proteins     | – | – | – | – | 36 | – |
NF56 ZZ gonad in comparison to stage NF53 gonad (Suppl. Table 10, and chosen genes are presented in Table 9). Functional analysis grouped these genes into several categories (Table 8).

Comparison of gene expression level in the ZZ gonads between stages NF62 and NF56 showed that at stage NF62 gonad, there were 334 genes with the upregulated expression (Suppl. Table 11, and chosen genes are presented in Table 10). Functional analysis grouped these genes into several categories (Table 8). Around stage NF56-NF62, cells group into the testis cords (Fig. 1H). Genes involved in this process are not known, and presumably, the genes upregulated at this stage may be responsible for the formation of testis cords. There were also 831 genes downregulated in ZZ gonad at stage NF62 in comparison to stage NF56 (Suppl. Table 12, and chosen genes are presented in Table 10, and the gene categories, which were analyzed functionally are shown in Table 8).

**Genes with sexual dimorphism of expression in ZW and ZZ gonads in different developmental stages**

The master sex-determining gene in *Xenopus* the *dm-w* was discovered in 2008 (Yoshimoto et al. 2008), but the molecular machinery of sex determination is certainly very complex and contains many other genes. We previously published the expression profile of known genes involved in sex determination and sexual differentiation in the *Xenopus* gonads (Piprek et al. 2018). We showed that the *gata4, sox9, dmrt1, amh, fgf9, ptgds, pdgf, fshr,* and *cyp17a1* had upregulated expression in testes, while *dm-w, fst, foxl2,* and *cyp19a1* had upregulated expression in the ovary (Piprek et al. 2018).

Here, we compared gene expression level between ZW and ZZ gonads at different stages of gonad development. These analyses showed that at stage NF50 (undifferentiated gonads during sex determination period), there were 820 genes with upregulated expression in ZW gonad (Suppl. Table 13, and chosen genes are shown in Table 12). Functional analysis grouped these genes into several categories (Table 11). Many genes upregulated in this period are uncharacterized. Among known genes upregulated in ZW gonad at stage NF50 is *chordin* (*chrd*). Chordin is a secreted protein responsible for several developmental processes such as dorsalization, head development, and gastrulation (Susai et al. 1994; Pappano et al. 1998; Bachiller et al. 2000); our study indicates that it may play a crucial role in female sex determination (Table 12, Suppl. Table 13). Other genes upregulated in ZW gonad at NF50 are two protease inhibitors, *serpin A3* and *serpin I2*, extracellular glycoprotein vitronectin, metalloproteinases *mmp7* and *adam27*, retinol-binding protein *rbp4*, signaling molecules *wnt10b, wnt11b,* and *igf3*, helicase *ddx25,* and transcription factors *foxa2* and *ilx8*. A role of these factors in sex determination in *Xenopus* is unknown and requires further study.

There were 372 genes with higher expression level in the ZZ (genetic males) gonads at stage NF50 (Suppl. Table 14, and chosen genes are shown in Table 13, and the functional groups are shown in Table 11). Among these upregulated genes are known genes such as epithelium markers keratin 5, 12, and 14, coiled-coil domain containing 50 (*ccdc50*) that acts as an effector in EGF signaling and negative regulator of NF-kB factor (Tsukiyama et al. 2012), signaling molecules: *wnt3a, wnt7b,* growth differentiation factor 3 (*gdf3*), fibroblast growth factor–binding protein 1 (*fgfbp1*), proteases cathepsin K and H, extracellular matrix molecules lumican, collagen IX and I, and decorin. A role of these genes in male sex determination and early testis development remains unknown.

There are 193 genes with a higher expression in ZW (genetic females) gonad at stage NF53 (the onset of sexual differentiation of gonads) (Suppl. Table 15, and chosen genes are shown in Table 14). Functional analysis did not link these genes...
genes to any specific pathway. Among these upregulated
genes, there are the following known genes: retinol-binding
protein 2 (rbp2), protease calpain 8, synuclein gamma with
unknown function, cell adhesion gene claudin 6,
metalloproteinases mmp1 and adam21, and galectin-la in-
volved in cell adhesion and signaling.

There were 890 genes with higher expression in ZZ (ge-
netic males) gonad at stage NF53 (the onset of sexual

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**Table 6** Chosen genes up- and downregulated in ZW (genetic females) gonads at NF62 in relation to NF56 stage

| Probe name | Gene symbol | Gene name | Log FC |
|------------|-------------|-----------|--------|
| A_10_P00661 | spdyc-b | Speedy/RINGO cell cycle regulator C | 5.91483 |
| A_10_P041271 | pabpn1l-a | Poly(A) binding protein, nuclear 1-like | 5.78779 |
| A_10_P078660 | rnf138 | Ring finger protein 138 | 5.43076 |
| A_10_P004355 | pou5f3.3 | POU class 5 homeobox 3, gene 3 | 4.82381 |
| A_10_P002029 | zar1 | Zygote arrest 1 | 4.7962 |
| A_10_P038461 | LOC398389 | Survivin | 4.75826 |
| A_10_P027361 | vegt-a | vegt protein | 4.68137 |
| A_10_P007276 | aldh3b1 | Aldehyde dehydrogenase 3 family, B1 | 4.65557 |
| A_10_P032511 | cldn6.1 | Claudin 6, gene 1 | 4.50308 |
| A_10_P162298 | zp2 | Zona pellucida glycoprotein 2 | 4.43055 |
| A_10_P009533 | gdf1 | Growth differentiation factor 1 | 4.40831 |
| A_10_P002027 | velo1 | velo1 protein | 4.36483 |
| A_10_P027280 | zpd | Zona pellucida protein D | 4.2713 |
| A_10_P205908 | foxh1 | Forkhead box H1 | 4.2256 |
| A_10_P031016 | foxr1 | Forkhead box R1 | 4.10517 |
| A_10_P008731 | wnt11b | Wingless-type MMTV integration site family, member 11B | 4.0833 |
| A_10_P033516 | zpy1 | Zona pellucida protein Y1 | 4.00754 |
| A_10_P117061 | ddx25 | DEAD box helicase 25 | 3.98223 |
| A_10_P040816 | sye3 | Synaptosomal complex protein 3 | 3.70889 |
| A_10_P071715 | lhc8 | LIM homeobox 8 | 3.56271 |
| A_10_P056732 | dppa2 | Developmental pluripotency-assoc 2 | 3.51303 |
| A_10_P027530 | adam21 | ADAM metallopeptidase domain 21 | 2.89064 |

