A study of thermally-induced sex reversal in casque-headed lizards

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ABSTRACT

Non-avian reptiles, unlike mammals and birds, have undergone numerous sex determination turnovers. For example, casque-headed lizards replaced the ancestral XY system shared across pleurodonts with a new pair of XY chromosomes. However, the evolutionary forces that triggered this transition have remained unclear. An interesting evolutionary hypothesis suggests that species with intermediate states, with sex chromosomes but also thermal-induced sex reversal at specific incubation temperatures, could be more susceptible to sex determination turnovers. We contrasted genotypic data (presence/absence of the Y chromosome) against the histology of gonads of embryos from stages 35-37 incubated at various temperatures, including typical male-producing (26°C) and female-producing (32°C) temperatures. We observed perfect concordance between genotype and phenotype at all temperatures. However, analysis of transcriptomic data from embryos incubated at 26°C and 32°C identified transcript variants of the chromatin modifiers *JARID2* and *KDM6B* that have been linked to temperature-dependent sex determination in other reptiles. Besides, our work reports for the first time to our knowledge the histology of gonads, including morphological changes, from stages 35-37 of development in the *Corytophanidae* family. We also observed that all embryos developed hemipenes, suggesting sex-linked developmental heterochrony.Our work tested the validity of a mixed sex determination system in the *Corytophanidae* family. We showed that XY chromosomes are dominant, however, our work supports the hypothesis of a conserved transcriptional response to incubation temperatures across non-avian reptiles that could be the reminiscence of an ancestral sex determination system.

INTRODUCTION

Although squamate reptiles are the most species-rich group among non-avian reptiles (96.3% of the diversity [1]) their sex-determination mechanisms remain poorly understood. In squamate reptiles, sex determination occurs by two general strategies: genotypic sex determination (GSD, the norm for Squamata) and temperature-dependent sex determination (TSD). In the first strategy, specific sex chromosomes control the development of the gonads, whereas in the second strategy, external cues, typically ambient temperature, regulate the sexual differentiation of ovaries and testis [2]. In many TSD species, ~26°C produces 100% of males (i.e., male-producing temperature), whereas ~32°C produces 100% of females (i.e., female-producing temperature). For many years it was assumed that GSD and TSD were mutually exclusive; however, as more non-avian reptiles have been

studied in detail, we now understand that GSD and TSD represent the endpoints of a continuum [3, 4] with some lizards showing temperature-induced sex reversal where ambient temperature can alter the genetic pathways of gonad differentiation [5]. Several squamate reptiles such as *Niveoscincus ocellatus* [6], *Eulamprus heatwolei* [7, 8], *Pogona vitticeps* [9, 10], and *Bassiana duperreyi* [4, 11] show intermediate states where the sex of the offspring is controlled by sex chromosomes under medium incubation temperatures but sex-linked genes can be overridden by alternative signaling cascades at elevated (*P. vitticeps* [10]) or lowered incubation temperatures (*B. duperreyi* [12]).

Mammals and birds have stable sex chromosomes. Non-avian reptiles, however, have undergone numerous sex determination turnovers with lineages shifting from TSD to GSD and vice versa [13]. A general explanation of why sex determination turnovers are more common in reptiles is lacking. Analyses of ancestral states indicated that the last common ancestor of all reptiles had TSD [14, 15] and, subsequently, GSD systems originated in some lineages [14, 15]. It is possible, therefore, that the TSD systems we observe nowadays in turtles, crocodiles, and some lizards derive from the ancestral TSD system [8, 16].

Non-avian reptiles are thought to have evolved temperature-dependent sex determination because their embryos develop in a close relationship with the environment [17]. An interesting evolutionary hypothesis suggests that species with intermediate states showing sex chromosomes and thermal-induced sex reversal may be more susceptible to sex determination turnovers [18] because the ancestral TSD system is still present but has been overridden by, for example, sex chromosomes [8]. This hypothesis is supported by observations in *P. vitticeps* where TSD becomes the dominant sex determination system when the ZW chromosomes are lost [9]. Moreover, three phylogenetically distant reptiles, a turtle (*Trachemys scripta*), a crocodile (*Alligator mississippiensis*), and an acrodont lizard (*P. vitticeps*), showed the same temperature-dependent spliced isoforms of two members of the *Jumonji* family of chromatin modifiers, *KDM6B* and *JARID2* [16]; *KDM6B* plays an important role in temperature-dependent sex determination signaling cascade because its knockdown results in a male-to-female sex reversal at male-producing temperatures in *T. scripta* [19]. *JARID2* has been proposed to play an important role in the temperature-dependent sex determination pathway in non-avian reptiles [20].

