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Impact of low temperature during embryonic development on the sex determination and *de novo* DNA methyltransferases in the androdiecious fish *Kryptolebias marmoratus*

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ABSTRACT

The teleost fish *Kryptolebias marmoratu* (mangrove rivulus) presents an unusual reproductive system as it is one of only two vertebrates in which hermaphroditic individuals can reproduce by self-fertilization but also outcross with males. Between different populations of rivulus, sex ratios are variable as sex determination processes are based on genetic and environmental factors. In laboratory, isogenic lineages that avoid genetic diversity can be produced, allowing to study the effect of environment only. Even though low temperature is known to impact the sex ratio by inducing male development, and epigenetic mechanisms are suspected to make the connection between environment and sexual gene expression, most of the mechanisms behind sex determination of *K. marmoratus* by temperature are still unknown. To better understand these processes, we compared the expression level of the *de novo* DNA methyltransferases dnmt3aa and dnmt3ab as well as those of the sexual genes sox9a and cyp19b in rivulus embryos incubated at 18°C and 25°C. Even though we could not show a correlation between dnmt3a and sexual genes expression, we found that, while dnmt3aa and dnmt3ab are similarly expressed for a same temperature, the level expression of both dnmt3a variants seems to increase at a faster rate at 18°C compared to 25°C, at least during the early stages of embryonic development. In parallel, we observed that in the last stages of embryonic development, sox9a was overexpressed at 18°C while cyp19b was overexpressed as 25°C. This study may be a starting point for further research to prove a correlation, or even a causality, between *de novo* methyltransferases and sexual genes as a pathway to explain temperature-dependent sex determination in mangrove rivulus.

Key words: androdioecy; DNA methylation; *Kryptolebias marmoratus*; masculinization; sex determination

1. Introduction

Sexual reproduction is an ancient and intrinsic feature of eukaryotic life (Speijer et al.., 2015). Yet, sexual systems, i.e. the pattern of distribution of the male and female function among the individuals of a given species, are diverse and complex (Bachtro et al.., 2014; Pla et al.., 2022). While common systems, like gonochorism or hermaphroditism, are well known, androdioecy, i.e., the presence of male and hermaphroditic individuals, is rare and still poorly understood (Weeks et al.., 2006; Moreira-Pinto & Pires-daSilva, 2018; Pla et al.., 2022). The first cases of documented androdiecious species only appeared in the '60s – '70s, mainly in plants (Charlesworth, 1984). Since then, other examples of androdiecious species have been found in plants and animals, especially in nematodes and crustaceans (Pannell, 2002; Weeks, 2012; Subranomian, 2017; Moreira-Pinto & Pires-daSilva, 2018). Even though this sexual pattern appears to be restricted taxonomically, extended research on androdiecious metazoans provided evidence that it has evolved many.(Weeks 2012; Leonard, 2018). The most common form of androdioecy in animals, which seems to have evolved from dioecy, involves hermaphrodites that either self-fertilize or outcross with males, like the nematode *Caenorhabditie elegans* and the fish *Kryptolebias marmoratus* (Harrington, 1967; Weeks 2012; Leonard, 2018; Moreira-Pinto & Pires-daSilva, 2018).

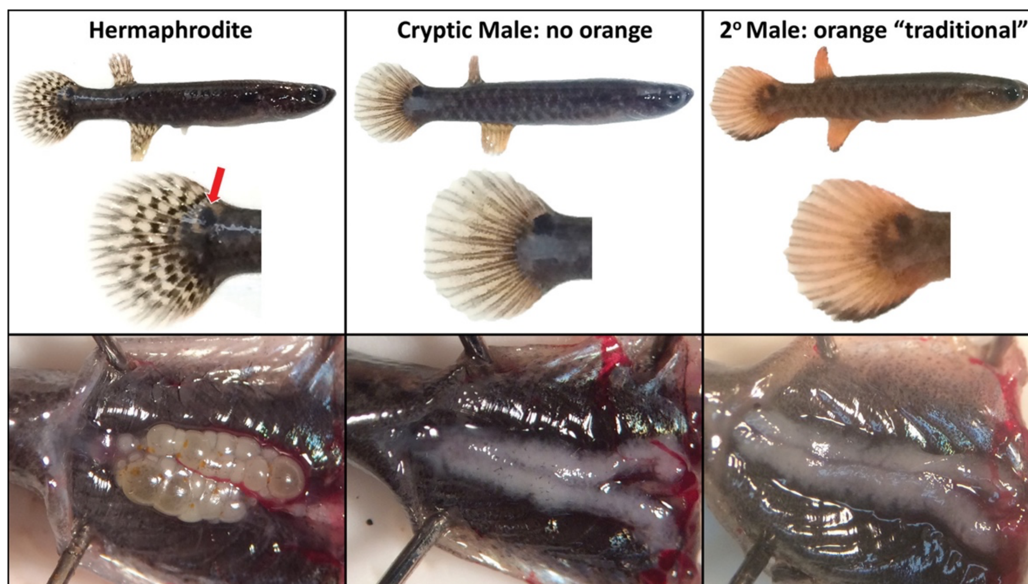


Figure 1 - Representatives of the three sexual phenotypes encountered in *K. marmoratus*. full bodies, caudal fin, and ocellus beneath. Bottom panels show a ventral view of a dissected animal. The ocellus is shown at the red arrow for the hermaphrodite (Marson *et al.*., 2019)

Kryptolebias marmoratus (Poey, 1880), also called mangrove rivulus or mangrove killifish, is a teleost fish mostly known for being one of the only androdiecious vertebrates (Harrington, 1960). This species is characterized by self-fertilizing hermaphrodites, primary males, which directly develop testicular tissues, and secondary males, which correspond to hermaphroditic adults characterized by a resorption of the ovarian tissue and a colour change (Figure 1) ((Weeks et al.., 2006; Kelley et al.., 2016; Marson *et al..*, 2019). Present in the tropical and subtropical West Atlantic, the rivulus distribution range extent from Florida to the north-eastern coast of Brazil, through the Yucatan Peninsula and the Bay of Campeche in Mexico (Taylor, 2012; Costa, 2016). This species, associated to mangroves, can colonize a large variety of micro-habitat, such as shallow water, crab burrows or mangrove litter (Tayler, 2000; Taylor *et al..,* 2008). The great phenotypic plasticity of the rivulus allows it to thrive in the large range of physico-chemical parameters like mangroves thatare a highly variable habitat in time and space, (Taylor, 2012). The hard living conditions might have led to low density populations during *K. marmoratus* life history, and thus mating difficulties. Androdioecy could have appeared as an potential strategy of reproductive assurance, as self-fertilizing individuals do not have to find a mate anymore and can produce almost homozygotic populations, which can be highly adapted to their environment, as multiple generations of selfing, starting from a heterozygote individual, can lead to the fixation of sets of alleles that are particularly advantageous in a specific biotope. (Taylor et al.., 2001; Weeks; 2012; Ellison et al.., 2013). On the other hand, rivulus populations that have low genetic diversity could be more sensitive to environmental variation as less polymorphism might prevent the emergence of new phenotypes in case of changing environments (Tatarenkov et al.., 2012). In the wild, not all population of rivulus are isogenic. Sex ratio, which is the result of complex interactions between genetic and environmental factors, finally resulting in the expression of the hermaphroditic or male phenotype at the individual level, is dependent on the population localization and varies from 1-2% to 45% (Turner et al.., 1990; Taylor et al.., 2001; Pers. com. Chapelle & Silvestre). The sexual phenotype is influenced by the developmental plasticity of the rivulus, as primary males are induced by incubation temperatures of around 18-20°C and hermaphrodites by temperatures of around 25-29°C, but also by the phenotypic flexibility in adults, as hermaphrodites can turn into secondary males due to environmental factors (Harrington, 1967; Harrington & Kallman,1968; Taylor, 2012; Ellison *et al..*, 2015; Kelley *et al..*, 2016). As more heterozygous individuals lead to a lower chance of inbreeding depression, sex ratio plays an important role in population fitness, and thus, species evolution (Taylor et al.., 2001; Piferrer *et*

al.., 2012; Ellison et al.., 2013). Unravelling the mechanisms behind sex determination for *K. marmoratus* is thus essential to better understand its evolution.