| Probe name | Gene symbol | Gene name | Log FC |
|------------|-------------|-----------|--------|
| A_10_P047196 | LOC100037217 | Uncharacted LOC100037217 | 6.582348 |
| A_10_P180718 | hrg | Histidine-rich glycoprotein | 6.249551 |
| A_10_P00453 | rbp4 | Retinol-binding protein 4 | 5.794043 |
| A_10_P034336 | serpina1 | Serpin peptidase inhibitor, A1 | 5.541168 |
| A_10_P006319 | sag | Arrestin | 5.153979 |
| A_10_P075910 | serpin2 | Serpin peptidase inhibitor, 12 | 4.285183 |
| A_10_P030976 | LOC398504 | Villin-1-like | 3.897723 |
| A_10_P068493 | fetub | Fetuin B | 3.871496 |
| A_10_P110124 | ker12 | Keratin 12 | 3.5294 |
| A_10_P006916 | emx1.2 | Empty spiracles homeobox 1, gene 2 | 3.505748 |
| A_10_P002103 | mmp7 | Matrix metallopeptidase 7 | 3.50358 |
| A_10_P153143 | igfbp4 | Insulin-like growth factor 3 | 3.452683 |
| A_10_P003787 | igfbp1-a | Insulin-like growth factor-binding 1 | 3.06992 |
| A_10_P005507 | ctsl | Cathepsin L | 2.882569 |
| A_10_P137683 | gata2 | GATA binding protein 2 | 2.5687 |
| A_10_P053899 | cdh26 | Cadherin 26 | 2.529154 |
| A_10_P126889 | rdh16 | Retinol dehydrogenase 16 (all-trans) | 2.355785 |
| A_10_P174228 | chr1 | Chordin | 2.347432 |
| A_10_P007857 | timp2 | TIMP metallopeptidase inhibitor 2 | 2.066979 |
differentiation of gonads) (Suppl. Table 16, and chosen genes are shown in Table 15). Functional analysis grouped these genes into several categories (Table 11). The upregulated known genes are coiled-coil domain containing 50 (ccdc50), retinol-binding protein 4 (rbp4), signaling molecules igf1 and igf3, estrogen receptor 2 (esr2), transcription factors, Kruppel-like factor 9 (klf9), Kruppel-like factor 15 (klf15), and foxo1 (forkhead box O1), enzyme glycerophosphodiesterase

Table 7  Chosen genes up- and downregulated in ZZ (genetic males) gonads at NF53 in relation to NF50 stage

| Probe name | Gene symbol | Gene name | Log FC |
|------------|-------------|-----------|--------|
| A_10_P030946 | rbp4 | Retinol-binding protein 4 | 4.97523 |
| A_10_P056207 | vtn | Vitronectin | 4.51992 |
| A_10_P075910 | serpin12 | Serpin peptidase inhibitor, clade I. 2 | 4.34831 |
| A_10_P041856 | igf3 | Insulin-like growth factor 3 | 4.34284 |
| A_10_P03882 | timp3 | TIMP metallopeptidase inhibitor 3 | 2.37097 |
| A_10_P007964 | serpinf2 | Serpin peptidase inhibitor, F2 | 2.32024 |
| A_10_P030126 | esr2 | Estrogen receptor 2 (ER beta) | 2.28938 |
| A_10_P058537 | col9a1-b | Collagen, type IX, alpha 1 | 2.24147 |
| A_10_P048579 | ocn | Oncomodulin | 2.22878 |
| A_10_P002931 | matn2 | Matrilin 2 | 2.06945 |

Genes downregulated (higher expression at NF50 than at NF53)

| Probe name | Gene symbol | Gene name | Log FC |
|------------|-------------|-----------|--------|
| A_10_P017957 | ocn | Oncomodulin | 6.204741 |
| A_10_P140568 | krt5.6 | Keratin 5, gene 6 | 4.866387 |
| A_10_P138508 | krt15 | Keratin 15 | 4.154655 |
| A_10_P126949 | mmp1 | Matrix metallopeptidase 1 | 2.745253 |
| A_10_P008082 | fgfbp1 | Fibroblast growth factor–binding 1 | 2.68819 |
| A_10_P203798 | lum | Lumican | 2.460273 |
| A_10_P222743 | isyna1-b | Inositol-3-phosphate synthase 1 | 2.399986 |
| A_10_P002391 | capn8-a | Calpain 8 | 2.388139 |
| A_10_P040276 | wnt7b | Wingless-type MMTV integration site family, member 7B | 2.038541 |

Table 8  Number of genes assigned to functional groups up- and downregulated in ZZ (genetic male) gonads

| Functional gene groups | ZZ (genetic males) |
|------------------------|--------------------|
|                        | NF53 vs NF50 | NF56 vs NF50 | NF62 vs NF56 |
|                        | Up   | Down | Up   | Down | Up   | Down |
| Signaling factors      | 48   | –    | 13   | 43   | 17   | –    |
| Calcium-binding proteins | –    | –    | 5    | –    | –    | –    |
| Metal-binding proteins | 30   | –    | –    | 21   | –    | –    |
| Monoxygenases          | –    | –    | –    | –    | 3    | 8    |
| Oxidoreductases        | 9    | –    | 8    | –    | 5    | 14   |
| Metalloproteases       | 4    | –    | 3    | –    | –    | –    |
| EGF-like domain–containing proteins | 4    | –    | –    | –    | –    | –    |
| Proteases              | 13   | –    | 14   | –    | 9    | –    |
| Hydrolyses             | 20   | –    | –    | 25   | 12   | –    |
| Disulfide bond–containing proteins | 35   | –    | –    | 31   | 13   | –    |
| Secreted proteins      | –    | –    | –    | 12   | –    | –    |
| Transport proteins     | –    | 3    | –    | –    | –    | –    |
| Steroid hormone synthesis pathway | 4    | –    | –    | 2    | –    | –    |
| Insulin signaling pathway | 4    | –    | –    | 7    | –    | –    |
| PPAR signaling pathway | 4    | –    | 3    | –    | –    | –    |
| Adipocytokine signaling pathway | 5    | –    | –    | 6    | –    | –    |
| Mitochondrial proteins | –    | 7    | –    | –    | –    | –    |
| Ion transport          | –    | 5    | –    | –    | –    | –    |
| Terpenoid backbone biosynthesis pathway | –    | 3    | –    | –    | –    | 3    |
| ER protein processing pathway | –    | 5    | –    | –    | –    | –    |
| Receptors              | –    | –    | 6    | –    | –    | –    |
| Metabolic pathway      | –    | –    | 23   | 8    | –    | –    |
| FoxO signaling pathway | –    | –    | 7    | –    | –    | 45   |
| Cell membrane proteins | –    | –    | –    | –    | –    | 48   |
| Intercellular transport | –    | –    | –    | –    | –    | 21   |
| Retinol metabolism     | –    | –    | –    | –    | –    | 5    |
phosphodiesterase 1 (gde1) responsible for synthesis of signaling molecule lysophosphatidic acid (LPA), cell adhesion proteins gap junction protein alpha 3 (gja3), occluding (ocln), and extracellular matrix component vitronectin (vtn).