Pleurodonts, including iguanas, spiny lizards, and *Anolis* have a common XY system [21, 22]. In *Anolis carolinensis*, researchers characterized a highly degenerated Y chromosome and a perfect dosage compensation mechanism that over-expresses the X chromosome in males [23]. In the pleurodont clade, however, casque-headed lizards (*Corytophanidae* family) replaced the ancestral XY system with a new pair of XY chromosomes [24, 25]. Moreover, initial observations performed in our laboratory with clutches of the Brown Basilisk (*Basiliscus vittatus*), a casque-headed lizard, indicated that female-biased offspring may occur at medium-high incubation temperatures (~29°C). In this study, we explored the proximate mechanisms of sex determination in a squamate reptile, tested whether *Basiliscus vittatus*, a casque-headed lizard, showed temperature-dependent sex reversal. Moreover, we also explored the possibility that *B. vittatus* exhibited genetic signatures of an ancestral temperature-dependent sex determination system.

MATERIALS AND METHODS

Animal collection

22 gravid females of *B. vittatus* were collected from a population that inhabits the tropical rainforest habitat at the community of "La Selva del Marinero" in Veracruz, México (18°26'36.3"N, 94°37'81.9"W, ca. 170 m a.s.l.; SEMARNAT Scientific Collector Permit 08-043). We sampled from April to July of 2018 and females were captured manually and with help of a noose. To evaluate the reproductive condition, both visual assessment and an abdominal palpation were performed (females with eggs showed multiple contours in the abdomen area). Gravid females showed the following morphometric data (mean ± standard error): LHC = 128.1 ± 1.4 mm, and weight 59.6 ± 2.3 g.

Laboratory conditions

Gravid females were placed inside terrariums (100 cm width x 50 cm depth x 50 cm height) until the termination of oviposition. One female per terrarium at a thermal gradient between 20–40°C, a photoperiod of 12/12 h (photofase/scotophase), constant humidity, live insects as food, and water *ad libitum*. The terrariums were monitored daily. 82 eggs were collected and randomly assigned to three incubation temperatures, 26°C, 29°C, and 32°C. Eggs were incubated at the three temperatures in three Percival L-30 incubators until they reached relatively late developmental stages (35-37 according to the development table established by Dufaure and Hubert [26]). We chose these stages because gonads should be fully determined. 48 eggs (12, 18, 18 for 26°C, 29°C,

and 32°C, respectively) reached stages 35-37. The posterior part of the embryos was dissected and fixed using Bouin solution for 30 minutes, washed with water for 30 minutes, and stored in 10% formaldehyde for further histological analysis. The rest of the embryo's body was stored in DNA/RNA shield buffer by Zymo Research (Cat. No. R1200-125) for further genetic analysis.

Microscopy analysis

Conventional histological techniques were performed on each sample: dehydration via graded ethanol, clearing tissues in xylene, and embedding tissues in Paraplast (Sigma-Aldrich, Cat. No. 145686-99-3). Histological sections were made at five microns and stained with Ehrlich-Eosin Hematoxylin (Sigma-Aldrich, Cat. No. 17372-87-1) to facilitate the description of the structures. Samples were then viewed and imaged via a compound microscope including a digital camera.

DNA extraction and Y-specific PCR analysis

We collected 25 mg of tissue and we purified DNA using the QIAamp Fast DNA Tissue Kit from QIAGEN (Cat. No. 51404). We verified the integrity of the DNA using 1% agarose gels. All DNA samples were tested for integrity (260/280 and 260/230 ratios >1.8), using a NanoDrop 2000 spectrophotometer (Thermo Scientific), and quantified in a Qubit 4 fluorometer (Thermo Scientific, MA, USA) with the Qubit dsDNA BR Assay kit from the same supplier. We confirmed the presence or absence of a Y chromosome in the samples using Y-specific primers designed previously [24]: *CAMSAP1Y,* forward: AGT CTC AGT CTG CAC CAG TGA AAG, reverse: TGA TTT CTG AGC CCA GGC AGT T. *GOLGA2Y,* forward: AGG CTG TCA GTC TCA CTC AGT AAG, reverse: CCC CAT ATT CCC AGG TTC TGT CA. We verified that PCR reactions worked using primers against *COL1A1* (autosomal/control) forward: TTT CGT GCA GGG TGG GTT CTT T, reverse: TCT GAA CTG GTG CAG CTT CAC A. We used the Phusion Flash High Fidelity from Thermo Fisher Scientific (Cat. No. F548L) with the following program: first 98°C - 10s, then 30 cycles of 98°C - 2s, 66°C - 5s and 72°C - 10s, with a final elongation step at 72°C - 30s. We confirmed the size of the PCR products and the presence of single amplicons in a 1% agarose gel.

