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# Sex-specific transcriptome of the chicken chorioallantoic membrane

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ABSTRACT

Dimorphism between male and female embryos has been demonstrated in many animal species, including chicken species. Likewise, extraembryonic membranes such as the chorioallantoic membrane (CAM) are likely to exhibit a sex-specific profile. Analysis of the previously published RNA-seq data of the chicken CAM sampled at two incubation times, revealed 783 differentially expressed genes between the CAM of male and female embryos. The expression of some of these genes is sex-dependant only at one or other stage of development, while 415 genes are sex-dependant at both developmental stages. These genes include well-known sex-determining and sex-differentiation genes (DMRT1, HEGM, etc.), and are mainly located on sex chromosomes. This study provides evidence that gene expression of extra-embryonic membranes is differentially regulated between male and female embryos. As such, a better characterisation of associated mechanisms should facilitate the identification of new sex-specific biomarkers.

## 1. Introduction

As in mammals, sexual dimorphism in birds begins with the inheritance of sex chromosomes (ZZ in males and ZW in females). However, in birds, the female determines the sex, unlike mammals. The Z chromosome contains 816 coding genes and 421 non-coding genes, while the W chromosome possesses 103 coding genes and 20 non-coding genes (GRCg6a (GCA_000002315.5)). During early incubation, some embryonic genes are differentially expressed between males and females, and result in the sexual differentiation of gonads that initiates around day 3 of incubation [1]. Male and female gonads become distinctly different by day eight of incubation, with the male gonad exhibiting two tubular structures, while the female gonad is characterised by a well-developed left tubular structure and an atrophied right gonad. Chickens are different from mammals in that their sexual phenotype is not solely governed by gonadal hormones. Recently, the proteome analysis of male and female avian primordial germ cells (PGCs) collected at pre-gonadal stages revealed that PGCs possess an inherent molecular sexual identity [2]. Later in the incubation but before the onset of gonadal development, several genes have been shown to be differentially expressed between male and female embryos. It was observed that the sex-difference in gene expression varies depending on both the tissues and the genes considered. Some of these genes were reported to be more dimorphic in gonads compared with the blastoderm, while the expression of others is similarly dimorphic in both tissues [3]. Experiments analysing mix-sex chimeric gonads or lateral gynandromorph chickens (in which one side of the animal appears male and the other female) also corroborated that some sexual dimorphism traits do result from a cell autonomous sex identity (termed CASI), independently of gonadal hormones [4–6].

Many publications have suggested that gonadal development in chicken is governed by one pivotal regulator gene that is *Z*-linked Doublesex and Mab3 Related Transcription Factor 1 (DMRT1). This gene triggers a cascade of molecular events associated with gonadal sex determination and in particular, testis development. Indeed, the over-expression of DMRT1 in female embryos results in their masculinisation [7,8]. In contrast, the knockout of one copy of DMRT1 ($DMRT1^{-/Z}$) in male chickens induces gonadal feminisation [9]. Although these two

*Abbreviations:* AMH, anti-Mullerian hormone; CAM, chorioallantoic membrane; CASI, cell autonomous sex identity; CYP19A1, Cytochrome P450 Family 19 Subfamily A member 1; DMRT1, Doublesex- and mab-3-related transcription factor 1; FOXD1, Forkhead box protein D1; FOXL2, Forkhead Box L2; GLDC, Glycine dehydrogenase (decarboxylating), mitochondrial precursor; HEMGN, hemogen; HOMER1, Homer scaffolding protein 1; sex-DEG, differentially expressed genes between males and females; EID, embryonic incubation day; FC, fold-change; LncRNA, long non-coding RNA; MAP1B, Microtubule-associated protein 1B; miRNA, microRNA; padj, adjusted *p*-value; PGC, primordial germ cell; PRLR, prolactin receptor; RLN3, Relaxin-like peptide locus B; SOX9, SRY-box; SPEF2, sperm flagellar 2.

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examples illustrate the major role of DMRT1 in gonad differentiation, DMRT1 disruption has been shown to be associated with incomplete testis feminisation in male chickens, from day 8 of incubation onwards [9]. Moreover, male and female DMRT1-disrupted chickens both exhibit reproductive organs that are immature at the adult stage [9]. In males, the double dose of DMRT1 factor stimulates downstream activation of the expression of several genes such as SOX9 (SRY-box, chromosome 18), AMH (anti-Mullerian hormone, chromosome 28) [10], and *Z*-linked HEMGN (hemogen) genes which are necessary for testes development [1,11]. All published data suggest that two functional DMRT1 copies are required to determine the male phenotype. However, the presence of one copy of DMRT1 is not sufficient to determine the adult secondary female characteristics [5], which suggests a role for other genes. The female phenotype is characterised by the expression of FOXL2 (Forkhead Box L2, located on chromosome 9) that induces the expression of Cytochrome P450 Family 19 Subfamily A member 1 (CYP19A1) also termed aromatase, located on chromosome 10 [1,11]. Aromatase catalyses the formation of C18 oestrogens from C19 androgens [12], thereby promoting the development of the ovarian cortex. In addition to these genes, several DMRT1-independent genes including long-chain RNAs and microRNAs are suspected to participate in sex determination [9,13,14]. In contrast, there is no evidence that some W-linked genes, including HINTW (histidine triad nucleotide binding protein W) that was one of the most promising candidates [15], contribute to sex determination. Nevertheless, the W chromosome seems to be crucial to maintain the female phenotype [5,16,17], as it is supposed to regulate the activation of *Z*-linked genes and early gonadal development [18].