Sex determination (SD) can be defined as the physiological processes that lead to the differentiation of the reproductive system under the influence of genetic or environmental factors (Hayes, 1998; Sandra & Norma, 2010; Santi *et al..,* 2016). SD is fundamental for sexual reproduction, species survival and evolution. As one of the most plastic evolutionary processes, many different mechanisms exist (Haag & Doty, 2005; Capel, 2017; Bertho *et al..*, 2018). In vertebrates alone, SD mechanisms are highly variable, ranging from strict genotypic sex determination (GSD) to strict environmental sex determination (ESD), including combinations of genetic and environmental determination (GSD+ESD) (Penman & Piferrer, 2008; Barchtog *et al..*, 2014; Capel, 2017). At first, sex determination was thought to be strictly control by genetic (Barchtog *et al..,* 2014). Most vertebrates have either a XX/XY-system in which the gene SRY, located on Y-male chromosome, act as a trigger for testis formation (mammals), or a ZZ/ZW-system in which the overexpression of the Z-linked gene dmrt1 leads to male sex determination (birds) (Matsuda *et al..*, 2002; Smith *et al..*, 2009; Yoshimoto & Ito, 2011; Barchtog *et al..*, 2014; Li & Gui, 2018). In fish and other non-mammal vertebrates, dmrt1 is involved in testis formation (Nagahama, 2005; Barchtog *et al..,* 2014; Capel, 2017). In certain species of fish, reptiles, and amphibians, rather than GSD, the incubation temperature of the eggs induced the sex of the progeny. For these species, sex is strictly determined by the environment, and in most cases by the temperature (Korpelainen, 1990; Ospina-Alvarez & Piferrer, 2008). Other species present a gradient between GSD and ESD. Environmental parameters, especially temperature, can override genotypic control (Barchtog *et al..*, 2014; Li & Gui, 2018). For example, it has been shown that, in the Nile Tilapia *Oreochromis niloticus*, high temperatures lead to the masculinization of the embryos, resulting in XX males (Baroiller, 1995; Baroiller *et al..,* 2009a). Sex determination is mainly studied in gonochoric species, but combination between GSD and ESD also exists in other sexual systems. In our model, *K. marmoratus*, masculinization is induced by low temperature and a modification of the level of expression of the sexual genes cyp19a, associated with the ovarian development, and sox9a, associated with the development of testicular tissues (Ellison *et al..*,2015). This is also the case for the Nile tilapia in which temperature regulates the expression level of genes included in sex determination pathways, and induces male phenotype through DNA methylation (Baroiller *et al..*, 2009a; Baroiller *et al..*, 2009b)

DNA methylation is an epigenetic mechanism that seems to bridge the gap between GSD and ESD (Ellison *et al..*, 2015; Fellous et al.., 2018). Epigenetics examines heritable

changes in gene expression that occur without modification of the DNA sequence (Berger *et al.*., 2009). Among epigenetic mechanisms, histone modifications and DNA methylation seem to be involved in the regulation of sex-related gene expression by the environment. Histone modifications regulate gene transcription by altering the structure of the chromatin (Bollati & Baccarelli, 2010; Bannister & Kouzarides 2011). For example, histone demethylase kdm6B regulates temperature-dependent sex determination in the red-eared slider turtle *Trachemys scripta elegans*. In this species, incubation temperature determines the turtle's sex. When kdm6b is knocked down and the embryos incubated at 26°C, females are sired instead of the expected males (Ge *et al.*., 2018). In the same species, DNA methylation of dmrt1 is also involved in the sex determination by temperature during embryo development (Ge *et al.*., 2017). As we saw before, DNA methylation is also involved in sex determination of fish. More precisely, masculinization by high temperature in the Nile tilapia is correlated with an increase of the DNA methylation level of the sexual gene cyp19a1a and a reduction of its expression level (Wang *et al.*., 2017). DNA methyltransferases (dnmts) catalyze the transfer of a methyl-group onto a cytosine, which usually prevents transcription and thus reduces or even suppresses gene expression (Moore & Fan, 2013). DNA methylation is also thought to be involved in sex determination in *K. marmoratus*. Ellison *et al.*. (2015) have shown that DNA methylation could be the intermediary through which temperature modulates sexual identity. In the rivulus, dnmt3a variants, responsible for *de novo* methylation, were shown to be differentially expressed between in male and hermaphrodite adult tissues, with dnmt3ab overexpressed in males and dnmt3aa overexpressed in hermaphrodites. The dnmt3a gene family plays a role in the establishment of DNA methylation patterns that occurs during early development, mainly during the reprogramming (Okano *et al.*., 1999; Santos & Dean 2004). In the rivulus, the two dnmt3a variants are differentially expressed during the early development as dnmt3ab is overexpressed during the last stages of development, i.e., after the DNA methylation reprogramming event, compared to dnmt3aa (Fellous *et al.*., 2018). As it can be seen as an erasure of epigenetic marks required for correct development of the embryo and establishment of DNA methylation patterns in the new individual, the reprogramming represents a window in which the genome is particularly sensitive to the environment (Santos & Dean, 2004). In *K. marmoratus,* this window is later and longer than in other vertebrates, especially compared to the zebrafish *Danio rerio* (Fellous *et al.*., 2018). This characteristic could be one of the reasons that allow the important phenotypic plasticity in the rivulus in a highly variable habitat as a wider window could provide sufficient time to environmental cues to be assimilated in the epigenome during embryonic development, and thus to impact genetic level expression (Fellous

et al., 2018). In our model species, DNA methylation as a potential explanation for the influence of the environment could explain maintenance of variable selfing rates by modulating mate availability (Ellison *et al.,* 2015; Fellous *et al.*., 2018). To better understand the impact of environment on sex ratio in *K. marmoratus,* interactions between GSD, ESD and epigenetic mechanisms require further study.

As an emergent model for research, the origin, the evolution, and the determination of *K. marmoratus* reproduction mode are still unknown. The main objective of this study is to investigate the impact of low temperature on sex determination during *Kryptolebias marmoratus*' embryonic development. To achieve this, we compared the expression of the *de novo* DNA methyltransferases dnmt3aa and dnmt3ab at 18°C and 25°C, as well as of the sexual genes sox9a and cyp19b. The relative expression of sox9a and cyp19b were analyzed during the last stages of development, i.e., after the reprogramming period (stage 28 to 33). Comparing the relative expression of dnmt3a variants and sex-related genes during the embryonic development will allow to assess whether dnmt3a could be involved in the regulation of sexual genes by temperature, which is in turn an important mechanism allowing the existence of androdioecy in the rivulus. In a further extent, this can give an insight into the significance of epigenetic mechanisms as driver of evolution in *K. marmoratus*, as environment is known to have an impact on sex determination, and thus on sex ratio, species fitness and species evolution.

2. Materials and methods

2.1 Ethic statement

All rivulus husbandry were performed in accordance with the Belgian animal protection standards and were approved by the University of Namur Local Research Ethics Committee (UN 21 367 KE). The agreement number of the laboratory for fish experiments is LA1900048.

2.2 Experimental animals and housing

The individuals used in this study were offsprings from adult hermaphrodites of the EPP population of *Kryptolebias marmoratus* located at the University of Namur (UNamur). The EPP stock population was initially obtained from fish sampled by Kristy Marson, Frédéric Silvestre, and Valentine Chapelle in 2019 in the Florida Keys (Emerson Point Preserve; N27°53'29.80", W82°62'55.01"). They are currently housed at 26 ± 1 C° in 12 ± 1 parts per

thousand (ppt) saltwater (Instant OceanTM sea salt), 12:12 light:dark cycle and fed every day *ad libitum* with live *Artemia salina* nauplii. Spawning cottons were added to the tanks to provide a substrate for oviposition. These housing conditions are kept for the rest of the experiment.