RNA extraction and RNA-seq analysis

We generated strand-specific RNA-seq libraries (using the Illumina TruSeq Stranded mRNA Library protocol) for a total of six samples obtained from whole embryos incubated at 26°C (four samples; three females and one male) and 32°C (two samples; two females). Embryos were sexed using the

above-mentioned Y-specific primers. Each library was sequenced on Illumina HiSeq 2500 platforms at the Macrogene facility in Korea (100 nucleotides, paired-end). We generated 31,910,378 million reads on average (± 3,753,014 million reads). We reconstructed a full transcriptome using Trinity (v2.0.2, default k-mer of 25 bp) [27]. Then, RNA-seq reads from 26°C and 32°C samples were mapped to the reconstructed transcriptome using Kallisto (100 bootstraps) [28]. We obtained the estimated counts per transcript and we used the EdgeR package [29] to perform differential expression analyses of transcripts between incubation temperatures (26°C versus 32°C, and vice versa). We used the *edgeR* and *splines* R libraries, TMM (Trimmed Mean of M-values) normalization, FDR (False Discovery Rate) set at 0.0001 given the limited number of replicates at 32°C, from which non-degraded RNA was difficult to obtain. We downloaded from the Ensembl database (https://www.ensembl.org/; v.92) the cDNAs and lncRNAs from *A. carolinensis* and we used BLASTn [30] to assign gene identities to the differentially expressed transcripts. When various transcripts for the same gene were differentially expressed, count estimates were added to obtain single values per gene. Enrichment analyses were carried out using Webgestalt (http://www.webgestalt.org/), specifying over-representation analysis, the genontology database, and the Biological Process category. RNA-seq data have been deposited to the NCBI-SRA database under BioProject PRJNA766022.

RESULTS

Histological analysis showed that ovaries and testes were well-differentiated in stages 35-37 of development

To evaluate the effect of temperature on the embryo's sex, first, we established the developmental stages where gonads were well-differentiated. We observed clear testis-specific and ovary-specific structures in stages 35-37 of embryonic development. During stage 35, the female ovary showed an oval morphology (Fig. 1a). The cortex and the medullary zone were well-differentiated. The cortex is structured by epithelial and germ cells, and the presence of some oogonia were visible. The testicle in stage 35 showed testicular cords in the medullary region together with epithelial cells, the future Sertoli cells (Fig. 1b). Some of these cords presented spermatogonia in the lumen, around the medullary area. In stage 36, the ovary size increased, the cortical zone became thicker showing 2-3 layers of germ cells, closely related to somatic cells or future follicular cells in their thickest part, delimited by a basal lamina of connective tissue, in addition to a greater number of oogonia than in the previous stage (Fig. 1c). Testicles in stage 36 were more elongated, testicular cords increased in

192 density in the medullary region (Fig. 1d), and spermatogonia were common inside the testicular
193 cords. Finally, in stage 37, connective tissue fibers in the ovaries delimited the cortical zone and the
194 medullary region (Fig. 1e). In the cortical region, the proliferation of oogonia covered the entire
195 region. As for the testicles, in stage 37, the shape was elongated and curved and the entire testicle
196 is made up of testicular cords, which were located more closely to the central area of the spinal
197 cord. Large spermatogonia became abundant inside the testicular cords, while Sertoli cells are
198 peripheral to the testicular cords (Fig. 1f).
199
200
201 **Fig. 1. Ovaries and testes at stages 35-37 of development in *B. vittatus* embryos.** a) histology of
202 the ovary in stage 35. b) histology of the testis in stage 35. c) histology of the ovary in stage 36. d)
203 histology of the testis in stage 36. e) histology of the ovary in stage 37. f) histology of the testis in
204 stage 37.
205
206
207 **No effect of incubation temperatures on the sex of *B. vittatus* embryos**
208 Next, we performed experiments to evaluate whether the sex of the embryos was affected by
209 different incubation temperatures, including typical male-producing (26°C) and female-producing
210 (32°C) temperatures. To do so, we contrasted genotypic data (i.e., presence/absence of the Y
211 chromosome) against the histology of the gonads from stages 35-37 (i.e., presence of testicular or
212 ovarian structures) at three incubation temperatures (26°C, 29°C, and 32°C). If *B. vittatus* presented
213 temperature-dependent sex reversal, specific genotypes would not necessarily develop the
214 expected gonads. For example, in a male-to-female sex reversal, individuals with a Y chromosome
215 would develop ovaries.
216
217 We analyzed a total of 48 embryos. At 26°C we observed eight embryos with a Y chromosome and
218 four embryos without a Y chromosome (Fig. 2). At 29°C we observed 11 embryos with a Y
219 chromosome and seven embryos without a Y chromosome (Fig. 2). At 32°C we observed nine
220 embryos with a Y chromosome and nine embryos without a Y chromosome (Fig. 2). We detected
221 the same frequency of males and females at the three temperatures (X^2 test, $P > 0.05$). Moreover,
222 after contrasting the genotype of the embryos against their gonads, we found that embryos with a
223 Y chromosome developed testes in all instances. Similarly, embryos without a Y chromosome
224 always developed ovaries (Fig. 2).