Most articles published on sexual dimorphism and sex determination during embryonic development focus on the embryo (blastoderm, gonads, brain, etc.) [3,19] but only a few articles have reported that extra-embryonic structures may also exhibit molecular differences between male and female embryos. Extra-embryonic structures (the allantoic sac, the yolk sac, and the chorioallantoic membrane) are crucial in that they ensure vital functions to accompany the development of the embryo during incubation. Interestingly, the chicken chorioallantoic membrane (CAM) that develops from day 5 of incubation, has been shown to synthesise and respond to steroid hormones in reptiles and birds [20]. Knowing that the CAM is involved in mineral metabolism, innate immunity, and gaseous exchanges, such a differential expression may also reflect sex-dependent differences in the metabolism and development of embryos [21]. Recently, we performed and published statistical analysis of the CAM transcriptome between two stages of development that initially aimed to explore the physiological functions of this extra-embryonic structure, and revealed an interaction between sex and stage of development for four genes [22]. All four genes are located on sex chromosomes (GRC6a chicken assembly): tropomodulin 1 (ENSGALG00000002125, chromosome Z), heterogeneous nuclear ribonucleoprotein K-like (ENSGALG00000040086, chromosome W), E3 ubiquitin-protein ligase KCMF1-like (LOC431003, chromosome W) and a lncRNA (ENSGALG00000048050, chromosome Z).

In the present article, we performed a new statistical analysis of the raw data [22] (supplementary data S1, Complete) to specifically explore the differentially expressed genes between the CAM from male and female embryos (sex-DEG), and how they are regulated between 11 and 15 days of incubation. The expression of ten selected sex-DEG was further studied from 6 days of incubation to 15 days of incubation by RT-qPCR, to investigate the onset of their sex-dependent expression. This study provides additional candidates to complete the repertoire of sexually dimorphic markers that characterise eggs containing male and female embryos during embryonic development. It also highlights the potential role of the chorioallantoic membrane in the phenotypic determination of future male and female chickens, via the activation of lncRNAs, and Z and W-linked genes.

## 2. Materials and methods

### 2.1. Ethics statement, handling of fertilised eggs, preparation of CAM samples, RNA extraction and sequencing

The procedure used to incubate fertilised eggs, collect CAM tissue and perform RNA sequencing is detailed in [22]. As previously mentioned, the protocol was in compliance with the European legislation on the "Protection of Animals Used for Experimental and Other Scientific Purposes" (2010/63/UE). It followed the guidelines approved by the institutional animal care and use committee (IACUC). All experiments were conducted in accordance with the ARRIVE essential 10 and Recommended Set of ARRIVE guidelines [23]. The datasets supporting the results and the discussion of the article are available at the NCBI Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/geo) using GSE199780 as the accession number. As previously described [22], one hundred and fifty fertilised eggs produced by 33-week broiler hens (ROSS 308) were purchased from a French hatchery (Boyé Accouvage, La Boissière en Gâtines, France) and one hundred fertilised eggs from 33-week old layer hens (ISA Brown) were obtained from INRAE, PEAT, Centre Val de Loire, France (DOI: 10.15454/1.5572326250887292E12). They were further maintained in the Poultry Experimental Facility (PEAT) UE1295 (INRAE, F-37380 Nouzilly, France, DOI: 10.15454/1.5572326250887292E12). Eggs were stored for three days at 75% relative humidity, 16 °C and incubated for 11 or 15 days under standard conditions (45% relative humidity, 37.8 °C, automatic turning every hour, Bekoto B64—S, Pont-Saint-Martin, France). In the present study, we performed an additional experiment using 33-week-old layer hens (hens, ISA Brown, Hendrix Genetics, St Brieuc, France) where fertilised eggs were incubated for 6 or 8 days (EID6 and EID8, respectively), under the same aforementioned conditions. This latter experiment aimed at investigating whether genes that were shown to be differentially expressed in CAM at EID11 and EID15 in both broiler and layer strains, were sex-DEG at earlier stages (EID6 and EID8, ISA Brown layer strain only).

### 2.2. RNA-seq analysis

All the procedures for collection of CAM samples, RNA extraction and RNA-seq analysis and molecular sexing are described in [22]. RNA-seq analysis was performed on EID11 and EID15 CAMs collected from broiler ROSS 308 eggs (i.e. 40 samples; $n = 20$ including ten male CAMs and ten female CAMs for EID11 and EID15). Samples were analysed by RNA sequencing (HiSeq PE150, Novogene, Cambridge, CA, UK), according to the internal procedures of the manufacturer [22]. After the quality check procedure, mRNA was enriched using oligo(dT) beads and fragmented randomly using fragmentation buffer. cDNA synthesis was performed using random hexamers and reverse transcriptase. The second strand synthesis was synthetized by nick translation using a custom second-strand synthesis buffer (Illumina, San Diego, CA, USA), dNTPs, RNase H and *Escherichia coli* polymerase I. The final cDNA library was ready after a round of purification, terminal repair, A-tailing, ligation of sequencing adapters, size selection, and PCR enrichment. Library concentration was first quantified using a Qubit 2.0 fluorometer (Life Technologies, CA, USA). The insert size was checked (Agilent 2100 bioanalyzer, Santa Clara, CA, USA) and quantified using quantitative

PCR. Libraries were analysed using Illumina technology (San Diego, CA, USA). The original raw data were transformed to Sequenced Reads by base calling. Raw data were recorded in a FASTQ file, which contains sequence information (reads) and corresponding sequencing quality information. Raw reads were then filtered to remove reads with adapter contamination or reads with low quality. Gene expression level was assessed by transcript abundance. The gene expression levels were estimated by counting the reads that mapped to genes or exons. Alignments to the reference *Gallus gallus* genome (GRCg6a (GCA_000002315.5), [24]) were performed with HISAT2 [25] that uses a graph-based alignment. HTSeq software [26] was used to quantify the gene expression levels using the union mode.

Resulting data are shown in Supplementary data S1 (S1_complete).

### 2.3. RT-qPCR of ten selected candidate genes

Total RNA samples (1 μg) from layer CAM samples collected at EID6 ($n = 2$ males and n = 2 females, this study), EID8 ($n = 4$ males and n = 4 females, this study), EID11 and EID15 (n = 4 males and n = 4 females, samples from [22]), and from broiler CAM at EID11 and EID15 (n = 4 males and n = 4 females, samples from [22]) were reverse transcribed using RNase H-MMLV reverse transcriptase (SuperscriptTM II RT, Invitrogen, Cergy Pontoise, France) without RNaseOUT®.