There were 75 genes with higher expression in ZW (genetic females) gonad at stage NF56 (Suppl. Table 17, and chosen genes are shown in Table 16, and the functional groups are shown in Table 11). Among known genes are retinoic binding protein 4 and vitronectin.

There were 346 genes with higher expression in ZZ (genetic males) gonad at stage NF56 (Suppl. Table 18, and chosen genes are shown in Table 17, and the functional groups are shown in Table 11). Among known genes are keratin 14 and 15, cell molecule gap junction protein, alpha (gja3), endophilin B2 (sh3glb2) and coiled-coil domain containing 50 (ccdc50).

There were 594 genes with higher expression in ZW (genetic females) gonad at stage NF62 (Suppl. Table 19, and chosen genes are shown in Table 18, and the functional groups are shown in Table 11). Many genes expressed at this stage such as zona pellucida glycoprotein 4 (zp4) and zona pellucida C glycoprotein (xlzpc) are involved in ovarian follicles and oocytes formation and development. Other genes with upregulated expression at this stage were enzyme arachidonate 12-lipoxygenase 12R type (alox12b) responsible for metabolism of a signal compound—arachidonic acid (ARA), signaling factors such as growth differentiation factor 1 (gdf1), Wnt11b, cell adhesion molecules claudin 6 and

| Probe name   | Gene symbol | Gene name                  | Log FC  |
|--------------|-------------|----------------------------|---------|
| A_10_P079665 | rbp2        | Retinol-binding protein 2, cellular | 6.44222 |
| A_10_P043951 | ptger3      | Prostaglandin E receptor 3  | 2.75218 |
| A_10_P036706 | sdf2l1      | Stromal cell-derived factor 2-like 1 | 2.32111 |
| A_10_P000364 | p75NTRa     | p75 neurotrophin receptor a-1| 2.02373 |

| Probe name   | Gene symbol | Gene name                  | Log FC  |
|--------------|-------------|----------------------------|---------|
| A_10_P036346 | LOC100189571 | Uncharacterized LOC100189571 | 8.899836 |
| A_10_P102465 | rbp4        | Retinol-binding protein 4   | 7.96364 |
| A_10_P056207 | vtn         | Vitronectin                 | 7.657184 |
| A_10_P027027 | ptx         | Pentraxin                   | 7.367071 |
| A_10_P041856 | igf3        | Insulin-like growth factor 3| 3.440657 |
| A_10_P002182 | serpincl    | Serpin peptidase inhibitor C1| 2.826371 |
| A_10_P094993 | krt12       | Keratin 12                  | 2.032246 |

| Probe name   | Gene symbol | Gene name | Log FC  |
|--------------|-------------|-----------|---------|
| A_10_P049320 | prss1       | Protease, serine, 1 | 6.7897  |
| A_10_P045961 | prss3       | Protease, serine, 3  | 6.64558 |
| A_10_P259137 | tip11       | Tuftelin-interacting protein 11 | 6.14112 |
| A_10_P027545 | mnp11       | Matrix metallopeptidase 11 | 3.31378 |
| A_10_P027246 | klf9-a      | Kruppel-like factor 9   | 2.7011  |
| A_10_P203798 | lum         | Lumican    | 2.10476 |

| Probe name   | Gene symbol | Gene name | Log FC  |
|--------------|-------------|-----------|---------|
| A_10_P032408 | ocm.2       | Oncomodulin | 7.54298 |
| A_10_P004053 | rbp4        | Retinol-binding protein 4 | 5.65705 |
| A_10_P000084 | krt5.5      | Keratin 5, gene 5     | 4.410092 |
| A_10_P003972 | mnp28-b     | Matrix metallopeptidase 28 | 3.191715 |
| A_10_P044151 | fgfr4-b     | Fibroblast growth factor receptor 4 | 3.091348 |
| A_10_P002657 | isyna1-a    | Inositol-3-phosphate synthase 1 | 3.030967 |
| A_10_P094993 | krt12       | Keratin 12      | 2.838533 |
Table 11  Number of genes assigned to functional groups expressed at higher level in ZW and ZZ gonads

| Functional gene groups                        | NF50 ZW | NF62 ZW | NF50 ZZ | NF62 ZZ |
|-----------------------------------------------|---------|---------|---------|---------|
| Signaling factors                             | 64      | 18      | 50      | 18      |
| Calcium-binding proteins                      | 28      | 3       | 26      | 3       |
| Metal-binding proteins                        | 7       | 3       | 5       | 3       |
| Metalloproteinases                             | 7       | 3       | 5       | 3       |
| Progesterone-mediated oocyte maturation pathway | 20      | 3       | 9       | 3       |
| Proteases                                     | 28      | 3       | 21      | 3       |
| Hydrolases                                    | 42      | 3       | 34      | 10      |
| Disulfide bond–containing proteins            | 15      | 7       | 14      | 6       |
| Extracellular matrix proteins                 | –       | 3       | –       | 3       |
| Markers of epithelial differentiation         | –       | 2       | –       | 2       |
| Meiosis regulation factors                    | –       | –       | –       | –       |
| Oocyte meiosis                                | –       | –       | –       | –       |
| RNA-binding proteins                          | –       | –       | –       | –       |
| Phosphoproteins                               | –       | –       | –       | –       |
| Proteins involved in development              | –       | –       | –       | –       |
| Cytoplasmic proteins                          | –       | –       | –       | –       |
| Oocyte meiosis                                | –       | –       | –       | –       |
| Nuclear proteins                              | –       | –       | –       | –       |
| p53 signaling                                 | –       | –       | –       | –       |
| Secreted proteins                             | –       | –       | –       | –       |
| Transport proteins                            | 14      | 3       | 33      | 5       |
| Metabolic pathway                             | –       | –       | –       | –       |
| Intermediate filaments                        | –       | –       | –       | –       |
| Mitochondrial proteins                        | –       | –       | –       | –       |
| Insulin signaling pathway                     | –       | –       | –       | –       |
| Steroid hormone synthesis                     | –       | –       | –       | –       |
| Adipocytokine signaling pathway               | –       | –       | –       | –       |
| FoxO signaling pathway                        | –       | –       | –       | –       |
| Cell membrane proteins                        | –       | –       | –       | –       |
| Cell division proteins                        | –       | –       | –       | –       |
| Mitotic proteins                              | –       | –       | –       | –       |
| Wnt signaling pathway                         | –       | –       | –       | –       |