Fig. 2. Number of embryos according to the incubation temperature, genotype, and histology. Histogram showing the number of *B. vittatus* embryos with a Y chromosome that developed testes (*Ychr+ts*; no sex reversal), the number of embryos lacking a Y chromosome that developed ovaries (*noY+ov*; no sex reversal), the number of embryos with a Y chromosome that developed ovaries (*Ychr+ov*; male-to-female sex reversal), and the number of embryos without a Y chromosome that developed testes (*noY+ts*; female-to-male sex reversal).

Differential expression analysis of transcriptomic data

We performed a differential expression analysis of RNA-seq data from embryos incubated at 26°C versus embryos incubated at 32°C. We found 272 genes that were over-expressed at 26°C and 136 genes that were over-expressed at 32°C (S1 Table). Enrichment analysis of GO terms showed that differentially expressed genes were associated, as expected, with biological processes that are common during embryonic development. Interestingly, however, genes over-expressed at 26°C were more frequently associated with neuron development (Table 1), whereas genes over-expressed at 32°C were mostly associated with muscle development (Table 2).

Table 1. GO terms enrichment of genes over-expressed at 26°C

Gene Set	Description	Size	Expect	Ratio	P Value	FDR
GO:0048666	neuron development	1068	15.382	3.251	7.57E-14	6.88E-10
GO:0010975	regulation of neuron projection development	475	6.8411	4.678	3.48E-13	1.58E-09
GO:0030030	cell projection organization	1522	21.92	2.692	9.84E-13	2.98E-09
GO:0120036	plasma membrane bounded cell projection organization	1488	21.431	2.66	4.33E-12	9.83E-09
GO:0120035	regulation of plasma membrane bounded cell projection organization	665	9.5775	3.759	6.61E-12	1.20E-08
GO:0022604	regulation of cell morphogenesis	473	6.8123	4.404	9.31E-12	1.26E-08
GO:0031344	regulation of cell projection organization	674	9.7072	3.709	9.72E-12	1.26E-08
GO:0031175	neuron projection development	940	13.538	3.176	1.23E-11	1.39E-08
GO:0030182	neuron differentiation	1313	18.91	2.697	4.67E-11	4.72E-08
GO:0000902	cell morphogenesis	982	14.143	2.97	1.81E-10	1.55E-07

Table 2. GO terms enrichment of genes over-expressed at 32°C

Gene Set	Description	Size	Expect	Ratio	P Value	FDR
GO:0003012	muscle system process	423	3.1984	8.754	0	0
GO:0006936	muscle contraction	339	2.5633	9.753	0	0

GO:0030239	myofibril assembly	67	0.5066	25.66	2.44E-15	7.40E-12
GO:0010927	cellular component assembly involved in morphogenesis	106	0.80149	17.47	5.62E-14	1.28E-10
GO:0031032	actomyosin structure organization	184	1.3913	11.5	6.27E-13	1.14E-09
GO:0097435	supramolecular fiber organization	640	4.8392	5.373	1.61E-12	2.44E-09
GO:0055001	muscle cell development	166	1.2552	11.95	1.97E-12	2.56E-09
GO:0030240	skeletal muscle thin filament assembly	14	0.10586	66.13	3.92E-12	4.46E-09
GO:0055002	striated muscle cell development	153	1.1569	12.1	9.45E-12	9.54E-09
GO:0014866	skeletal myofibril assembly	16	0.12098	57.86	1.29E-11	1.17E-08

Although we did not detect thermal-induced sex reversal in *B. vittatus* within the 26°C-32°C temperature range, we examined the differentially expressed transcripts in further detail. We found that specific isoforms of *JARID2* and *KDM6B* were over-expressed at 26°C (Table 3; S1 Table). Remarkably, *JARID2* was the top-second gene with the highest expression difference between 26°C and 32°C (LogFoldChage = -5.2891, FDR = 8.96E-12; Table 3). For both genes, the isoforms that were differentially expressed appeared to have retained an intron. In *JARID2*, the retained intron corresponds to intron 15, the third to last intron (Fig. 3), which is the same retained intron that was previously reported for *T. scripta*, *A. mississippiensis*, and *P. vitticeps* [16]. In contrast, *KDM6B* retained the last intron, intron 18, instead of the second to last that was reported in the other species [16]. Careful examination of *KDM6B* using blastn alignments indicated, contrary to *JARID2*, that the isoform did not retain the full sequence of intron 18. Instead, three shorter sections of intron 18 were included in the *KDM6B* isoform (Fig. 3).

Fig. 3. Exon and intron structure of differentially expressed isoforms of *JARID2* and *KDM6B*. a) Diagram of the exon/intron structure of the *JARID2* isoform from *B. vittatus* that is over-expressed at 26°C. Exons are shown as dark blue rectangles, introns are shown as light blue lines. Blastn matches to exonic sequences are shown as yellow rectangles, whereas matches to intronic sequences are indicated by pink rectangles. The green bar represents the reference genome of *A. carolinensis*. Blastn alignments were performed against this reference genome. b) Same as in a) but for the *KDM6B* isoform from *B. vittatus* that is over-expressed at 26°C.