Primers to detect the expression of ten candidate genes and four housekeeping genes (beta-actin, stromal antigen 2 (STAG2), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) and TATA box binding protein (TBP)) were designed using Primer-BLAST (NCBI, https://www.ncbi.nlm.nih.gov/) and synthesised (Eurogentec, Seraing, Belgium). The sequences and characteristics of each pair of primers used to explore gene expression in male and female samples are given in Supplementary data S2.

The gene expression was analysed by RT-qPCR using LightCycler® 480 SYBR Green I Master and LightCycler® 480 instrument II (Roche, Basel, Switzerland). Four to eight biological replicates were used, and three technical replicates were performed for each sample. GenNorm® software was used for validation of housekeeping gene stabilities [27]. The normalised expression of candidate genes were calculated using the following formula: gene efficiency^(ctcalibrator – ctsample) / geometric average quantity of housekeeping genes.

### 2.4. Statistical analyses

For RNA-seq analysis, all statistical analyses were performed on raw data [22] using the R software [28], version v.4.2.1, and the edgeR package v.3.38.4 [29] in Bioconductor project v.3.17 [30]. Briefly, after the filtering and the normalisation steps, the negative binomial generalized linear model (GLM) [29] with the factors "day of incubation" (EID11, EID15) and "sex", and the "day of incubation" x "sex" interaction effect, was fitted for each gene. *P*-values were adjusted by controlling the false discovery rate (<0.05) using Benjamini-Hochberg correction. Genes differentially expressed between male and female CAM were obtained by testing three comparisons: differentially expressed genes between male and female CAMs at EID11 (Supplementary data S1, Sex_DEG_EID11), differentially expressed genes between male and female CAMs at EID15 (Supplementary data S1, Sex_DEG_EID15), and differentially expressed genes between male and female CAMs (Supplementary data S1, Sex_DEG (EID11_EID15)). The compilation of sex-DEG obtained with the three comparisons is available in supplementary data S1 (Sex_DEG_all).

For RT-qPCR analysis, depending on the normality of the data, statistical analyses to explore differential gene expression between male and female CAMs were performed using a *t*-test or a Mann-Whitney test ($p < 0.05$).

## 3. Results

### 3.1. Overview of differentially expressed genes (RNA-seq data)

As shown in Fig. 1, 539 genes are differentially expressed between male and female CAMs (sex-DEG) after 11 days of incubation (EID11) (Supplementary data S1, Sex_DEG_EID11, Fig. 1) and 510 genes are sex-DEG after 15 days of incubation (EID15) (Supplementary data S1, Sex_DEG_EID15, Fig. 1). Most sex-DEG at EID11 are also sex-DEG at EID15 (415 genes) but some of them are uniquely sex-DEG at EID11 (124 genes) or EID15 (95 genes) (Supplementary data S1, Sex_DEG_all, Fig. 1). The statistical analysis of sex-DEG from the three comparisons obtained at EID11 and EID15, indicated 714 sex-DEG with 149 sex-DEG that are uniquely statistically different when compiling data from both stages (Supplementary data S1, Sex_DEG_all; Fig. 1). Of interest, 40 genes are only sex-DEG at EID11 and 29 genes are only sex-DEG at EID15 (Supplementary data S1, Sex_DEG_all; Fig. 1). To summarise, the statistical analyses performed on EID11 samples, EID15 samples or when combining EID11 and EID15 samples gave a total of 783 genes that were found to be differentially expressed between male and female CAMs after 11 and 15 days of incubation (Supplementary data S1, Sex_DEG_all). These sex-DEG include 223 genes that are overexpressed in female CAMs and 560 genes in male CAMs (Fig. 1). They represent 5.6% of the 14,078 genes identified in the CAM by RNA sequencing (supplementary data S1, Complete; [22]).

### 3.2. Features of CAM sex-DEG (RNA-seq data)

Regarding genes that are overexpressed in female CAMs, the $\log_2$ fold-change ranges from −0.11 (copine 1, CPNE1, located on the chromosome 20) to −10.86 (zinc finger protein 532-like, LOC425347/ENSGALG00000035785, located on the W chromosome) (Supplementary data S1, Sex_DEG (EID11_15)). For genes that are overexpressed in male CAMs, the $\log_2$ fold-change ranges from 0.12 (transmembrane p24 trafficking protein 7, TMED7, located on the Z chromosome) to 1.71 (CD180 molecule, CD180, located on the Z chromosome). Most sex-DEG are linked to Z and W chromosomes (60.7% and 5.4%, respectively)

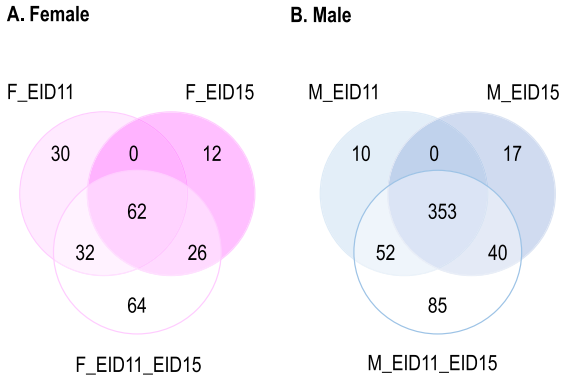


**Fig. 1.** Venn diagrams of the number of genes differentially expressed between male and female CAMs. A) Genes overexpressed in female CAMs. B) Genes overexpressed in male CAMs.
F_EID11, genes overexpressed in female CAMs after 11 days of incubation (EID11); F_EID15, genes overexpressed in female CAMs after 15 days of incubation (EID15); F_EID11_EID15, genes overexpressed in female CAMs;
M_EID11, genes overexpressed in male CAMs after 11 days of incubation (EID11); M_EID15, genes overexpressed in male CAMs after 15 days of incubation (EID15); M_EID11_EID15, genes overexpressed in male CAMs.
Venn diagrams were prepared with JVenn tool [53].