Table 12  Chosen genes upregulated in ZW (genetic females) in relation to ZZ (genetic males) gonads at NF50 stage [higher gene expression level in ZW than in ZZ gonads]

| Probe name        | Gene symbol | Gene name                          | Log FC  |
|-------------------|-------------|------------------------------------|---------|
| A_10_P174228      | chrd        | Chordin                            | 11.30213|
| A_10_P007346      | MGC85508    | MGC85508 protein                   | 8.151194|
| A_10_P008816      | serpina3    | Serpin peptidase inhibitor, clade A3 | 6.774417|
| A_10_P075910      | serpin2     | Serpin peptidase inhibitor, clade I2 | 6.378762|
| A_10_P233398      | vtn         | Vitronectin                         | 5.368433|
| A_10_P187778      | wnt11b      | Wingless-type MMTV integration site family, member 11B | 5.00604|
| A_10_P004053      | rbp4        | Retinol-binding protein 4, plasma  | 4.876474|
| A_10_P065884      | wnt10b      | Wingless-type MMTV integration site family, member 10B | 4.20504|
| A_10_P027350      | adam21      | ADAM metalloproteinase domain 21   | 3.851196|
| A_10_P009298      | igf3        | Insulin-like growth factor 3       | 3.848738|
| A_10_P202038      | MGC69070    | Matrix metalloproteinase 7         | 3.690095|
| A_10_P006376      | arxa13      | Annexin A13                        | 3.483353|
| A_10_P003549      | MGC69070    | Matrix metalloproteinase 7         | 3.459862|
| A_10_P003038      | ddx25       | DEAD box helicase 25               | 3.239557|
| A_10_P082395      | foxa2       | Forkhead box A2                    | 3.049031|
| A_10_P003648      | lhx8        | LIM homeobox 8                     | 2.965778|
transcription factors foxr1 and foxh1, and survivin—an inhibitor of apoptosis.

There were 2630 genes with upregulated expression in ZZ (genetic males) gonad at stage NF62 (Suppl. Table 20, and chosen genes are shown in Table 19). Functional analysis grouped these into many categories (Table 11). Among known genes with upregulated expression were factors involved in signaling and signaling pathways: igf1, desert hedgehog (dhh), sonic hedgehog (shh), indian hedgehog (ihh), wnt3a, wnt8b, wnt7b, Janus kinase 2 (jak2), frizzled receptor 4 and 10 (frz4; frz10), cellular retinoic acid–binding protein 2 (crabp2), SMAD family member 4 (smad4); proteases: serine protease 3 (prss3), cathepsin H (ctsh), peptidase inhibitor—serpin2; transcription factors: LIM homeobox 1 (lhx1), homeobox a9, d10, and d13 (hoxa9, hoxd10, hoxd13), foxf1, foxa2, gata2; extracellular matrix components: collagen III (col3a1), collagen I (col1a1), fibrillin 3 (fbn3); extracellular matrix enzymes: mmp2, mmp16, cell adhesion molecule 3 (cadm3); and intermediate filaments: keratin 15 and nestin (nst).

Genes identified here that showed sexual dimorphism of expression can be categorized into several functional groups: (1) signaling molecules: chordin (upregulated in ⇑♂), wnt3a (upregulated in ⇑♂), wnt7b (⇑♂), wnt8b (⇑♂), wnt10b (⇑♀), wnt11b (⇑♀), igf1 (⇑♂), igf3 (⇑♂ and ⇑♀), gdf1 (⇑♂), gdf3 (⇑♀), cdc50 (effector in EGF pathway) (⇑♀), including hedgehog factors (⇑♂); dhh, shh, ihh; (2) retinoic binding proteins: rbp2 (⇑♀), rbp4 (⇑♀ and ⇑♂); (3) enzymes involved in signaling: enzyme glycerolphosphodiester phosphodiesterase 1 (gde1) responsible for synthesis of signaling molecule lysophosphatidic acid (LPA) (⇑♀), enzyme arachidonate 12-lipoxygenase 12R type (alox12b) responsible for metabolism of a signal compound—arachidonic acid (⇑♀); (4) receptors of wnt signaling: fzd4 (⇑♀), fzd10 (⇑♀); (5) proteases: cathepsin H (⇑♂), cathepsin K (⇑♂), calpain 8 (⇑♀); (6) protease inhibitors: serpin A3 (⇑♀),

### Table 13

| Probe name | Gene symbol | Gene name | Log FC |
|------------|-------------|-----------|--------|
| A_10_P136703 | krt14 | Keratin 14 | 7.50258 |
| A_10_P183185 | ccdc50 | Coiled-coil domain containing 50 | 6.57626 |
| A_10_P140568 | krt5.6 | Keratin 5, gene 6 | 5.84154 |
| A_10_P003366 | lum | Lumican | 5.75697 |
| A_10_P193923 | krt14 | Keratin 14 | 5.1494 |
| A_10_P008082 | fgfbp1 | Fibroblast growth factor–binding protein 1 | 3.66899 |
| A_10_P002950 | col9a1 | Collagen, type IX, alpha 1 | 3.07046 |
| A_10_P046256 | ctsk | Cathepsin K | 2.60816 |
| A_10_P036156 | dcn | Decorin | 2.60212 |
| A_10_P244713 | colla1 | Collagen, type I, alpha 1 | 2.56712 |
| A_10_P040276 | wnt7b | Wingless-type MMTV integration site family, member 7B | 2.3506 |
| A_10_P026995 | wnt5a | Wingless-type MMTV integration site family, member 3A | 2.25544 |
| A_10_P094993 | krt12 | Keratin 12 | 2.23416 |
| A_10_P002727 | gdf3 | Growth differentiation factor 3 | 2.07545 |
| A_10_P046876 | ctsk | Cathepsin H | 2.01352 |