Table 3. Estimated read counts for *JARID2* and *KDM6B* (differentially expressed isoforms)

		embryos at 32°C		embryos at 26°C			
FoldChange	FDR	rep1	rep2	rep1	rep2	rep3	rep4

JARID2	-5.2891	8.96E-12	307	380	3635	2143	8742	5135
KDM6B	-3.3684	0.000159	150	125	1305	933	5716	1964

All embryos had hemipenes; sex has to be established through histology of gonads or genotypic analysis

It should be noted that 100% of the embryos presented intrusive organs (hemipenes), regardless of their genotype (presence/absence of the Y chromosome) or whether the embryos carried testes or ovaries. The presence of copulatory organs was observed from stage 35 (Fig. 4a,d). The morphology in this stage corresponded to a prominent lobe that becomes slightly larger. Hemipenes became bilobed in stage 36, with blood supply throughout the entire phallus (Fig. 4b,e). By stage 37, hemipenes were bifurcated and bilobed with blood supply only in the apical part (Fig. 4c,f).

Fig. 4. Hemipenes are present in both male and female embryos. Females showing hemipenes in a) stage 35, b) stage 36, c) and stage 37. Males showing hemipenes in d) stage 35, e) stage 36, and f) stage 37. Black arrows point at the hemipenes.

DISCUSSION

Casque-headed lizards are the only group of pleurodonts that transitioned from the ancestral XY system to a more recent pair of XY chromosomes. The evolutionary forces that triggered this transition have remained unclear. Here, we tested whether *B. vittatus* showed temperature-dependent sex reversal and whether we could detect genetic signatures of an ancestral TSD system; having a latent sex determination system could facilitate transitions between sex determination systems. We contrasted genotypic data and histological data of embryos from stages 35-37 incubated at three different temperatures. We found no effect of incubation temperatures on the development of gonads: embryos with a Y chromosome developed testes, whereas embryos without a Y chromosome developed ovaries. Thus, *B. vittatus* does not appear to show temperature-dependent sex reversal within the 26-32°C temperature range. We selected these incubation temperatures because they are common male-producing or female-producing temperatures in non-

avian reptiles and because they represent average temperatures during the reproductive season of *B. vittatus* [31].

Remarkably, we observed that *JARID2* was the top-second gene with the highest expression difference between low and high incubation temperatures. We found that at a lower temperature (26°C) *JARID2* retained the same intron that was previously reported for *T. scripta*, *A. mississippiensis*, and *P. vitticeps* at male-producing temperatures [16]. Our results suggest that the alternative splicing of *JARID2* is a common trait across non-avian reptiles and appears to be a reminiscence of an ancestral response to temperature [32]. We showed that the new pair of XY chromosomes are dominant, however, it is still not clear whether the two XY systems co-existed or whether a potentially latent TSD system facilitated the transition. Future work could explore whether more extreme incubation temperatures may trigger sex reversal and the nature of the signaling cascades controlled by the temperature-specific isoforms of *JARID2*.

In contrast, the differentially expressed isoform of *KDM6B* did not show the expected pattern since it retained shorter sections of the last intron rather than the complete sequence of the second to last intron. This pattern is rather consistent with *KDM6B* showing alternative 3'-UTR and could indicate that only the *JARID2*-dependent pathway is still sensitive to temperature changes in pleurodonts.

Differential expression analyses showed that genes related to neuron development were over-expressed at lower temperatures, whereas genes related to muscular development were over-expressed at higher incubation temperatures. Embryonic development is boosted at elevated incubation temperatures, however, we found that tissues responded differently to temperature. Finally, our work examined for the first time to our knowledge the development of gonad during stages 35-37 in a member of the *Corytophanidae* family. We found that gonads were well-differentiated and increased in size and cellular density during these stages. Intriguingly, we found that all embryos showed hemipenes during stages 35-37. This result suggests heterochrony in the regression of hemipenes in females (i. e., both gonad and genital structures development may not be closely coordinated), as it occurs in just three phylogenetically distant lizards [33-35].

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Authors' contributions

G.S.-V., D.C., and O.H.-G. designed the study. G.S.-V., E.M.-C., A.A., M.V.-S.-C., and D.C. performed the analyses. All authors contributed to the interpretation of the results. G.S.-V., D.C. and O.H.-G. wrote the article. All authors read and approved the final article.

Supporting information

S1 Table: Differentially expressed genes found in this study.

Data availability

RNA-seq data have been deposited to the NCBI-SRA database under BioProject PRJNA766022.

Ethics declarations

Permission for fieldwork and sampling was granted by the Secretaría del Medioambiente y Recursos Naturales (SEMARNAT Scientific Collector Permit 08-043).