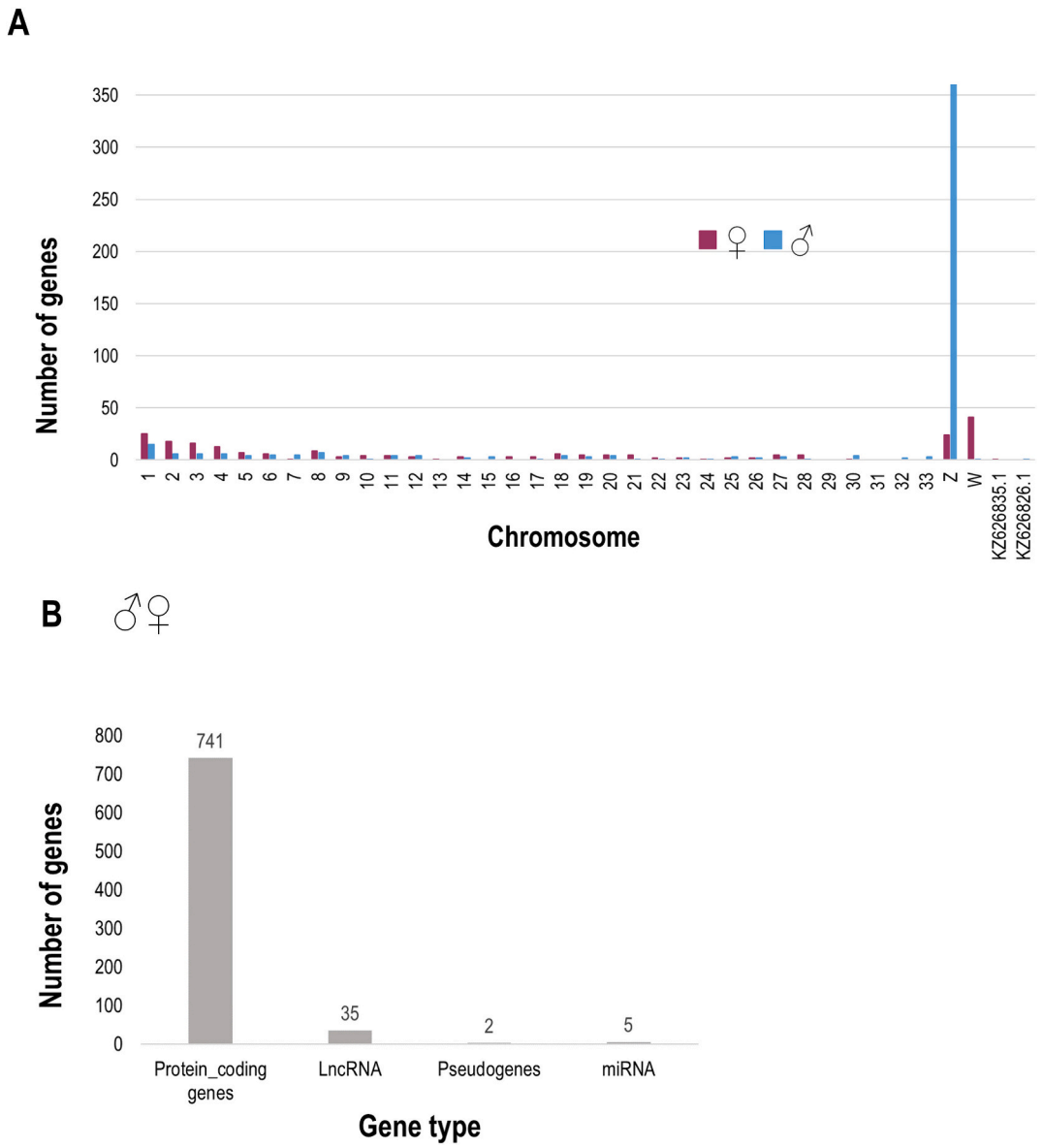


**Fig. 2.** Chromosomal location and gene type of 783 sex-DEG in CAM after 11 and 15 days of incubation (Supplementary data S1, Sex_DEG_all). A) Chromosome location. B) Gene type associated with sex-DEG. LncRNA: long non-coding RNAs; miRNA. This nomenclature was extracted from the Ensembl website [54].

(Fig. 2A). As expected, W-linked DEG are associated with overexpression in female CAMs except for the coding W-linked gene ENSGALG00000047434 that is overexpressed in male CAMs. However, this discrepancy may be due to a misplacement of this latter gene in the GRC6a chicken assembly (GCA_000002315.5) as the accession number is no longer referenced in the last released database (GCA_016699485.1). Most sex-DEG are protein coding genes but a significant number of long non-coding RNAs (lncRNAs) and micro-RNAs (miRNAs) were also identified (Fig. 2B).

Table 1 and Table 2 list the top-ten genes that are DEG between male and female CAMs, respectively. The top-ten genes overexpressed in male CAMs include two lncRNA and eight genes that are located on the Z chromosome (Table 1). Concerning the top-ten genes overexpressed in female CAMs, they all locate on the W chromosome (Table 2). Consequently, they are not expressed in male CAMs, regardless of the developmental stage (Supplementary data S1).

### *3.3. Expression of ten sex-DEG in CAM from a broiler strain (validation of RNA-seq data) and a layer strain, by RT-qPCR*

To validate the RNA-seq data, seven genes that exhibited an overexpression in female CAMs (LOC100859072, RPTC15L, LOC107049046, HINTW, LOC431003, NIPBLL, MAP1B) and three genes showed to be overexpressed in male CAMs (PPIC, FAM172A, and ALDH7A1) (Supplementary data S2) were further analysed by RT-qPCR using the same EID11 and EID15 CAM samples that were used for RNA sequencing (ROSS 308, broiler strain). Some of these genes (PPIC, NIPBLL, RPTC15L, and LOC431003) were chosen from the top-ten DEG lists (Table 1 and Table 2). All these genes are located on the W or Z chromosomes (Supplementary data S2). The expression of these genes was also investigated in CAM samples collected at EID6, EID8, EID11 and EID15 on a layer strain (ISA Brown). This second study allowed the exploration of the potential strain-specificity of their sex differential

**Table 1**
Top-ten genes that are overexpressed in male CAMs (highest male *versus* female $Log_2$ Fold Change, Supplementary data S1; Sex_DEG (EID11_EID15). F, female; M, male.