2.3 Egg collection and temperature treatment

From April 2022 to November 2022, eggs were manually collected from adult hermaphrodite individuals. The developmental stage before incubation was determined according to the criteria described in Mourabit *et al.*. (2011) (Table 1). Once identified, the eggs were individually incubated at 18°C or 25°C for 3 days. The stage of development after incubation was determined again and embryos that reached a stage between 16 and 33 were selected. Eggs were pooled by 2 or 3 depending on the stage to ensure a sufficient amount of RNA for the extraction, frozen in liquid nitrogen and conserved at -80°C.

Table 1 – Number, corresponding name and hours post-fertilization (hpf) at 25°C for stage 16 to stage 33 of *K. marmoratus* embryotic development (Mourabit *et al.*., 2011)

Stage Number	Stage Name	HPF (25°C)
16	Recognition of head an tail regions	34,5
17	Optic vesicle and somite formation	36
18	Otic vesicle formation	43,5
19	Lens formation	53
20	Heart beats	55,5
21	Body movements	58
22	Circulation	62
23	Increased vitelline circulation	71
24	Otolith formation	73
25	Pectoral fin development and erythrophore formation	77
26	Liver formation	90
27	Increased pigmentation and body movement	105
28	Caudal fin formation	140
29	Air bladder and anal fin formation	180
30	Jaw formation	211
31	Pectoral fin movement	240
32	Hatching	310
33	Diapause	> 310

2.4 Sample preparation, RNA extraction and RNA quality assessment

RNA was extracted using a TRIzol reagent. Briefly, pooled eggs were crushed using a mortar and homogenized with 150 µl of TRI Reagent. Another 150µl of TRI Reagent was then added to reach a total volume of 300 µl. After a 5 minute incubation at room temperature, 35 µl of 1-bromo-3-chloropropane were added to separate the samples in 3 phases (aqueous phase containing the RNA, interphase consisting of DNA and organic phase containing the proteins) after incubation and centrifugation. The clear supernatant, containing the RNA, was extracted, and transferred into a new Eppendorf. 175 µl of room temperature isopropanol were added to the supernatant to precipitate the RNA. After an incubation at room temperature and a centrifugation, the isopropanol was removed, and samples were rinsed with 350 µl of ice-cold ethanol (75%). Samples were centrifuged, the ethanol removed, and the remaining RNA pellet dried out. After evaporation of the ethanol, pellets were resuspended in 30 µl of nuclease-free water and frozen at -80°C. ARN concentration and sample contamination were checked using a Nanodrop spectrophotometer.

2.5 Quantitative reverse transcription PCR (RT-qPCR)

2.5.1. DNase and Reverse Transcription

First, samples were treated with DNase to remove residual genomic DNA that could interfere with the technique. The RQ1 RNase-Free Dnase kit ® (PROMEGA) was used. Briefly, 2.5 µl of 10x Reaction Buffer, 0.5 µl of RNase-free DNase and nuclease-free water were added to a maximum of 5 µg of RNA to obtain a final volume of 25 µl. After an incubation at 37°C for 25 min, 2.5 µl of DNase Inactivation Reagent was added to stop the activity of the DNase and thus, the degradation of residual genomic DNA. After a 2 min incubation at room temperature and a centrifugation, 11 µl of RNA was transferred to a fresh tube to perform the reverse transcription. 11 µl were transferred to a second tube to subsequently serve as negative control for the qPCR. The DNA-free RNA from the first tube was reverse-transcribed using the RevertAid RT Reverse Transcription kit ® (Thermo Fisher Scientific). Briefly, 1 µl of Random Hexamer primer was added to each sample and incubated at 65°C for 5 min. 8 µl of a MasterMix composed of 4 µl of 5x Reaction Buffer, 2 µl of 10 mM dNTP Mix, 1 µl of RiboLock Rnase Inhibitor and 1 µl of RevertAid RT was added to each sample to reach a final volume of 20 µl. The samples were incubated at 25°C for 5 min, 42°C for 60 min and then, 70°C for 5 min to

catalyze the cDNA synthesis. After cooling down to 4°C, cDNA samples were conserved at -20°C.

2.5.2 Primer design

Specific primers have been designed to amplify the cDNA of genes of interest. The ARNm sequence of each gene was downloaded from https://www.ncbi.nlm.nih.gov in FASTA format (exons). Pairs of primers were designed using AmplifX ®, the specificity was tested via Primer-Blast (Ye *et al.*., 2012) and Multiple Primer Analyzer (Breslauer, 1986) was used to detect potential undesirable primer-dimers. The primer efficiency was tested via a standard qPCR. To do so, cDNA from each sample is pooled together and serially diluted (5x, 25x, 125x, 625x, 3125x). Triplicates were done for each sample. Two negative controls (nuclease-free water and DNA-free RNA) were also carried out in triplicate. 2.5 µl of sample (cDNA, nuclease-free water or DNA-free RNA), 2.5 µl of primer (2.5 mM) and 5 µl of SYBER green (Bio Rad ®) are added in each well. The qPCRs were performed with a QuantStudio 5 Real-Time PCR System ® (Thermo Fisher Scientific), and the results were retrieved and analyzed via QuantStudio Design and Analysis ® (Thermo Fisher Scientific). Only primers with an efficacity between 95% and 105% and a R2 > 0,98 were selected (see appendix 1).

2.5.3 qPCR amplification

After the primer assessment, the RT-qPCR was carried out. The cDNA samples were diluted 100x and each sample was analyzed in triplicate. 2.5 µl of sample (cDNA, nuclease-free water or DNA-free RNA), 2.5 µl of primer (2,5 mM) and 5 µl of SYBER green (Bio Rad ®) were added in a 384-well plate, centrifuged and placed in the Real-Time qPCR system.

2.6 Data analysis

To calculate the relative gene expression, we used the ΔΔCt method with 18S as housekeeping gene. The best housekeeping gene was chosen by using RefFinder (Xie *et al.*.,2012) (See appendix 1). To do so, we calculated the ΔCt by subtracting the Ct of the housekeeping gene from the Ct of the gene of interest for the same condition. We calculated the ΔΔCt by subtracting the ΔCt of the reference group, corresponding to the treatment 25°C for the earliest stage

analyzed (Stage 16 or Stage 28). We calculated the fold change using $2^{-\Delta\Delta Ct}$. Samples with a Ct standard deviation between replicates superior to 0,5 were removed. A linear model was done on fold change values to eliminate any outliers. For each gene and temperature, the function outlierTest() (package *car*; R version 4.0.3) was used on the model *lm (FC ~ Stage)*. One dnmt3aa sample (p-value < 0,05) was removed (See appendix 2).

2.7 Statistic analysis

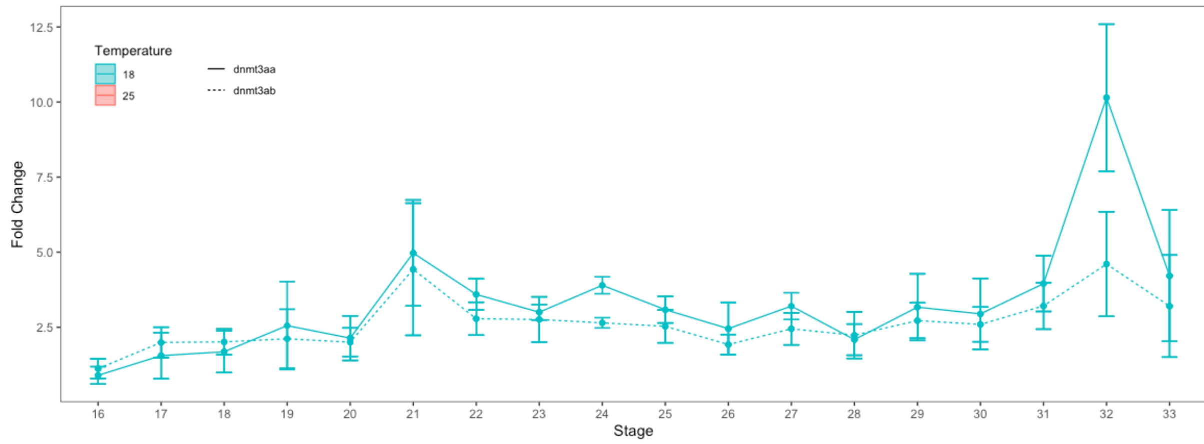
Normality and homogeneity hypotheses were tested with Shapiro-Wilk and Levene's test respectively (See appendix 3). Data for dnmt3aa and dnmt3ab were log2 transformed to achieve normality. A two-way ANOVA was used to test the interaction between temperatures and stages. A post-hoc Tukey's test was used to assess differences across temperature and stages. For all statistical analyses, a p-value $p < 0,05$ was considered significant. All analyses were performed on R (version 4.0.3).