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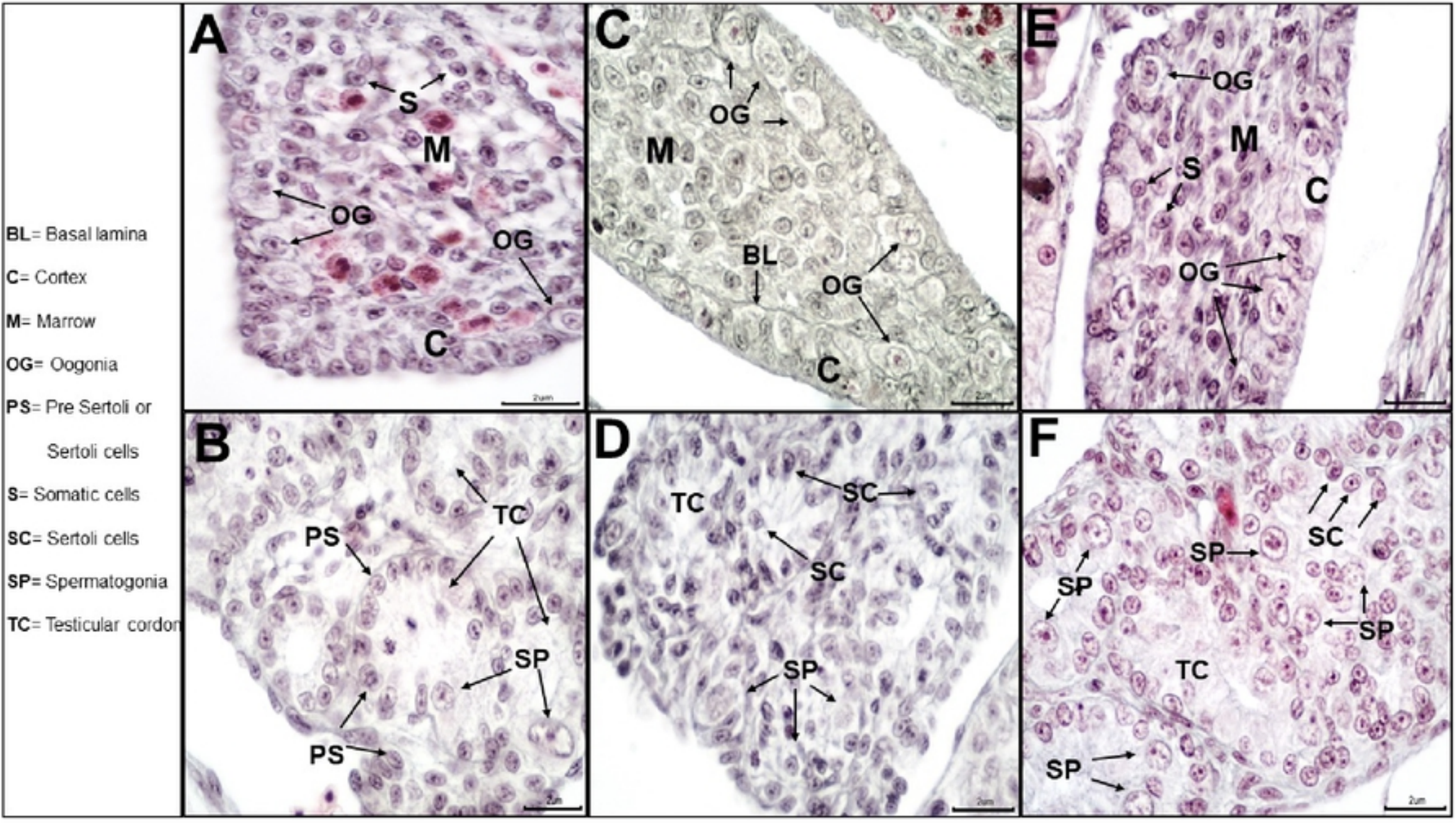
BL= Basal lamina
C= Cortex
M= Marrow
OG= Oogonia
PS= Pre Sertoli or Sertoli cells
S= Somatic cells
SC= Sertoli cells
SP= Spermatogonia
TC= Testicular cordon
A
S
M
OG
OG
C
C
OG
M
BL
OG
C
E
OG
M
S
C
OG
B
TC
PS
SP
PS
D
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SC
SC
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F
SC
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SP
SP
TC
SP