| Ensembl gene ID (GRC6a)/NCBI gene ID | Gene symbol | Gene name | Chromosome location (GRC6a/GRC7b assemblies)[a] | $Log_2$ Fold Change |
|---|---|---|---|---|
| ENSGALG00000023411 /431584 | CD180 | CD180 molecule | Z/Z | 1.7 |
| ENSGALG00000047764 /431593 | HSPB3 | heat shock protein family B (small) member 3 | Z/Z | 1.6 |
| ENSGALG00000052282 | LncRNA | N/A | Z/? | 1.5 |
| ENSGALG00000023552 /427435 | SPEF2 | sperm flagellar 2 | Z/Z | 1.5 |
| ENSGALG00000002125 /395883 | TMOD1 | tropomodulin 1 | Z/Z | 1.3 |
| ENSGALG00000044243 | LncRNA | N/A | Z/? | 1.2 |
| ENSGALG00000015145 /427255 | TMC1 | transmembrane channel like 1 | Z/Z | 1.2 |
| ENSGALG00000025649 /112530794 | LOC112530794 | small nucleolar RNA SNORA47 | Z/Z | 1.2 |
| ENSGALG00000045282 /101750451 | LOC101750451 | coiled-coil domain-containing protein 81-like | Z/Z | 1.1 |
| ENSGALG00000005346 /768427 | PPIC | peptidylprolyl isomerase C | Z/Z | 1.1 |

[a] Bioinformatic analysis of RNA sequencing data, including chromosomic location, was performed using GRC6a chicken genome assembly as the reference. The location of these genes was further verified using the GRC7b chicken genome assembly. N/A, non-applicable.

**Table 2**
Top-ten genes that are overexpressed in female CAMs (lowest male *versus* female $Log_2$ Fold Change, Supplementary data S1; Sex_DEG_(EID11_15)).

| Ensembl gene ID (GRC6a)/NCBI gene ID | Gene symbol | Gene name | Chromosome location (GRC6a/ GRC7b)[a] | $Log_2$ Fold Change |
|---|---|---|---|---|
| ENSGALG00000035785/425347 | LOC425347 | zinc finger protein 532-like | *W*/W | −10.9 |
| ENSGALG00000039023/427025 | LOC427025/ NIPBL/ NIPBLL | Nipped-B homolog-like | W/W | −10.7 |
| ENSGALG00000031327/374195 | LOC374195/ CHD1 | chromodomain helicase DNA binding protein 1 | W/W | −10.5 |
| ENSGALG00000038064/426615 | LOC426615/ MIER3L | mesoderm induction early response protein 3-like | W/W | −10.4 |
| ENSGALG00000034488/427010 | LOC427010/ ZFRL1 | zinc finger RNA-binding protein-like | W/W | −10.4 |
| ENSGALG00000033705/430766 | LOC430766/ TERAL2 | transitional endoplasmic reticulum ATPase-like | W/W | −10.1 |
| ENSGALG00000035780/431003 | LOC431003 | E3 ubiquitin-protein ligase KCMF1-like | W/W | −10.1 |
| ENSGALG00000040086/426516 | HNRNPKL | heterogeneous nuclear ribonucleoprotein K-like | W/W Unlocalised Scaffold | −9.9 |
| ENSGALG00000027170/100859602 | LOC100859602 | zinc finger SWIM domain-containing protein 6-like | W/W | −9.9 |
| ENSGALG00000034905/107055444 | LOC107055444/ RPTC15L | activated RNA polymerase II transcriptional coactivator p15-like | W/W | −9.8 |

[a] Bioinformatic analysis of RNA sequencing data, including chromosomic location, was performed using GRC6a chicken genome assembly as the reference. The location of these genes was further verified using the GRC7b chicken genome assembly.

expression, and whether this sex difference in expression is detectable at earlier developmental stages.

The seven genes shown to be overexpressed in female CAMs by RNA sequencing (six located on the W chromosome and one on the Z chromosome), were all shown to be overexpressed in female CAMs by RT-qPCR of the broiler strain (Fig. 3A, C, D, E, F, G, I), which validates RNA-seq data. For LOC100859072, LOC107049046, and HINTW, the expression was higher at EID15 compared with EID11 (Fig. 3A, E, and G), while that of MAP1B was lower at EID15 compared with EID11 (Fig. 3F). The expressions of RPT15L (Fig. 3C), NIPBLL (Fig. 3D) and LOC431003 (Fig. 3I) remain comparable between the two stages. The expression of all these genes in the layer CAMs was detectable as early as EID6, corresponding to the onset of CAM formation. However, some significant differences ($p < 0.05$) between the layer and the broiler strains could be noticed for some genes: at both EID11 and EID15 stages for LOC100859072 ($p = 0.039$ and $p = 0.0048$, respectively) and RPTC15L ($p = 0.023$ and $p = 0.015$, respectively), at EID11 for LOC1070449046 ($p = 0.0436$) and MAP1B ($p = 0.0329$), and at EID15 for LOC431003 ($p = 0.0001$). The expression of HINTW and NIPBLL genes was not statistically different between the two strains. Thus, the overall pattern of expression of these ten genes is the same in broilers and layers but the level of expression may differ for some genes at some developmental time points.

The three genes that were shown to be overexpressed in the male CAMs by RNA sequencing (PPIC, FAM172A, and ALDH7A1) have a comparable overexpression in male CAMs (broiler CAMs) by RT-qPCR (Fig. 3B, H and J). These genes are located on the Z chromosome (Supplementary data S2) and are expressed by both male and female CAMs (Fig. 3B, H, and J). For PPIC, FAM172A, and ALDH7A1 genes, the expression remains the same level at EID11 and EID15, regardless of the strain. Conversely, the expression of PPIC in male CAMs is higher in the layer strain compared with the broiler strain, at EID11 and EID15 ($p = 0.006$ and $p = 0.0001$, respectively). In addition, in the male CAM, the expression of PPIC and FAM172A increases from EID6 to EID15 (Fig. 3B and H, respectively), while the expression of ALDH7A1 remains stable during incubation (Fig. 3J).