3. Results

3.1 de novo DNA methylation gene expression

3.1.1 dnmt3aa

The two-way ANOVA showed that development stage and temperature had a significant influence on the gene expression of dnmt3aa. The interaction between the developmental stage and the temperature was also significant (Table 2). At 18°C, dnmt3aa expression increased from stage 16 (FC = 0.90 ± 0.29) to reach a first peak at stage 21 (FC = 4.97 ± 1.77). Even though the maximum expression level is obtained during stage 32 (FC = 10.15 ± 2.45), it is not significantly different from stage 21. The expression of dnmt3aa stayed constant from stage 21 to stage 33. At 25°C, dnmt3aa expression increased from stage 16 (reference group; FC = 1.04 ± 0.38) to reach a maximum during stage 30 (FC = 4.36 ± 1.45), without reaching a peak at stage 21. In the first stages of the development, the increase of dnmt3aa expression was slower at 25°C compared to 18°C. Regarding the impact of temperature on gene expression, dnmt3aa was overexpressed at 18°C compared to 25°C at stage 21 only (Figure 2; Figure 3; Table 1).



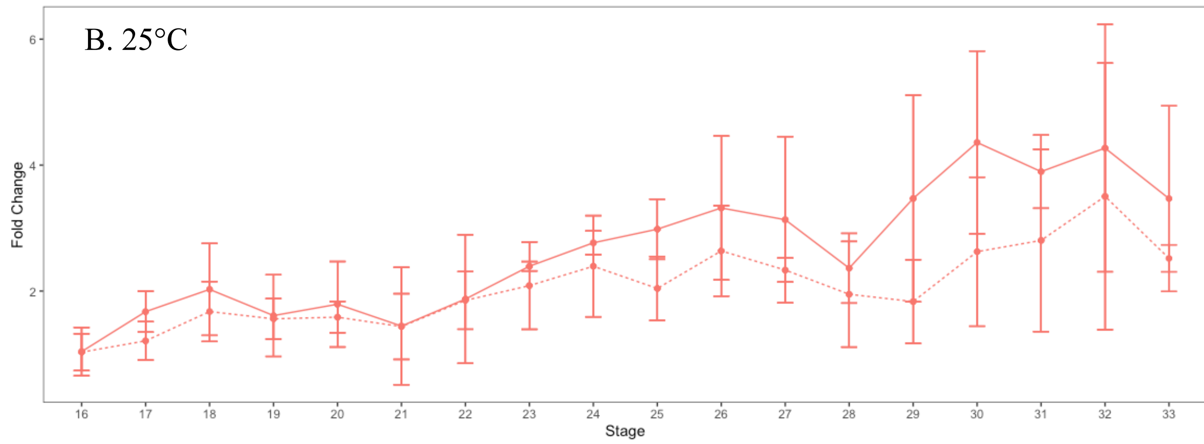


Figure 2 – Fold change of dnmt3aa (solid line) and dnmt3ab (dash line) during embryo development at (A) 18°C and (B) 25°C.

Table 2 – Results of the ANOVA II and significant results of post hoc Tukey's tests for the gene dnmt3aa (p-value < 0.05). For the Tukey's test, the group name corresponds to stage – temperature.

ANOVA II

	F	p-value	
Stage	8.884	4.36e-13	***
Temperature	6.954	9.80e-03	**
Stage : Temperature	2.771	9.23e-04	***

Tukey's test

Group 1	Group 2	p–value (Tukey's correction)
16–18	21–18	3.97e–05
16–18	22–18	2.13e–03
16–18	23–18	1.83e–02
16–18	24–18	1.51e–02
16–18	25–18	1.43e–02
16–18	29–18	1.52e–02
16–18	30–18	4.44e–02
16–18	31–18	7.09e–04
16–18	32–18	7.70e–10
16–18	33–18	1.07e–03
16–25	25–25	3.65e–02
16–25	26–25	1.81e–02
16–25	27–25	3.51e–02
16–25	29–25	1.55e–02
16–25	30–25	1.60e–03
16–25	31–25	4.28e–03
16–25	32–25	2.90e–03
17–18	21–18	1.07e–02
17–18	32–18	9.31e–08
18–18	21–18	3.48e–02
18–18	32–18	4.87e–07
19–18	32–18	1.21e–02
20–18	32–18	3.07e–05
21–18	21–25	9.71e–04
21–25	30–25	1.86e–02
21–25	31–25	4.28e–02
21–25	32–25	3.09e–02
23–18	32–18	9.74e–03
25–18	32–18	1.29e–02
26–18	32–18	2.95e–04
28–18	32–18	3.29e–05
29–18	32–18	1.20e–02
30–18	32–18	3.31e–03