Figure1

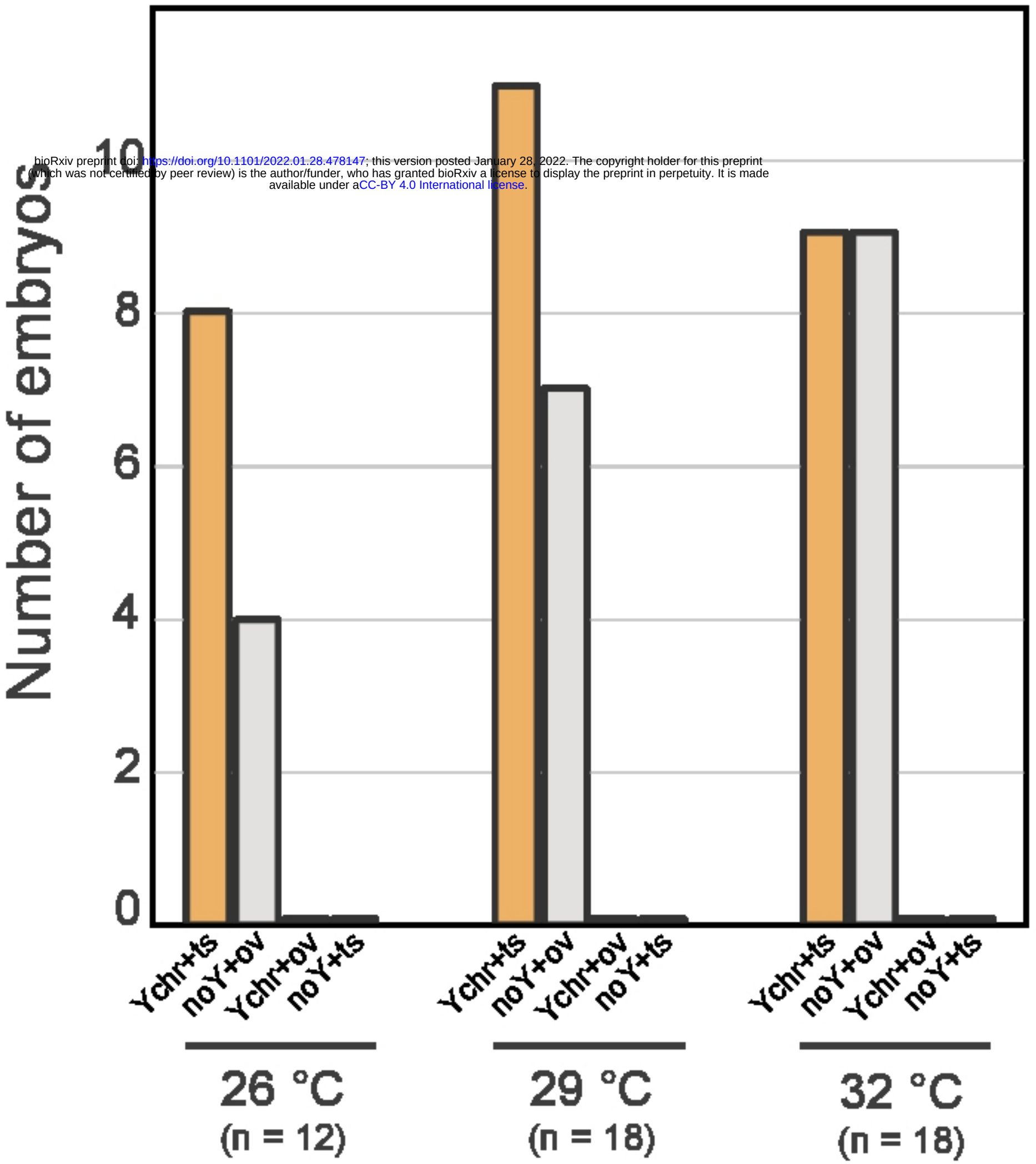
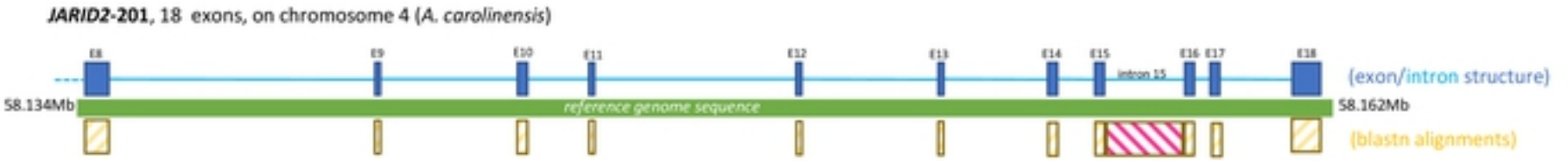


Figure2

a)


JARID2-201, 18 exons, on chromosome 4 (A. carolinensis)
E8
E9
E10
E11
E12
E13
E14
E15
intron 15
E16
E17
E18
(exon/intron structure)
58.134Mb
reference genome sequence
58.162Mb
(blastn alignments)

b)