### Table 14

| Probe name | Gene symbol | Gene name | Log FC |
|------------|-------------|-----------|--------|
| A_10_P079665 | rbp2 | Retinol-binding protein 2 | 5.229889 |
| A_10_P032636 | LOC100101274 | Uncharacterized LOC100101274 | 3.804135 |
| A_10_P062524 | lgalsia-a | Galectin-Ia | 2.939513 |
| A_10_P008579 | krt5.2 | Keratin 5, gene 2 | 2.846329 |
| A_10_P057292 | snog-a | Synuclein, gamma | 2.52171 |
| A_10_P002391 | capn8-a | Calpain 8 | 2.404349 |
| A_10_P032511 | cldn6.1 | Claudin 6, gene 1 | 2.207111 |
| A_10_P027350 | adam21 | ADAM metallopeptidase domain 21 | 2.177794 |
| A_10_P126949 | mmp1 | Matrix metallopeptidase 1 | 2.07391 |
serpin I2 (♀ and ♂); (7) transcription factors: foxa2 (♀), foxf1 (♂), foxh1 (♀), foxo1 (♂), foxr1 (♀), gata2 (♂), Kruppel-like factor 9 (klf9) (♂), Kruppel-like factor 15 (klf15) (♂); (8) helicase: ddx25 (♀); (9) cell adhesion molecules: occludin (♂), claudin 6 (♀), galecin-a (♀); (10) extracellular matrix components (mainly in ♂): collagen 1,3,9 (♂), vitronectin (♂), decorin (♂), lumican (♂), fibrillin 3 (♂); (11) extracellular matrix enzymes: mmp1 (♀), mmp2 (♂), mmp7 (♀), mmp16 (♂), adam21 (♀), adam27 (♀); (12) oocyte-specific proteins (♀): zp4, xlzpc; (13) epithelium-specific intermediate filaments (♂): keratins 5, 12, 14, 15.

The changes in the level of the expression of several genes listed above indicate that EGF signaling and lysophosphatidic acid (LPA) signaling may be involved in testis differentiation, arachidonic acid signaling may be involved in ovarian differentiation, while the wnt signaling, insulin-like growth factor signaling, and retinol signaling may be involved in gonad development in both sexes.

Interestingly, from the moment of sexual differentiation (after stage NF53), the genes encoding cytoplasmic and nuclear proteins are upregulated in ZW gonads (developing ovaries), while the genes encoding cell membrane proteins are upregulated in ZZ gonads (developing testes) (Fig. 4). The same trend was noted during gonad development in *Silurana tropicalis* (Haselman et al. 2015). This indicates that there are important molecular differences between developing ovaries and testes.

### Table 15

| Probe name | Gene symbol | Gene name | Log FC |
|------------|-------------|-----------|--------|
| A_10_P183185 | ccdc50 | Coiled-coil domain containing 50 | 7.79896 |
| A_10_P009082 | gde1 | Glycerophosphodiester phosphodiesterase 1 | 6.52222 |
| A_10_P030946 | rhbp4 | Retinol-binding protein 4, plasma | 5.8968 |
| A_10_P233398 | vtn | Vitronectin | 5.85368 |
| A_10_P009298 | igf3 | Insulin-like growth factor 3 | 3.50579 |
| A_10_P002488 | gia3 | Gap junction protein, alpha 3, 46 kDa | 3.2409 |
| A_10_P001965 | klf15 | Kruppel-like factor 15 | 2.9746 |
| A_10_P027246 | klf9-a | Kruppel-like factor 9 | 2.74415 |
| A_10_P030126 | esr2 | Estrogen receptor 2 (ER beta) | 2.42444 |
| A_10_P027093 | igfl | Insulin-like growth factor 1 | 2.40431 |
| A_10_P000763 | foxa1 | Forkhead box O1 | 1.21499 |
| A_10_P048579 | ocn-b | Occludin | 1.12035 |

### Table 16

| Probe name | Gene symbol | Gene name | Log FC |
|------------|-------------|-----------|--------|
| A_10_P306346 | LOC100189571 | Uncharacterized LOC100189571 | 5.456322 |
| A_10_P056207 | vtn | Vitronectin | 4.026518 |
| A_10_P030946 | rhbp4 | Retinol-binding protein 4, plasma | 3.555976 |

Comparison of sex-specifically expressed genes in developing gonads of *Xenopus* and other vertebrates

We compared *Xenopus* microarray data to the published microarray data of developing gonads in other vertebrates: mouse (Jameson et al. 2012), chicken (Ayers et al. 2015), a red-eared slider *Trachemys scripta* (Czerwinski et al. 2016), American alligator (Yatsu et al. 2016)—both species with temperature-dependent sex determination, and zebralfish (Sreenivasan et al. 2008). The comparison is shown in Tables 20, 21, and 22.

The transcriptome of developing mouse gonad did not show the expression of *Wnt3, Wnt7, Wnt8, Wnt10, Wnt11,* and chordin (Jameson et al. 2012), which were expressed in *Xenopus* developing gonads. The *Igf1* was expressed in XX (genetic females) mouse gonads at a higher level than in XY gonads (Jameson et al. 2012); however, in *Xenopus*, this gene was expressed in ZZ developing gonads (genetic males). In mouse, in contrast to *Xenopus* (data presented in this study), the developing gonads did not express the *Igf3, Gdf1,* and *Gdf3* (Jameson et al. 2012). The *Ccdc50* was expressed in the developing mouse gonads but did not show sexual dimorphism of expression (Jameson et al. 2012). In *Xenopus*, this gene had an upregulated expression in ZZ gonads. Among hedgehog growth factors, in developing mouse gonads, only the *dhh* was expressed (Jameson et al. 2012). In *Xenopus*, gonads *dhh* and also *shh* and *ihh* were expressed. In mice,
the Rbp1 (in XX) and Rbp4 (in XY gonads) were expressed (Jameson et al. 2012). In Xenopus, the rbp2 was expressed in ZW and rbp4 in ZZ and ZW gonads. Gde1 gene was expressed in developing mouse gonads; however, it did not show sexual dimorphism of expression (Jameson et al. 2012). In Xenopus, this gene had an upregulated expression in ZZ gonads.

Alox12b gene was not expressed in the developing mouse gonads (Jameson et al. 2012) but was upregulated in Xenopus ZW gonads. A subpopulation of fzd receptors was expressed in the developing mouse gonads. In Xenopus, fzd4 and fzd10 had an upregulated expression in developing ZZ gonads. The calpain 8 (Capn8) was not expressed in developing mouse gonads (Jameson et al. 2012) but was upregulated in Xenopus ZW gonads.