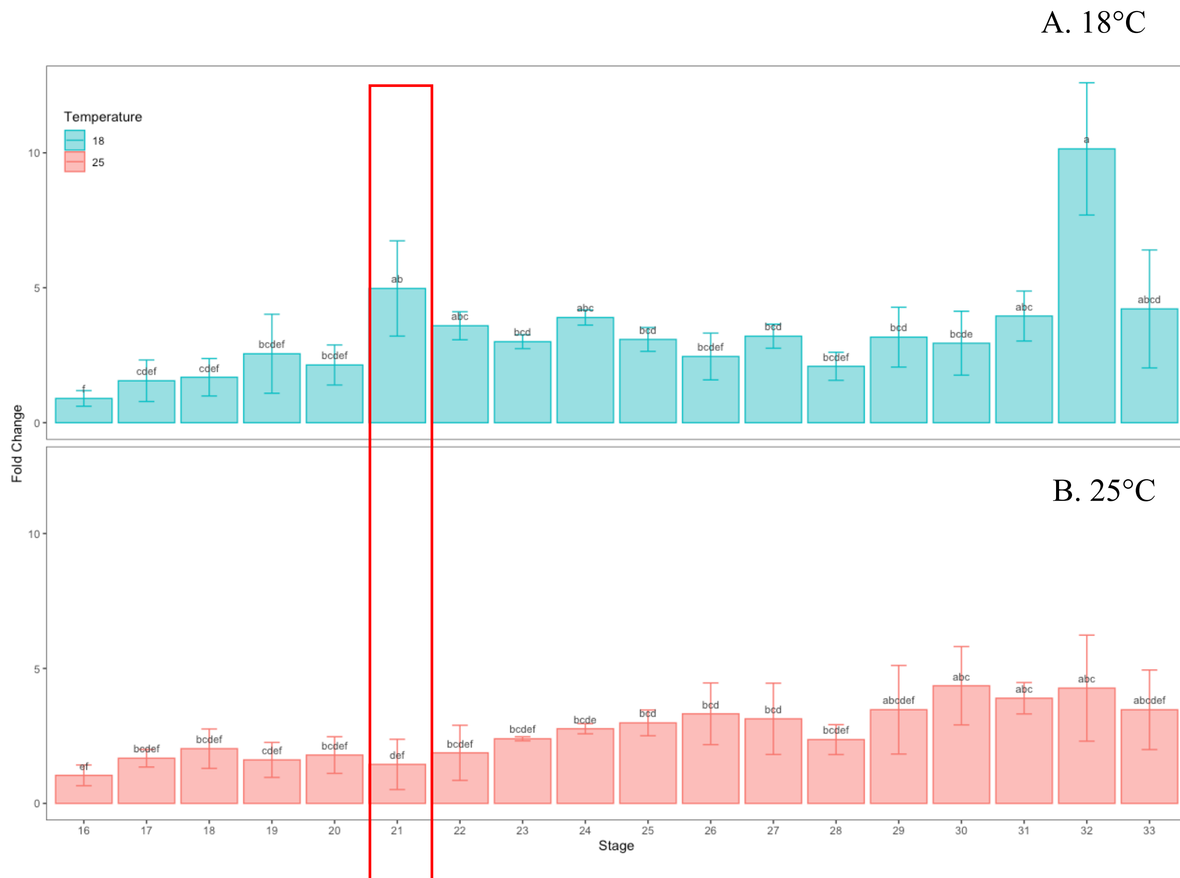


Figure 3 - Fold change of dnmt3aa during the emrbyo development of *K. marmoratus* at (A) 18°C and (B) 25°C. A two-way ANOVA showed that the interaction between the developmental stage and the temperature is significant (p-value = 9.23e-04). 18S was used housekeeping gene. The red box represents the stage for which temperature as a significant impact on gene expression. Significant differences between stages and temperature were checked using post hoc Tukey's test.

3.1.2 dnmt3ab

The development stage and the temperature had a significant influence on the gene expression of dnmt3ab, but no interaction was found between stages and the temperatures (Table 2). At 18°C, the gene expression rises from stage 16 (FC = 1.12 ± 0.32) to hit the first peak at stage 21 (FC = 4.43 ± 2.2). The maximum expression level is obtained at stage 32 (FC = 4.6 ± 1.74) but the difference with stage 21 was not significant. The expression level of dnmt3ab stayed constant from stage 21 to stage 33. At 25°C, stage 32 (FC = 3.5 ± 2.12) is the only one considered as significantly different from the reference group (T = 25°C; Stage = 16). As no

peak is reached at stage 21, the increase of dnmt3ab expression was slower at 25°C compared to 18°C during the first stages of development. Regarding the impact of temperature on gene expression, dnmt3ab was overexpressed at 18°C compared to 25°C at stage 21 only (Figure 4; Table 3).

Table 3 – Results of the ANOVA II and significant results of post hoc Tukey's tests for the gene dnmt3ab (p-value < 0.05). For the Tukey's test, the group name corresponds to stage – temperature.

ANOVA II

	F	p-value	
Stage	4.792	2.48e-07	***
Temperature	18.715	3.60e-05	***
Stage : Temperature	1.249	2.42e-01	

Tukey's test

Group 1	Group 2	p–value (Tukey's correction)
16–18	21–18	2.85e–04
16–18	31–18	1.30e–02
16–18	32–18	6.80e–05
16–18	33–18	3.20e–02
16–25	32–25	9.60e–03
21–18	21–25	1.08e–02

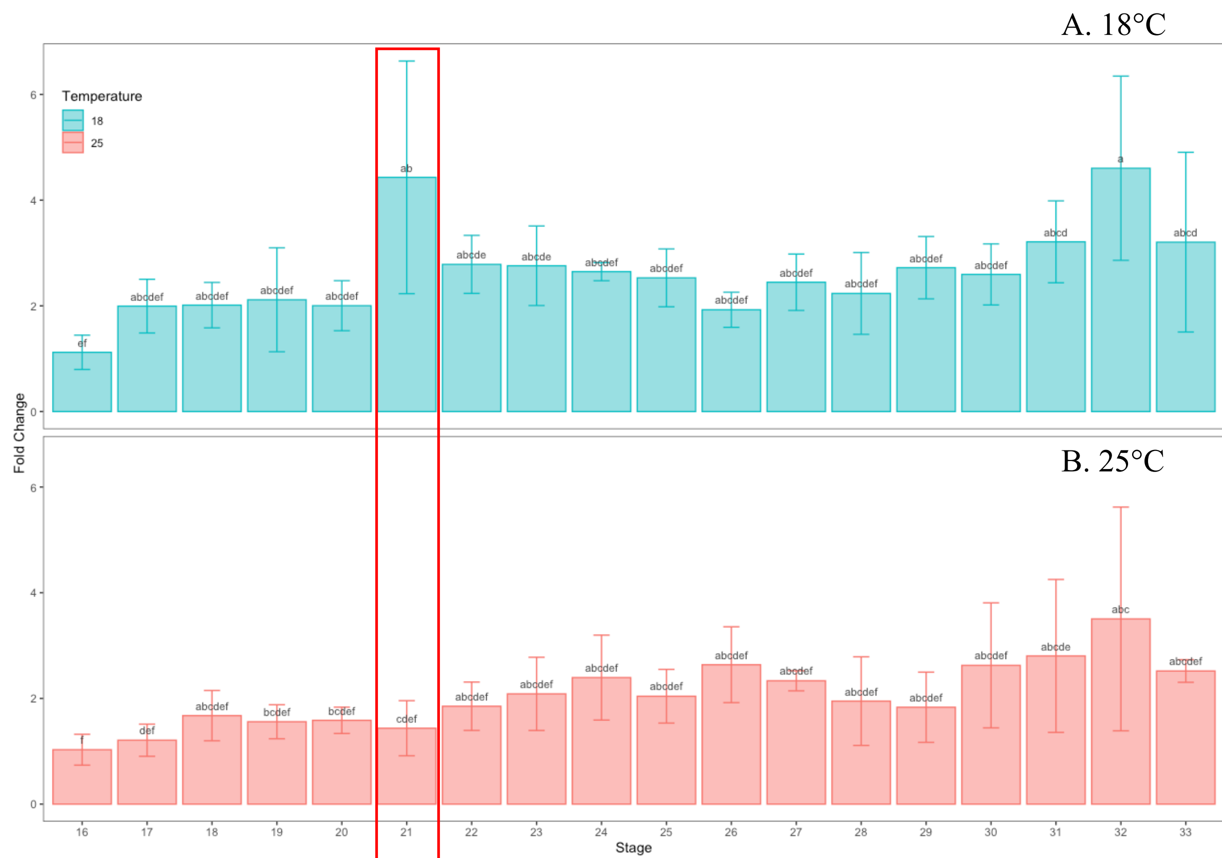


Figure 4 - Fold change of dnmt3ab during the embryo development of *K. marmoratus* at (A) 18°C and (B) 25°C. The interaction between the developmental stage and the temperature is not significant (p-value = 2.42e-01). 18S was used as housekeeping gene. The red box represents the stage for which temperature as a significant impact on gene expression. Significant differences between stages and temperature were checked using post hoc Tukey's test.

3.2 Sex-related gene expression

Regarding the sex-related genes, the stage and/or the temperature had a significant impact on gene expression (Table 4; Table 5). At 18°C, sox9a expression decreased from stage 28 (FC = 2.75 ± 0.5) to reach a minimum during diapause (FC = 0.62 ± 0.21). At 25°C, no significant differences could be highlighted. Regarding the impact of temperature on the gene expression, sox9a is overexpressed at 18°C during stage 28 only (Figure 5A; Table 4). For cyp19b expression, there were no significant differences between stages at 18°C. At 25°C, cyp19b expression increased from stage 28 (FC = 1.03 ± 0.03) to reach a maximum during diapause

(FC = 2.89 ± 0.08). No significant differences in cyp19b expression could be highlighted between temperatures (Figure 5B; Table 5).

Table 4 – Results of the ANOVA II and significant results of post hoc Tukey's tests for the gene sox9a (p-value < 0.05). For the Tukey's test, the group name corresponds to stage – temperature.