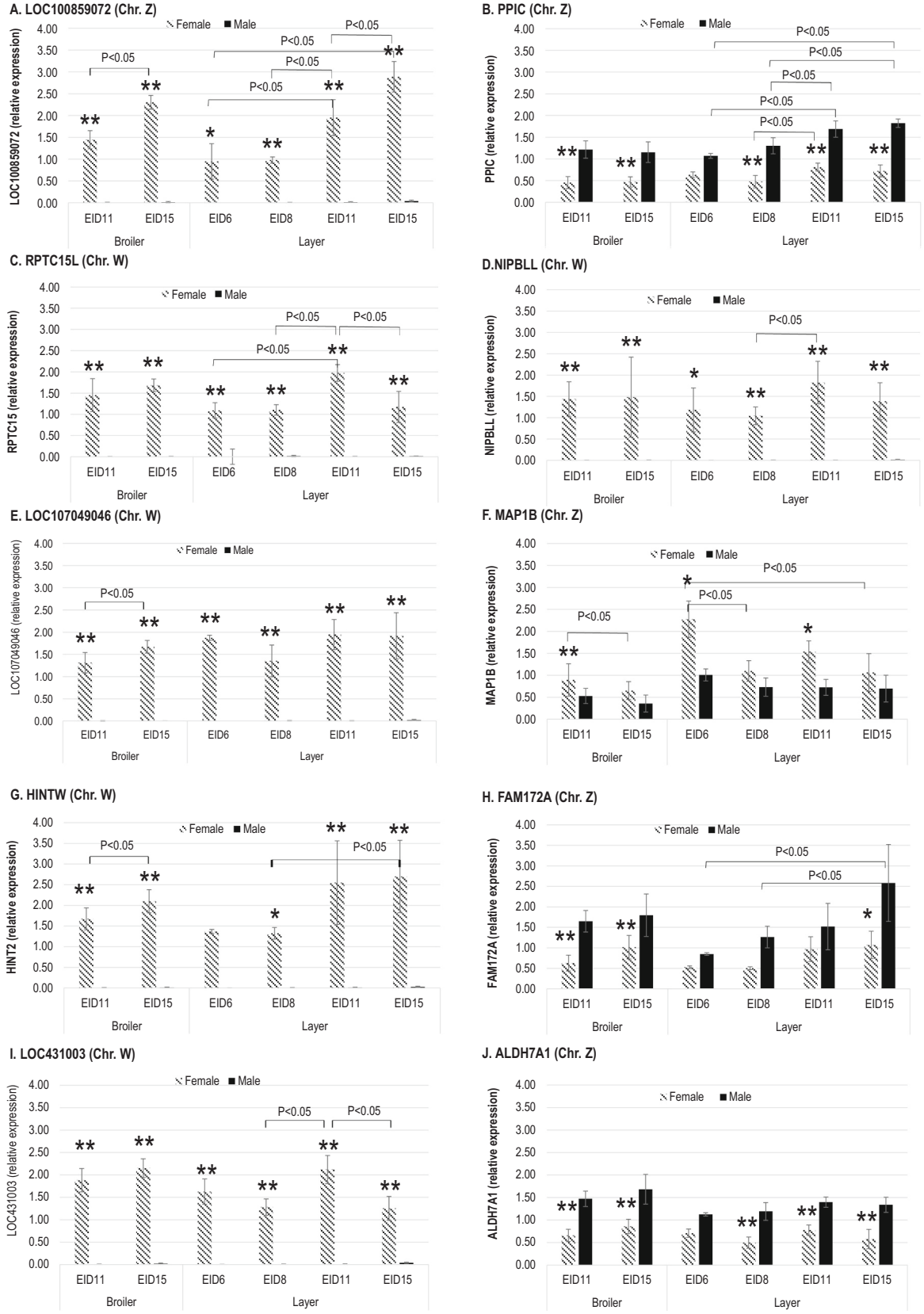


**Fig. 3.** Comparison of the expression of 10 sex-DEG in CAMs collected from two chicken strains (Ross 308 broiler strain and ISA Brown layer strain), by RT-qPCR. Statistical analyses were performed as described in Material and methods. N = 4 males and n = 4 females for EID8, EID11, and EID15. N = 2 males and n = 2 females for EID6. EID: embryonic incubation day. Statistical differences between broiler CAMs and layer CAMs are not indicated to avoid overloading the figure but are detailed in the text.

## 4. Discussion

Like embryos, extra-embryonic membranes are supposed to express genes that depend on the sex of the embryo. As functional supporting structures for the developing embryo, the analysis of the sex-specific profile of these membranes may contribute to explain the differences in the metabolism and the phenotype between male and female embryos. The analysis of the CAM transcriptome after sampling at a non-differentiated stage (EID11) and an active stage (EID15) revealed a total of 783 genes that are differentially expressed between male and female CAMs. Considering the 560 overexpressed genes in male CAMs and the 223 overexpressed genes in female CAMs, the number of genes overexpressed in male CAMs is 2.5 times higher than in female CAMs. About 57.6% of the genes overexpressed in male CAMs are located on the Z chromosome, which suggests that the acquisition of the phenotype and molecular profile of the male CAM strongly depends on *Z*-linked genes. The 475 genes overexpressed in male CAMs represent 28.4% of the 1671 genes identified on the Z chromosome (coding genes, non-coding genes, and pseudogenes). For female CAMs, *Z*-genes account for 10.8% of the 223 genes overexpressed in female CAMs (1.4% of the 1671 genes located on the Z chromosome), while W-genes represent 18.4% (22.9% of the 179 genes located on the W chromosome). Therefore, 29.2% of the genes that are overexpressed in female CAMs belong to sex chromosomes. Although many of sex-DEG are autosome-linked (42.4% and 70.8% for male and female CAMs, respectively), the genes undergoing the highest fold change between male and female CAMs are located on sex chromosomes (Table 1 and Table 2). Altogether, these data highlight that the chorioallantoic membrane exhibits a sex-specific transcriptome that is likely to be driven by Z and W-linked genes. Their expression is supposed to contribute to define, fix, and maintain the molecular and phenotypic dimorphism between male and female embryos, and presumably that of animals after hatching.