The serpins were not expressed in developing mouse gonad (Jameson et al. 2012), but they were expressed in Xenopus developing gonads. In developing mouse gonads, several cathepsins (Cts) were expressed; however, only cathepsin H (ctsh) was upregulated in XY gonads (Jameson et al. 2012), and this gene was also upregulated in ZZ Xenopus gonads. Among forkhead box factors, only Foxo1 was expressed in XY developing mouse gonads (Jameson et al. 2012) and in ZZ Xenopus gonads. Similarly, Lhx1 was expressed in XY developing mouse gonads (Jameson et al. 2012) and ZZ Xenopus gonads. Considering proteins of extracellular matrix, only collagen 9 and metalloproteinase Mmp2 were expressed in a similar manner in XY developing mouse gonads (Jameson et al. 2012) and ZZ Xenopus gonads.

Analysis of transcriptome of developing chicken gonads showed that calpain 5 (Capn5), Gpr56, and Fgfr3 were upregulated in ZW (female) gonads, which suggested that they may be involved in sexual differentiation (Ayers et al. 2015). Calpain 5 was expressed in developing Xenopus gonads, but not in a sex dimorphic manner. We showed the upregulation of calpain 8 in ZW (females) Xenopus gonads, which suggests a role of this group of proteases in sexual differentiation of vertebrate gonads. However, calpain 5 or 8 was not expressed in developing mouse gonads (Jameson et al. 2012). Gpr56 was upregulated in XY mouse and ZW chicken gonads (Ayers et al. 2015; Jameson et al. 2012), but it was not expressed in Xenopus developing gonads. Fgfr3 showed sexual dimorphism of expression in developing chicken gonads (upregulated in ZW) (Ayers et al. 2015) and was also expressed, equally in both sexes, in mouse (Jameson et al. 2012) and Xenopus gonads.

Analysis of transcriptome of a red-eared slider (T. scripta) developing gonads showed that Vwa2, Fdxr, Nov, Kdm6b, Rbm20, and Pcsk6 were upregulated in the male-producing temperature, while Fank1, Avil, Twist1, and Hspb6 were upregulated in the female-producing temperature (Czerwinski et al. 2016). Fdxr2 and Hspb6 were also upregulated in ZW (male) developing gonads of Xenopus, but the sexual dimorphism in

| Probe name | Gene symbol | Gene name | Log FC |
|------------|-------------|-----------|--------|
| A_10_P084685 | krt14 | Keratin 14 | 3.53568 |
| A_10_P171263 | sh3glb2 | SH3-domain GRB2-like endophilin B2 | 3.50581 |
| A_10_P183185 | cdc50 | Coiled-coil domain containing 50 | 3.3565 |
| A_10_P138508 | krt15 | Keratin 15 | 3.13432 |
| A_10_P002488 | gja3 | Gap junction protein, alpha 3, 46 kDa | 2.0898 |

Table 18 Chosen genes upregulated in ZW versus ZZ gonads at NF62 stage [higher gene expression level in ZW than in ZZ gonads]

| Probe name | Gene symbol | Gene name | Log FC |
|------------|-------------|-----------|--------|
| A_10_P009488 | alox12b | Arachidonate 12-lipoxygenase, 12R | 5.326002 |
| A_10_P035553 | zp4-a | Zona pellucida glycoprotein 4 | 4.350343 |
| A_10_P032511 | cldn6.1 | Claudin 6, gene 1 | 4.081705 |
| A_10_P038461 | LOC398389 | Survivin | 3.781997 |
| A_10_P034497 | kpna2 | Importin alpha 1b | 3.634424 |
| A_10_P048511 | foxl1 | Forkhead box H1 | 3.486778 |
| A_10_P009533 | gdf1 | Growth differentiation factor 1 | 3.42356 |
| A_10_P031016 | foxr1 | Forkhead box R1 | 3.378015 |
| A_10_P005051 | xtcp | Zona pellucida C glycoprotein | 2.893829 |
| A_10_P205908 | foxl1 | Forkhead box H1 | 2.859015 |
| A_10_P004066 | LOC397866 | Connexin 38 | 2.845325 |
| A_10_P008731 | wnt11b | Wingless-type MMTV integration site family, member 11B | 2.404891 |
the level of expression was not statistically significant. *Twist1* gene was slightly upregulated in ZZ gonads of *Xenopus*, but the sexual dimorphism in the level of expression was also not significant. We detected the expression of *Nov* and *Pcsk6* in *Xenopus* gonads but these genes did not show a sexual dimorphism of expression. Among *Kdms* genes, we detected only the expression of *kdm6a* but it did not show sexual dimorphism. We did not detect the expression of *Vwa2*, *Rbm20*, *Frankl*, or *Avil* in developing *Xenopus* gonads.

In American alligator, the expression of *Wnt11* was shown at male-producing temperature, which induces the development of the testes (Yatsu et al. 2016). We detected the expression of this gene in ZW (female) developing gonads in *Xenopus*. Analysis of transcriptome of developing testes (Sreenivasan et al. 2008). The ZZ developing *Xenopus* gonads also upregulated the expression of this gene.

This comparison indicates that there is a profound difference in the pattern of gene expression and sexual dimorphism of gene expression between *Xenopus* and other vertebrates. Only few genes indicated above show a similar pattern of expression between *Xenopus* and other vertebrates. This shows how complex and fast-evolving is a molecular regulation of gonad development.

### Table 19

| Probe name       | Gene symbol | Gene name                                                | Log FC |
|------------------|-------------|----------------------------------------------------------|--------|
| A_10_P077615     | MGC116439   | Uncharacterized protein MGC116439                        | 8.36828|
| A_10_P045961     | prss3       | Protease, serine, 3                                      | 7.894  |
| A_10_P075910     | sernpi2     | Serpin peptidase inhibitor, clade I2                     | 4.04603|
| A_10_P143593     | ctsb        | Cathepsin H                                              | 3.91699|
| A_10_P041916     | smad4.1     | SMAD family member 4, gene 1                             | 3.54869|
| A_10_P186858     | lhx1        | LIM homeobox 1                                            | 3.43296|
| A_10_P067362     | igf1        | Insulin-like growth factor 1                             | 3.21852|
| A_10_P037301     | dhh-b       | Desert hedgehog                                          | 3.07896|
| A_10_P004008     | hoxd10      | Homeobox D10                                             | 2.92652|
| A_10_P036201     | krt15       | Keratin 15                                               | 2.86458|
| A_10_P027055     | prss3       | Protease, serine, 3                                      | 2.84344|
| A_10_P047936     | hoxd13      | Homeobox D13                                             | 2.7812 |
| A_10_P026995     | wnt3a       | Wingless-type MMTV integration site family, member 3A    | 2.78056|
| A_10_P002038     | mmp16       | Matrix metalloproteinase 16                              | 2.77842|
| A_10_P137013     | col3a1      | Collagen, type III, alpha 1                              | 2.75957|
| A_10_P143748     | crabp2      | Cellular retinoic acid–binding protein 2                 | 2.74791|
| A_10_P116556     | wnt8b       | Wingless-type MMTV integration site family, member 8B    | 2.72865|
| A_10_P139638     | nes         | Nestin                                                   | 2.71329|
| A_10_P000674     | foxf1-a     | Forkhead box F1                                          | 2.69515|
| A_10_P232633     | fbn3        | Fibrillin 3                                              | 2.64504|
| A_10_P002666     | cadm3       | Cell adhesion molecule 3                                 | 2.53779|
| A_10_P040276     | wnt7b       | Wingless-type MMTV integration site family, member 7B    | 2.52685|
| A_10_P016774     | foxa2       | Forkhead box A2                                          | 2.48915|
| A_10_P050489     | jak2        | Janus kinase 2                                           | 2.48864|
| A_10_P000087     | fzd10-a     | Frizzled class receptor 10                               | 2.46006|
| A_10_P267657     | colla1      | Collagen, type I, alpha 1                                | 2.43227|
| A_10_P162773     | gata2       | GATA binding protein 2                                   | 2.41128|
| A_10_P141938     | hoxa9       | Homeobox A9                                              | 2.3827 |
| A_10_P000694     | fzd4        | Frizzled class receptor 4                                | 2.3253 |
| A_10_P027230     | mmp2        | Matrix metalloproteinase 2                               | 2.11478|