ANOVA II

	F	p-value	
Stage	11.634	5.62e-07	***
Temperature	0.012	9.13e-01	
Stage : Temperature	7.561	4.47e-05	***

Tukey's test

Group 1	Group 2	p–value (Tukey's correction)
28–18	28–25	4.73e–04
28–18	32–18	4.83e–06
28–18	33–18	1.65e–06
29–18	32–18	2.18e–04
29–18	33–18	7.09e–05
30–18	32–18	2.97e–02
30–18	33–18	1.13e–02
31–18	32–18	1.21e–02
31–18	33–18	4.68e–03

Table 5 – Results of the ANOVA II and significant results of post hoc Tukey's tests for the gene cyp19b (p-value < 0.05). For the Tukey's test, the group name corresponds to stage – temperature.

ANOVA II

	F	p-value	
Stage	6.073	2.34e-04	***
Temperature	0.517	4,76e-01	
Stage : Temperature	3.128	1,68e-02	*

Tukey's test

Group 1	Group 2	p–value (Tukey's correction)
28–25	32–25	1.76e–02
28–25	33–25	1.29e–03
29–25	33–25	8.71e–03
30–25	33–25	1.46e–02

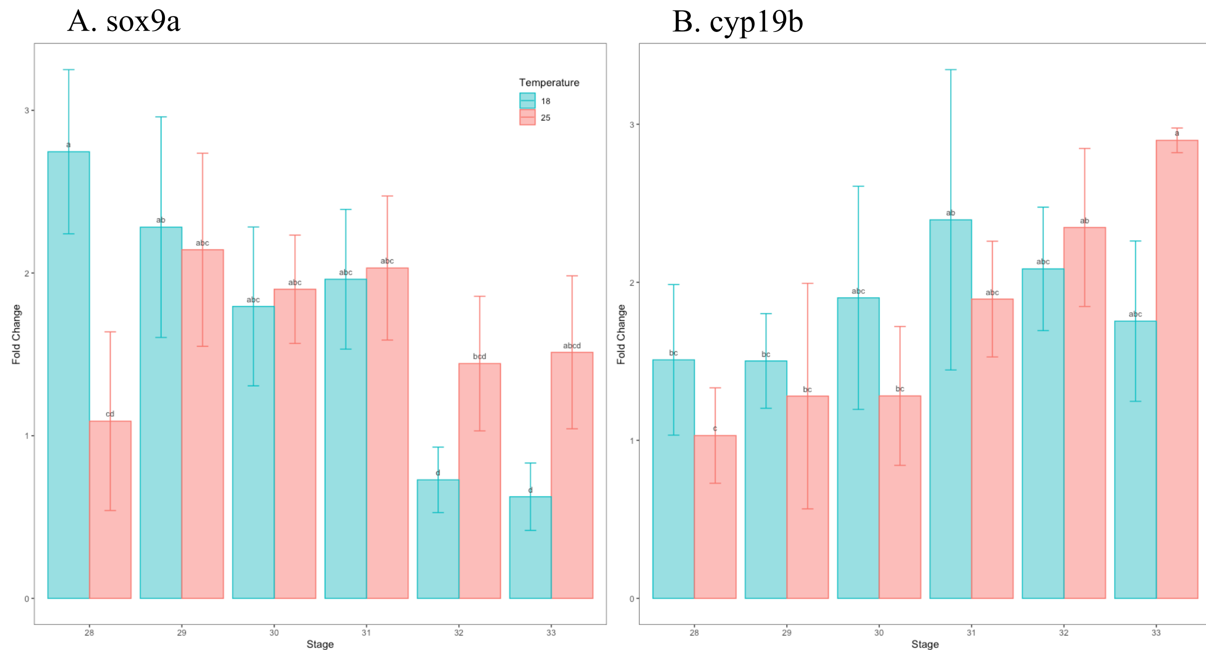


Figure 5 – Fold change of (A) sox9a and (B) cyp19b during the last stages of *K. marmoratus* embryo development at 18°C (blue) and 25°C (red). A two-way ANOVA showed that the interaction between the developmental stage and the temperature is significant ($p\text{-value}_{sox9a}$ = 4.47e-05; $p\text{-value}_{cyp19b}$ = 1.68e-02). 18S was used as housekeeping gene. Significant differences between stages and temperature were checked using post hoc Tukey's test.

4. Discussion

Previous studies on *K. marmoratus* showed that different populations have different sex ratio (Turner et al.., 1990; Taylor et al.., 2001) depending on the population's genetics and the environment (Harrington, 1967; Harrington & Kallman,1968; Taylor, 2012; Ellison *et al.*., 2015; Kelley *et al.*., 2016). Harrington (1967; 1968) discovered that temperature plays an important role in the sex determination of the rivulus and situated he thermolabile period between the stage 31 and 32. When incubated at 18°-20°, i.e., 5 to 7 degrees below their optimum of 25°C, *K. marmoratus* embryos have tendency to differenciate into males. Ellison *et al.*. (2015) also showed that masculinization is induced by low temperature, as well as a modification of the level of expression of the sexual genes cyp19a, associated with the ovarian development, and sox9a, associated with the development of testicular tissues, and that DNA methylation could be the intermediary through which temperature modulates sexual identity. DNA methylation is catalyzed by the activity of enzymes from the dnmts family, through which methyl-groups are added on DNA strands, and reduces or even suppresses gene expression by

blocking the binding of transcription factors (Moore & Fan, 2013). Fellous et al.. (2018) studied the expression level of different dnmts during the embryonic development of hermaphroditic embryos, and between male and hermaphroditic adult tissues. For embryos incubated at 25°C, they found that dnmt3ab was overexpressed during the last stages of embryonic development compared to previous stages and to dnmt3aa. According to them, that could indicate a potential role of rivulus dnmt3ab in oogenesis and in late DNA remethylation during embryonic development. They also found that dnmt3ab was overexpressed in male tissues and dnmt3aa was overexpressed in hermaphroditic tissues. In addition, Ellison *et al..* (2015) observed that a significant interaction between sex, temperature and methylation patterns. They were also able to identify several genes differentially methylated in males and hermaphrodites, namely sox9a and cyp19a. By highlighting differences in dnmt3a expression between males and hermaphrodites (Fellous *et al.*., 2018) and gene candidates for the temperature-mediated sex regulation (Ellison *et al.*., 2015), these studies incited us to look at the impact of temperature on sex determination during the embryonic development of *K. marmoratus* to try to identify a possible correlation between the expression of sexual genes and of the enzymes directly responsible for *de novo* methylation. In the current study, we therefore investigated the effect of a low temperature (18°C) on embryonic SD by looking at the expression of *de novo* DNA methylation genes (dnmt3aa and dnmt3ab) and sex-related genes (sox9a and cyp19b). Based on the work of Harrington (1967; 1968), we incubated eggs at 18°C and 25°C.

First, we investigate the expression pattern of dnmt3aa and dnmt3ab in embryos incubated at 18°C and 25°C. In most of teleost fish, two dnmt3a paralogs exist, dnmt3aa and dnmt3ab, both responsible for *de novo* DNA methylation (Campos *et al.*.,2012; Liu *et al.*., 2020). In this study, we found that the two variants were similarly expressed during embryo development of *K. marmoratus* when incubated at the same temperature. This result differs from what Fellous *et al..* (2018) found for *K. marmoratus* embryos incubated at 25°C, which is a differential expression between dnmt3aa and dnmt3ab during the last stages of embryonic development. This differential expression could indicate a potential role of rivulus dnmt3ab in oogenesis and in late DNA remethylation during embryonic development. It is important to note that the fish population that we use (EPP) differs from the one studied by Fellous et al.. (DC4). As sex determination of *K. marmoratus* depends on environmental but also on genetic factors (Ellison et al.., 2015), further studies would be needed to assess the part of genetic and/or environmental factors that influence the difference in the expression level of dnmt3aa in EPP and DC4 population. The similitude of expression levels of the two variants does not correspond to the differences found by Fellous et al.. (2018) in adults tissus. This may be due to the fact