DMRT1 and FOXL2 gene expression during incubation is crucial to determine the male and female phenotype, respectively [1,11]. Their expression triggers the expression of many other genes that are essential for primary sex determination. Our results showed that DMRT1 is overexpressed in female CAMs at EID11 while FOXL2 could not be found in the raw data of RNA sequencing (Table 3). SOX9 and AMH that are essential to male differentiation [31] were not sex-DEG, while HEGM was up-regulated in male CAMs (Table 3). None of the other genes known to be associated with female gonad differentiation (CYP19A, WNT4, RSPO and CTNNB1) [32] were sex-DEG in the CAM (Table 3). This observation confirms that the CAM is unlikely to participate in sexual differentiation of gonads that is initiated at EID3. It is noteworthy that the differential expression of ten sex-DEG of the CAM was observed as early as it forms, around day 6 of incubation (Fig. 3). The presence of 783 sex-DEG in the CAM transcriptome, many of them being *Z*- or W-linked, suggests that this structure might be involved in other traits of sexual dimorphism (metabolism, growth, development of secondary sexual traits, etc.).

*Z*-linked DMRT1 transcripts were previously reported to be at least two-fold higher in male gonads during early incubation [33,34]. Since male chicken embryos have two Z chromosomes, there was an initial assumption that Z chromosome compensation was necessary for sex determination [35–37]. However, dosage compensation seems to be gene-dependant [37,38]. In the CAM transcriptome, the comparison of the level of transcript of *Z*-DEG between male and female CAMs brings new evidence of a gene-dependant dosage compensation (Fig. 3). Most Z-DEG (355 genes) were found to be dosage compensated in male CAMs (Fig. 4; fold change = 1.1–1.7). Of particular interest, three Z-linked hydroxysteroid deshydrogenases (HSD) were overexpressed in male CAMs compared with female CAMs but not dosage compensated: HSD17B3, that is involved in synthesis of testosterone [39], HSD17B4 that regulates oestrogen level [40], and HSDL2 that is supposed to regulate hormone bioavailability through its sterol carrier domain [41] (supplementary data S1). In contrast, the expression of 92 genes is doubled in male CAMs (Fig. 4; fold change =1.8–2.5). Among these, we found several genes whose expression has previously been found to be also doubled in other male embryonic tissues compared with those of females (spindlin 1-Z, SPIN1Z [42]; histidine triad nucleotide binding protein, HINT [19]; Cyclin H, CCNH [19], etc.), suggesting an absence of dosage compensation for these genes. These not-compensated genes also include a microRNA, mir-2954, whose expression was reported to be significantly higher in male embryos and gonads than in females [43], in contrast to our CAM study where male to female fold change = 2.05 (supplementary data S1, Sex_DEG_(EID11_15)). Interestingly, four of Z-

**Table 3**
Data analysis of genes associated with male and female sex determination, and sex differentiation. Sex-DEG, differentially expressed genes between male and female CAMs.

| Gene ID | Gene symbol | Gene description | Expression in CAM transcriptome | Chromosome location |
|---|---|---|---|---|
| ENSGALG00000010160 / 769,693 | DMRT1 | doublesex and mab-3 related transcription factor 1 | up in female CAMs at EID11 | Z |
| ENSGALG00000004386 /374148 | SOX9 | SRY (sex determining region Y)-box 9 | not sex-DEG | 18 |
| ENSGALG00000036346 /395887 | AMH | anti-Mullerian hormone | not sex-DEG | 28 |
| ENSGALG00000045740 /427378 | HEGM | hemogen | up in male CAMs | Z |
| ENSGALG00000008121 / 425,056 | CYP17A1 | cytochrome P450 family 17 subfamily A member 1 | not sex-DEG | 6 |
| ENSGALG00000003242 /395421 | STAR | steroidogenic acute regulatory protein | not sex-DEG | 22 |
| ENSGALG00000029282 /503512 | FOXL2 | forkhead box L2 | not found | 9 |
| ENSGALG00000041708 /395561 | WNT4 | Wnt family member 4 | not sex-DEG | 21 |
| ENSGALG00000001946 /419613 | RSPO1 | R-spondin 1 | not sex-DEG | 23 |
| ENSGALG00000042607 /421715 | RSPO3 | R-spondin 3 | not sex-DEG | 3 |
| ENSGALG00000037203 /395964 | CTNNB1 | Beta-catenin | not sex-DEG | 2 |
| ENSGALG00000013294 /414854 | CYP19A1 | cytochrome P450 family 19 subfamily A member 1 | not found | 10 |
| ENSGALG00000016473 /100316920 | OSR1 | odd-skipped related transcription factor 1 | not sex-DEG | 3 |

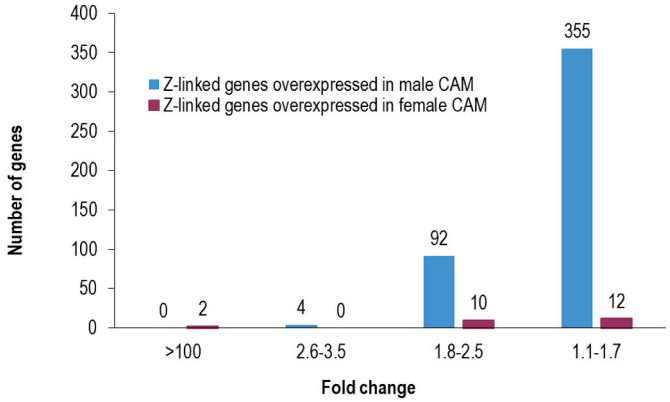


**Fig. 4.** Fold-change of the 475 sex-DEG linked to the Z chromosome. The Z-linked transcripts that are overexpressed in female CAM include two lncRNA (ENSGALG00000051419, ENSGALG00000050012, fold change >100), FOXD1, ALDH1A1, DMRT1, MAP1B, HOMER1, GDLC, BHMT2, RLN3, ENSGALG00000045548 lncRNA and ENSGALG00000048050 (Fold change =1.8–2.5), and 12 genes with fold changes ranging from 1.1 to 1.7. The Z-linked genes that are overexpressed in male CAM include CD180, HSPB3, ENSGALG00000052282 lncRNA, and SPEF2 (Fold change = 2.6–3.5), 94 genes with fold change = 1.8–2.5, and 354 genes with fold change = 1.1–1.7. The complete list of Z-DEG genes is available in supplementary data S1 (Sex_DEG_all).