**Conclusion**

In this study, we revealed genes representing many functional groups, which showed sexual dimorphism of expression in developing *Xenopus* gonads. Some of these genes are probably involved in sex determination and sexual differentiation of
the gonads. We also detected a sexual dimorphism of expression of many uncharacterized and unnamed genes. These genes should be characterized and studied further to discover if they are involved in sex determination and sexual differentiation. Comparative analysis of genes expressed in developing gonads of different classes of vertebrates showed striking inter-specific differences. Only few genes showed similarities of expression pattern between the species. This indicates how little we know and how complex, diversified, and evolutionary malleable are molecular mechanisms driving gonad development in vertebrates.

**Material and methods**

**Animals**

Tadpoles of the African clawed frog (*Xenopus laevis* Daudin, 1802) were raised in 10-L aquaria (30 tadpoles per 10 L) at 22 °C, fed daily with powder food Sera Micron (Sera), and staged according to Nieuwkoop and Faber (1956). The tadpoles at four stages (NF50, NF53, NF56, and NF62) were anesthetized with 0.1% MS222 solution, and the gonads were manually dissected under the dissecting microscope. All
Sex determination by PCR

The genetic sex of each tadpole was determined using PCR detection of female-specific dm-w gene. DNA was isolated from tadpole tails using NucleoSpin Tissue Kit (Macherey-Nagel, 740952.240C). The dm-w gene (W-linked female-specific marker) and dmrt1 gene (positive control) were used to determine ZZ or ZW status of tested animals. PCR was performed as previously described (Yoshimoto et al. 2008). Following pairs of primers were used: for dm-w, 5′-CCAC A C C C A G C T C A T G T A A A G - 3′ and 5′-GGGC AGAGTCATATACTG-3′, and for dmrt1, 5′-AAACA G A G C C C A A T T C T G A G - 3′ and 5′-A A C T GCTTGACCTCTAATGC-3′.

Histological analysis

Bouin’s solution-fixed and paraffin-embedded samples were sectioned at 4 μm. Sections were deparafinated, rehydrated, and stained with hematoxylin and picroaniline according to Debreuill’s procedure (Piprek et al. 2012). Sections were viewed under the Nikon Eclipse E600 microscope.

RNA isolation

Total RNA was isolated using Trizol and purified with Direct-zol RNA kit according to the manufacturer’s protocol (Zymo Research, R2061). The total RNA was quantified using NanoDrop 2000, and RIN (RNA Integrity Number) was assessed with Bioanalyzer 2100. All samples used in the study had RIN above 8. In order to obtain a sufficient amount of RNA, the samples from 10 individuals were pooled in each experiment as previously described (Piprek et al. 2018). Total RNA in RNase-free water was frozen at −80 °C until further use.

Microarray analysis

Microarray analysis was performed as previously described (Piprek et al. 2018). Total RNA was labeled with fluorescent dyes using Agilent One-Color Quick Amp Labeling Protocol. RNA isolated from ZW gonads were labeled with Cy3, and RNA from ZZ gonads with Cy5. Fluorescently labeled RNA samples were mixed with Agilent Hi-RPM Hybridization Buffer, and hybridized at 65 °C for 17 h in HybArray12 hybridization station (Perkin Elmer). RNA from ZW and ZZ

Table 20  Comparison of sex-specifically expressed genes in developing gonads of Xenopus and mouse

| Gene          | Xenopus laevis (this paper) | Mouse (Jameson et al. 2012) |
|---------------|-----------------------------|----------------------------|
| Wnt3, Wnt7, Wnt8, Wnt10, Wnt11, chordin | Sexual dimorphism           | No sexual dimorphism       |
| Igf1          | Higher in ZZ                | Higher in XX               |
| Gdf1          | Higher in ZW                | Not expressed              |
| Igf3, Gdf3    | Higher in ZZ                | Not expressed              |
| Cdc50         | Higher in ZZ                | No sexual dimorphism       |
| Dhh, Shh, Ihh | Higher in ZW                | Only Dhh expressed         |
| Rbp           | rbp2 higher in ZW and rbp4 in ZZ and ZW | Rbp1 (in XX) and Rbp4 (in XY) |
| Gde1          | Higher in ZZ                | No sexual dimorphism       |
| Alox12b       | Higher in ZW                | Not expressed              |
| serpins       | Several expressed           | Not expressed              |
| Cathepsin H (ctsh) | ctsh higher in ZZ        | Only Ctsh higher in XY     |
| Foxo1         | Higher in ZZ                | Higher in XY               |
| Lhx1          | Higher in ZZ                | Higher in XY               |
| Col9          | Higher in ZZ                | Higher in XY               |
| MMP2          | Higher in ZZ                | Higher in XY               |
| calpain 8 (Capn8) | Higher in ZW               | Not expressed              |

Table 21  Comparison of sex-specifically expressed genes in developing gonads of Xenopus and chicken

| Gene          | Xenopus laevis (this paper) | Chicken (Ayers et al. 2015) |
|---------------|-----------------------------|-----------------------------|
| calpain 5 (Capn5) | No sexual dimorphism      | Higher in ZW                |
| gpr56         | Not expressed               | Higher in ZW                |
| fgfr3         | Not expressed               | Higher in ZW                |
were mixed together and hybridized to the same chip. The RNA isolated from the gonads in different stages of development was labeled with the same fluorochrome (either Cy3 or Cy5) and hybridized individually to the separate chips. Samples were washed in Gene Expression Wash Buffer 1 (6X SSPE, 0.005% N-lauroylsarcosine; at RT) and Gene Expression Wash Buffer (0.06X SSPE, 0.005% N-lauroylsarcosine; at RT) for 1 min each and immersed in a solution of acetonitrile. Air-dried slides (custom-commercial Agilent-070330 X. laevis Microarray slides) were scanned in the Agilent Technologies G2505C Microarray Scanner at a 5-μm resolution. The microarray experiment was repeated three times.