that Fellous et al.. (2018) did the analyses on secondary males while we looked at potential primary males. Sex determination mechanisms and sex differentiation mechanisms might differ in *K. marmoratus.* Even though, understanding these differences needs further investigation, it has already been shown that steroid hormones are key regulators for sex differentiation (Godwin, 2010; Garcia *et al.*., 2016). Regarding the expression of dnmt3a variants according to the development temperature, we found that the expression level of both variants increased with the development stage, but the augmentation seems to start earlier at 18°C (male-producing temperature) compared to 25°C (hermaphrodite-producing temperature), with a maximal expression of dnmt3a at stage 21 in potential future primary males. The early expression of the dnmt3s in male embryos might increase the methylation and thus downregulate genes responsible for ovarian development. This corresponds to what was found in the zebrafish where the inhibition of dnmts by exposition to 5-aza-2′-deoxycytidine during embryonic development induces the feminization of the fish (Ribas *et al..,* 2017). In the Nile tilapia, dnmts might also have a potential role in the epigenetic control of gonadal development (Li et al.., 2014). If present in both testis and ovary, dnmt3aa and dnmt3ab will a higher level of expression in testis than in ovary. The difference of expression between gonads can be compared with the dnmt3 profile expression of *K. marmoratus* where dnmt3a expression increases earlier in potential males compared to hermaphrodites. In the Nile tilapia, the use of a dnmt inhibitor reduced dnmt3aa and dnmt3ab expression level leading to a significant augmentation in expression of cyp19a1a and dmrt1, key genes of gonadal development for females and males respectively. If embryos incubated at 18°C give a majority of males, DNA methyltransferase, and particularly dnmt3a, might have a potential role in gonadal development of *K. marmoratus.*

Then we looked at the level expression of sexual genes that might play a role in sex determination, namely sox9a and cyp19b. Sox9a is part of the SRY-related high mobility group box (SOX) genes family which corresponds to transcription factors that play crucial roles during reproduction and development (Hu *et al.*., 2021). While sox9 has been demonstrated to play a key role in sex determination and differentiation in vertebrates, it is not yet proven in teleost fish and its function is still debated (Chiang et al.., 2001; Nakamura et al.., 2012). In our study, embryos incubated at 18°C underwent a diminution of sox9a expression level during the last stages of development as well as an overexpression of sox9a at stage 28 compared to embryos incubated at 25°C, i.e. sox9a is overexpressed in males compared to hermaphrodites. In hermaphrodites, a constant expression level of sox9a was kept during the last stages of their development. Harrington (1968) showed that rivulus thermolabile stage was the second part of stage 31. Here, we found that a sexual gene involved in testis development is overexpressed

prior to stage 31. As for dnmt3a, the fish population analyzed here is different from the ones used by Harrington. The genetic differences between the two population might translate into an earlier thermolabile period in EPP population. Ellison et al.. (2015) found that sox9a was hypermethylated in males incubated at low temperature, which lead to a reduction of sox9a expression level in males compared to hermaphrodites, as opposed to what we observed in our analyses. Several scenarios could explain this Again, the genetic differences of the EPP population compared to the ones used by Ellison *et al.*., leads to differential expression of sox9a. If embryos incubated at 18°C do not yield 100% of males, the expression level observed might be superior to the expected one. Also, we see that, even though it is not significative compared to 25°C, expression level of sox9a drop significatively from stageb.28 to stage 33 at 18°C. It could also be interesting to look at the second paralogs of sox9, sox9b. In the Nile tilapia, the two paralogs do not have the same role in sex determination. While sox9a may play major role in the regulation of ovarian differentiation, sox9b seems to be involved in the regulation of testicular differentiation (Wei et al.., 2016).

The cyp19 gene encodes cytochrome P450 aromatase, which catalyzes the synthesis of estrogens (Chiang *et al.*., 2001). Two variants have been highlighted in fish, cyp19a which is expressed in the ovary, and cyp19b which is expressed in the brain (Chiang *et al.*., 2001). As the regulation of this gene dictates the ratio of androgens to estrogens, an appropriate expression of this enzyme is critical for reproduction and sex differentiation for most vertebrates (Trant *et al..,* 2001). Here, we found that expression level of cyp19b increases during the last stages of development for embryos incubated as 25°C, i.e. the hermaphrodites. In embryos incubated at male-producing temperature, expression level of cyp19b stays constant. Ellison *et al.*. (2015) showed that cyp19a could be involved in modulating sex ratio of the rivulus in response to temperature during the embryonic development as it is downregulated in the brain of fish incubated at 25°C. Here, we observed an augmentation of cyp19b expression at hermaphrodite producing temperature, which could indicate that this gene is indeed involved in the pathway leading to the differenciation of ovarian tissue in the rivulus, as it is in other species such as the zebrafish and the sea bass *Dicentrarchus labrax* (Trant *et al..,* 2001; Blázquez & Piferrer, 2005), but opposite to what Ellison et al.. (2015) suggest.. Cyp19b is however not always involved in sex determination or differenciation in all species, as for example for the Atlantic halibut *Hippoglossus hippoglossus*, where no dimorphic expression has been observed (van Nes *et al.*, 2005). In the Nile tilapia, another form of cyp19b, named tilapia cyp19b (tCyp19b) was shown to be exclusively expressed in female brain (Chang *et al.*., 2005). Further study still has to be done to determine the implication of cyp19b in sex determination by temperature.

Conclusion

Through this study, we manage to highlight the difference in level expression of dnmt3a between embryos incubated at 18°C and at 25°C. We also showed that sox9a is more expressed in male embryos. No significant differences could be observed for cyp19b. Regarding our main objective, no significant differences could be identified in dnmt3aa and dnmt3ab expression level, neither for embryos incubated at 18°C nor for those incubated at 25°C, during the last stages of embryonic development. No correlation could be found between dnmt3aa/ab and sexual genes sox9a/cyp19b in our study. In further study, it will be interesting to look at other sexual genes that might be involved in the sex determination by temperature like dmrt1, amh, foxl2 or fig-alpha, and to further investigate the methylation of those genes.

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Appendexes

Appendix 1 – Primer efficiency

Primers for 4 genes of interest (dnmt3aa, dnmt3ab, cyp19b and sox9a) and 3 housekeeping genes (18S, ß-actin and rpl8) presented an efficiency between 95% and 105% and $R^2 > 0,98$ (Table S1). Based on RefFinder, the housekeeping gene 18S was kept (Table S2). ß-actin could not be used because the number of replicates for certain stages was to low.

Table S1 – List of selected primers

Gene	Gene ID	Primers	Efficacity (%)	Standard Deviation	R^2
18S	108251219	F: GAACTCACCGACACCAGCA R: ATCATCGACGCTCCTGGA	100,911	0,062	0,996
ß-actin	108236185	F : CTTGCGGAATCCACGAGACC R : CCAGGGCTGTGATCTCCTTCTG	102,011	0,064	0,095
rpl8	108232351	F : TCGCTGGAGGCGGTCGTATTGATAAGC R : CCACCGAAGGGATGCTCAACAGGATTCA	103,665	0,091	0,99
dnmt3aa	108233456	F: CGACGCTGAAGCTGTGGAAATCCAGACTCG R: CGCCTTCTCTGCAGAGTTCTTGCGTGGT	101,56	0,056	0,996
dnmt3ab	108239073	F: TCTTTATGTGGTGTCCTGGTTCAAC R: TGTGCA TCAAACCTCACTTTGGA	100,149	0,095	0,99
sox9a	108241363	F: AGCATGGGCGAAGTGCACTC R: CAGCTCCAAAGTCGATATTGAGCTGC	102,359	0,083	0,992
cyp19b	108230104	F: TGTTCCTCAGGGTGCCTCTGAATGAG R: GAGCCATCCGAGCTTGAAGAAAACGT	102,914	0,106	0,987

Table S2 – RefFinder results for best housekeeping gene

	Ranking order		
	1	2	3
ΔCt	18S	ß-actin	rpl8
BestKeeper	18S	ß-actin	rpl8
Normfinder	18S	ß-actin	rpl8
Genorm	ß-actin|18S		rpl8
Recommended comprehensive ranking	**18S**	**ß-actin**	**rpl8**

Appendix 2 – Outlier elimination

To eliminate any potential outliers, a linear model *lm (FC ~ Stage*Temperature)* was done on fold change values for each gene. The *p-values* given by the fuction outlierTest() are presented in Table S3. Based on Cook's distance plot, the sample *25.28.19* was found to be an outlier in dnmt3aa data and removed. (Figure S1)

Table S3 – Results of the outlier test on the linear model *lm (FC ~ Stage*Temperature)* for each gene of interest.