DEG overexpressed in male CAMs are characterised by an expression that is about three times higher compared with female CAMs (SPEF2, ENGALG00000052282 lncRNA, HSPB3, and CD180). It is noteworthy that the sperm flagellar 2 (SPEF2) together with prolactin receptor (PRLR, fold change = 1.7) were reported to be associated with feathering. Their partial duplication that is observed in most modern breeds was shown to result in late feathering [44]. Similar to [37] who studied the expression of Z-linked genes in the embryo (VLDLR, ALDOB, FST, IREBP, BRM/SARCA2), we observed a dosage compensation in the male CAMs for these five genes, and no dosage compensation for the SCII/SMC2 gene. In addition to these Z-linked DEG, 166 Z-linked genes were shown to be not differentially expressed between male and female CAMs (supplementary data S1, complete), while 367 are sex-DEG with a fold change ranging from 1.1 to 1.7 (355 overexpressed in male CAMs and 12 overexpressed in female CAMs, Fig. 4). The fact that at least eight genes (DMRT1, Doublesex- and mab-3-related transcription factor 1; FOXD1, Forkhead box protein D1, HOMER1, Homer scaffolding protein 1; MAP1B, Microtubule-associated protein 1B; GLDC, Glycine dehydrogenase (decarboxylating), mitochondrial precursor; RLN3, Relaxin-like peptide locus B; and two lncRNAs) are overexpressed in the female CAM, with a fold change >1.8, suggests that in the male CAM, the presence of a double copy of these genes has repressed their expression (through a negative feedback loop, for example).

Although the role of W-linked genes remains uncertain to date [45], it has been demonstrated that the W chromosome was important for female fertility traits, and was shown to respond to female-specific selection that has been used for decades to produce the modern layer strains [46]. Our data highlight that 42 W-linked genes are overexpressed in the female CAM compared with the male CAM (Supplementary data S1, Table 2). These include known W-linked genes such as spindlin 1-W (SPIN1W), ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1 (ATP5A1W) [47] but also lncRNAs. None of these 42 genes possesses a signal peptide as assessed by Biomart (Ensembl, Supplementary data S1_Biomart_SignalP_TM), suggesting that they are likely to have an intracellular role, including the regulation of the expression of other genes [18]. Furthermore, we have shown that some W-linked genes (LOC100859072, RPTC15L, LOC1070449046, MAP1B, and LOC431003) are differentially expressed in the CAM of broiler and layer female embryos. However, although difference in expression may result from a female-specific selection in the layer breed as proposed previously [46], it might also illustrate some difference in embryonic metabolic growth between the two breeds. Indeed, broiler and layer embryos exhibit different developmental phenotypes, with broiler embryos being larger than layer ones, due to difference in yolk to white ratios and their intrinsic metabolic efficiency [21,48].

The biological significance of the sex-DEG in the CAM remains essentially unknown. The next challenge will be to understand the kinetics of expression of sex-DEG in the CAM and define their role throughout incubation. Further validation of the biological role and importance sex-DEG in sex differentiation and phenotype is needed, using gene knockout strategies [9], for example. An interaction between sex and developmental stage have been previously reported for four genes [22] while the present analysis has revealed 124 genes that are uniquely sex-DEG at EID11, 95 at EID15, and 415 at both developmental stages. Thus, the differential expression of genes between male and female CAMs is also regulated during incubation. The CAM is a highly vascularized structure that subserves many diverse functions including gaseous exchanges, mineral transport, homeostasis, and defence against pathogens. Difference in expression between male and female CAMs may contribute to difference in the susceptibility of male and female chicks and adults, to various metabolic and immune disorders. In addition, the identification of the functions associated with these sex-DEG genes and the precise analysis of their genomic organisation are likely to help in identifying sex-dependent phenotypic characteristics that could contribute to the development of *in ovo* sexing methods. Indeed, the characterisation of PRLR and SPEF2 genes whose partial duplication has been shown to be associated with late feathering [44] was used to generate chicken strains selected on this specific trait, which now facilitates sorting of male and female chicks at hatch and prior to hatch (*in ovo* sexing) [49]. Several other approaches based on phenotypic features (feathering, feather colour, gonad development) [50] are currently being studied and/or optimized to determine the sex of the embryo *in ovo*. Knowing that the CAM develops onto the inner eggshell, it would not be surprising that the difference in volatile organic compounds that has been observed at the surface of the eggshell between eggs containing a male or female embryo [51,52] correlates with sex-DEG of the CAM. It is likely that some molecules are produced by the CAM, following the expression of sex-specific genes, and diffuse through eggshell pores.

Most non-invasive *in ovo* sexing methods available today are effective from day 13 onwards, which is rather late in the time course of embryonic development and which constitutes the main ethical issue of these techniques. In addition, some of these methods are only effective on brown layer strains as they are based on hyperspectral techniques that detect differences in feather colour between male and female embryos [46]. The identification of molecules and features of sex dimorphism that are common to the brown and white strains of layers (egg production) and broilers (meat production), should enable the development of early *in ovo* sexing methods that could be used to detect the sex of the embryo at a early stage, regardless of the genetic origin of the eggs.

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## CRediT authorship contribution statement

**Maeva Halgrain:** Data curation, Investigation, Validation, Writing – review & editing. **Nelly Bernardet:** Investigation, Validation, Writing – review & editing. **Christelle Hennequet-Antier:** Data curation, Formal analysis, Writing – review & editing. **Sophie Réhault-Godbert:** Conceptualization, Funding acquisition, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no competing interests.

## Data availability

The datasets supporting the results and the discussion of the article are available at the GEO repository (GSE199780. https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE199780).

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