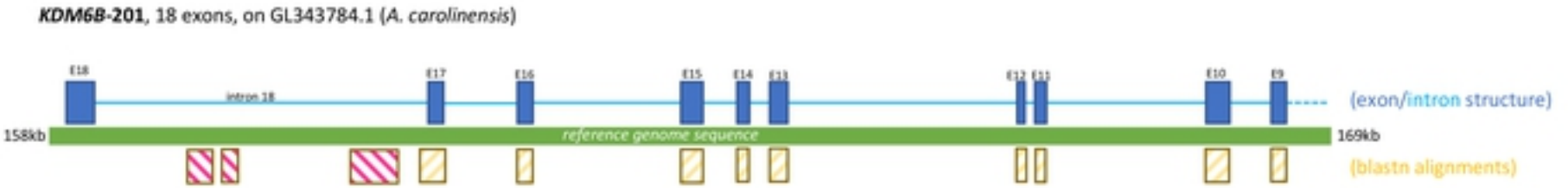

KDM6B-201, 18 exons, on GL343784.1 (A. carolinensis)
E18
intron 18
E17
E16
E15
E14
E13
E12
E11
E10
E9
(exon/intron structure)
158kb
reference genome sequence
169kb
(blastn alignments)

Figure3

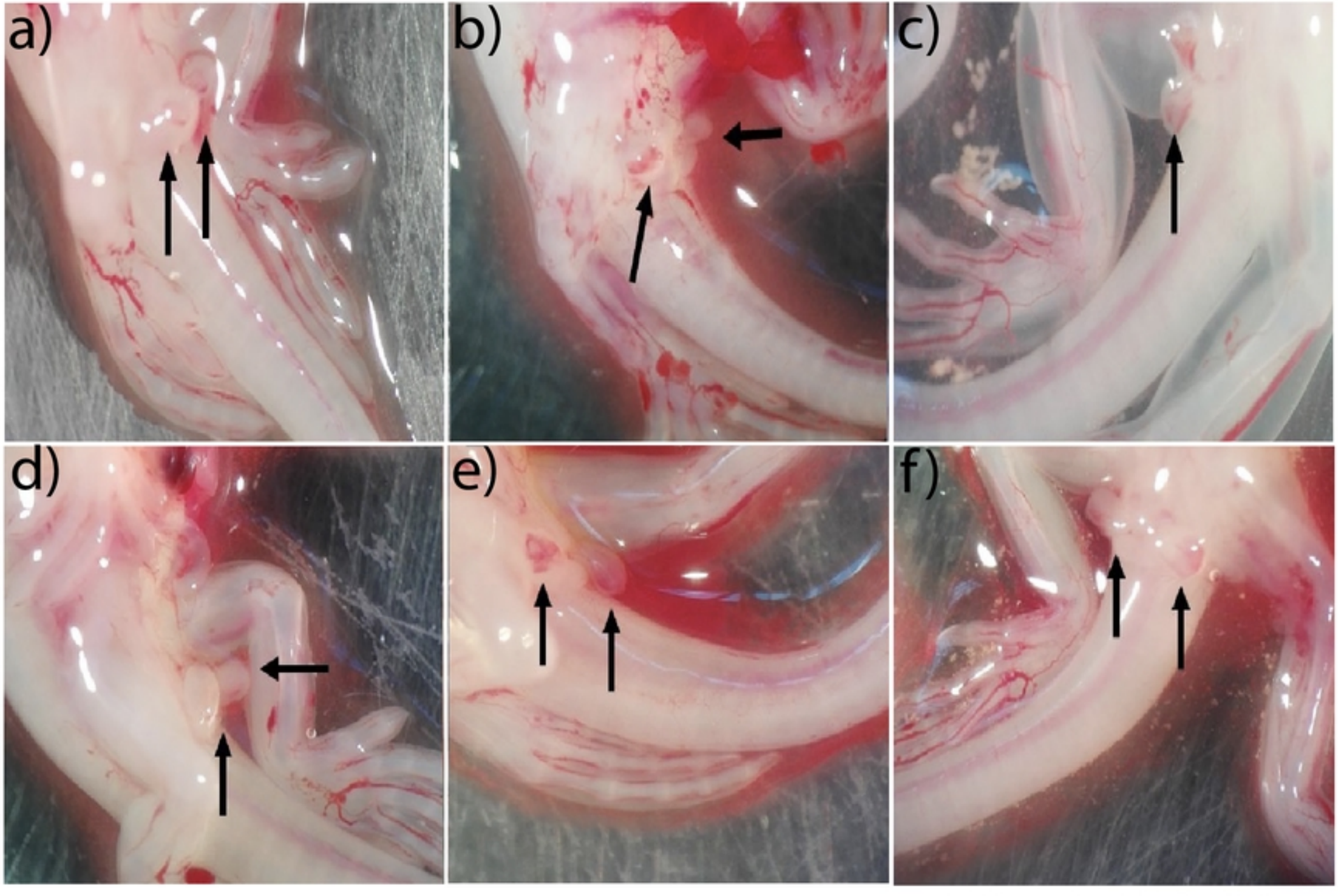


Figure4