Gene	Sample removed	unadjust p-value	Bonferroni p-value
dnmt3aa	None	0.00035914	0.046688
dnmt3aa	25.28.19	0.0013426	0.1732
dnmt3ab	None	0.00036868	0.050141
sox9a	None	0.0050525	0.26273
cyp19b	None	0.012533	0.70187

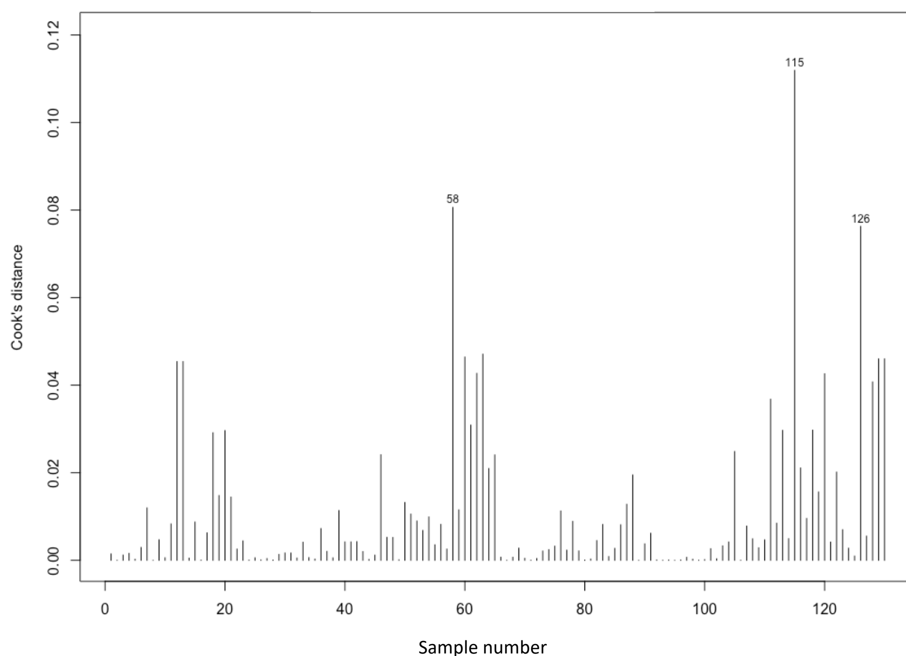

Figure S1 – Cook's distance of each sample from dnmt3aa dataset. Sample n°115, corresponding to sample ID 25.28.19, was considered an outlier, and removed.

Appendix 3 – Residuals normality and Homogeneity

To be able to perform a two-way ANOVA, residuals normality and homogeneity hypotheses have been tested for each gene with Shapiro and Levene's tests respectively (Table S4). A qqplot was carried out for each gene to visualize the residuals distribution (Figure S2). Based on the graphs and the p-values, data for dnmt3aa and dnmt3ab were log2 transformed.

Table S4 – Results of the Shapiro and Levene's tests for each gene of interest.

Gene	Data transformation	Test	F	p-value
dnmt3aa	None	Shapiro	0.990	0.437
		Levene	2.79	0.0000467
	Log2	Shapiro	0.982	0.0873
		Levene	1.22	0.227
dnmt3ab	None	Shapiro	0.959	0.000389
		Levene	2.43	0.000306
	Log2	Shapiro	0.985	0.154
		Levene	0.920	0.599
sox9a	None	Shapiro	0.982	0.598
		Levene	0.478	0.906
cyp19b	None	Shapiro	0.986	0.744
		Levene	0.951	0.503

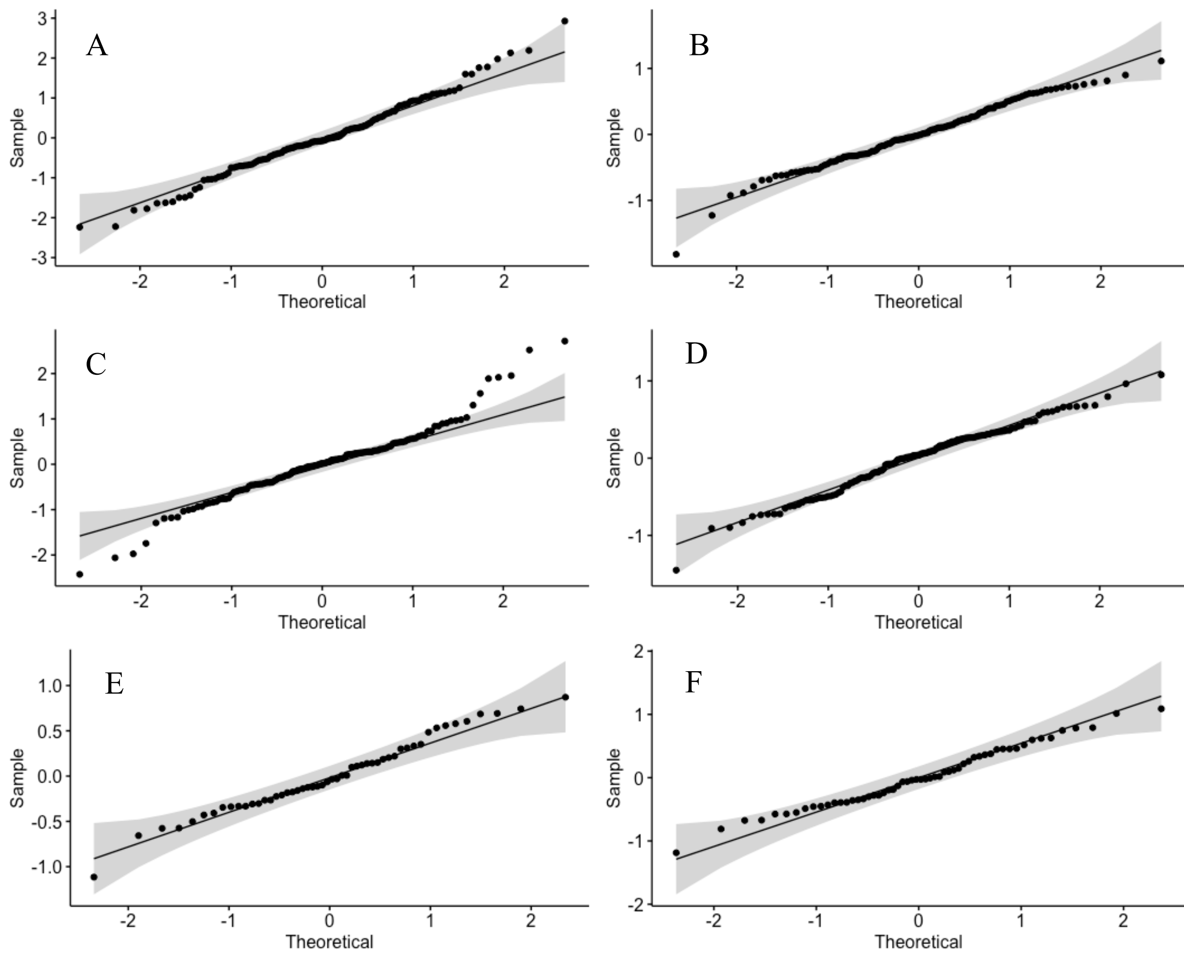

Figure S2 – qqplot of (A) dnmt3aa, (B) dnmt3aa (log2 transformed), (C) dnmt3ab,(D) dnmt3ab (log2 transformed), (E) sox9a, (F) cyp19b