Data processing

Data processing was performed as previously described (Piprek et al. 2018). TIF files obtained in microarray scanner were processed using Agilent Feature Extraction software version 10.5.1.1. Control and non-uniform features were removed; remaining values for each unique probe sequence were averaged. Log base 2 intensities were median centered between arrays. Differential gene expression was filtered using a statistical significance threshold (FDR < 0.05) and a fold change threshold (2-fold). The data were published in Gene Expression Omnibus (accession number GSE105103). Functional analysis and gene ontology were carried out using DAVID 6.8 (https://david.ncifcrf.gov/tools.jsp) and IPA (Ingenuity Pathway Analysis, Qiagen). First, we compared the level of gene expression between gonads in different stages of development within each sex. The gene expression level at each stage of gonad development was compared to the gene expression level at the previous developmental stage, i.e., the stage NF53 was compared to the stage NF50, the stage NF56 was compared to the stage NF53, and the stage NF62 was compared to the stage NF56. In each comparison, the level of gene expression in the younger stage of gonad development was arbitrarily designated as the reference level of expression. The results of these analyses gave us an overview of the pattern of gene expression in consecutive stages of gonad development. Subsequently, we compared the level of gene expression between genetic female (ZW) versus male (ZZ) gonads at each studied developmental stage.

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Compliance with ethical standards

All individuals used in the experiments were handled according to Polish legal regulations concerning the scientific procedures on animals (Dz. U. nr 33, poz. 289, 2005) and with the permission from the First Local Commission for Ethics in Experiments on Animals.

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References

Ayers KL, Lambeth LS, Davidson NM, Sinclair AH, Oshlack A, Smith CA (2015) Identification of candidate gonadal sex differentiation genes in the chicken embryo using RNA-seq. BMC Genomics 16: 704
Bachiller D, Klingensmith J, Kemp C, Belo JA, Anderson RM, May SR, McMahon JA, McMahon AP, Harland RM, Rossant J, De Robertis EM (2000) The organizer factors Chordin and Noggin are required for mouse forebrain development. Nature 403:658–661
Bar I, Cummins S, Elizur A (2016) Transcriptome analysis reveals differentially expressed genes associated with germ cell and gonad...
development in the Southern bluefin tuna (Thunnus maccoyii). BMC Genomics 17:217
Beverdam A, Koopman P (2006) Expression profiling of purified mouse gonadal somatic cells during the critical time window of sex determination reveals novel candidate genes for human sexual dysgenesis syndromes. Hum Mol Genet 15:417–431
Chen H, Palmer JS, Thiagarajan RD, Dinger ME, Lesieur E, Chiu H, Schulz A, Spiller C, Grimmond SM, Little MH, Koopman P, Wilhelm D (2012) Identification of novel markers of mouse fetal ovary development. PLoS One 7:e41683
Czerwinski M, Natarajan A, Barske L, Looger LL, Capel B (2016) A Xenopus laevis bipotential gonad into testis or ovary is driven by sex-specific cell-cell interactions, proliferation rate, cell migration and deposition of extracellular matrix. Dev Biol 432:298–310
Piprek RP, Damulewicz M, Kloc M, Kubiaj JZ (2018) Transcriptome analysis identifies genes involved in sex determination and development of Xenopus laevis gonads. Differentiation 100:46–56
Sasai Y, Lu B, Steinbeisser H, Geissert D, Gont LK, De Robertis EM (1994) Xenopus chordin: a novel doralizing factor activated by organizer-specific homeobox genes. Cell 79:779–790
Scheider J, Anfonso-Grunz F, Hoffmeier K, Horres R, Groher F, Rycak L, Oehlmann J, Winter P (2014) Gene expression of chicken gonads is sex- and side-specific. Sex Dev 8:178–191
Small CL, Shima JE, Uzunecu M, Skinner MK, Grisswold MD (2005) Profiling gene expression during the differentiation and development of the murine embryonic gonad. Biol Reprod 72:492–501
Sreemivasan R, Cał M, Bartfai R, Wang X, Christoffels A, Orban L (2008) Transcriptomic analyses reveal novel genes with sexually dimorphic expression in the zebrafish gonad and brain. PLoS One 3:e1791
Sun LX, Teng J, Zhao Y, Li N, Wang H, Ji XS (2018) Gonad transcriptome analysis of high-temperature-treated females and high-temperature-induced sex-reversed neomales in nile tilapia. Int J Mol Sci 19(3):E689
Toker A (2005) The biology and biochemistry of diacylglycerol signaling. EMBO Rep 6(4):310–314
Tsukiyama T, Matsuda-Tsukiyama M, Bohgaki M, Terai S, Tanaka S, Hatakeyama S (2012) Ymer acts as a multifunctional regulator in nuclear factor-κB and Fas signaling pathways. Mol Med 18:587–597
Xu D, Shen KN, Fan Z, Huang W, You F, Lou B, Hsiao CD (2016) The testis and ovary transcriptomes of the rock bream (Oplegnathus fasciatus): a bony fish with a unique neo Y chromosome. Genom Data 7:210–213
Yatsu R, Miyagawa S, Kohno S, Parrott BB, Yamaguchi K, Ogino Y, Miyakawa H, Lowers RH, Shigenobu S, Guillette LJ Jr, Ichugi T (2016) RNA-seq analysis of the gonadal transcriptome during Alligator mississippiensis temperature-induced sex-reversal. BMC Genomics 17:77
Yoshimoto S, Okada E, Umemoto H, Tamura K, Uno Y, Nishida-Yoshimoto S, Ikeda N, Izutsu Y, Shiba T, Takamatsu N, Ito M (2010) Opposite roles of DMRT1 and its W-linked parologue, DM-W, in sexual dimorphism of Xenopus laevis: implications of a ZZZW-type sex-determining system. Development 137:2519–2526

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