Expression profiles of *Dax1*, *Dmrt1*, and *Sox9* during temperature sex determination in gonads of the sea turtle *Lepidochelys olivacea*

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Abstract

Sex determination is controlled either by genetic or environmental factors. In mammals *Sry* initiates determination but no homologue of this gene exists in non-mammalian species. Other genes of the mammalian sex-determining pathway have been identified in gonads of different vertebrates. *Sox9*, *Dax1*, and *Dmrt1* are expressed at the onset of gonadal development in birds and reptiles. In the sea turtle *Lepidochelys olivacea*, a species with temperature sex determination (TSD), *Sox9* is expressed in undifferentiated gonads at male- (MPT) or female-promoting temperatures (FPT). At MPT, *Sox9* remains expressed in male gonads, but at FPT it is downregulated coinciding with the onset of the ovarian morphologic differentiation and female sex determination. At MPT however, male sex is determined early than at FPT in still undifferentiated gonads suggesting that other genes maintain *Sox9* expression in testis. Here we used RT-PCR to study the expression profiles of *Dax1*, *Dmrt1*, and *Sox9* in gonads of embryos of *L. olivacea* incubated at MPT or at FPT. The profiles were correlated with sex determination during and after the temperature-sensitive period (TSP). *Dax1* maintained similar levels at both temperatures during the TSP. The *Dax1* expression level increased significantly in ovaries compared to testes at stage 27, once they were morphologically distinct. The expression levels of *Dmrt1* were higher at MPT than at FPT at all stages, in contrast with *Sox9* levels which were similar at both temperatures at stages 23–25. Together, current results suggest that, whereas *Dax1* is not involved in TSD in *L. olivacea*, upregulation of *Dmrt1* and downregulation of *Sox9* may play a role in male and female sex determination, respectively.
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Keywords: Temperature sex-determination (TSD); *Dmrt1*; *Dax1*; *Sox9*

1. Introduction

Vertebrates exhibit at least three different sex-determining mechanisms. Whereas in mammals the male is the heterogametic sex (X or Y sperms), in birds the heterogametic sex is the female (Z or W oocytes). The third mechanism is found in several species of reptiles lacking sex chromosomes. In these species, temperature determines sex at a critical stage of development (Bull, 1980).

In mammals sex determination depends of the gene *Sry* (Koopman et al., 1991; Sinclair et al., 1990). In birds and reptiles no *Sry* homologue has been found, but homologues of several other mammalian sexual genes have been identified (Smith et al., 1999a; Spotila et al., 1998; Western et al., 1999). Among them *Dax1* has been studied in several vertebrates. *Dax1* encodes an atypical member of the nuclear receptor family that retains the conserved ligand-binding motif but lacks the zinc-finger DNA-binding motif (Zanaria et al., 1994). In the mouse, *Dax1* expression has been detected during the first stages of gonadal and adrenal development, its expression persists in the developing ovary but decreases in the testis coinciding with *Sry* activation and testis differentiation (Swain et al., 1996). *Dax1* gene homologues have

been found in the chicken (Smith et al., 1999a; Smith and Sinclair, 2001) and in *Alligator mississippiensis*, a species with temperature sex-determination (TSD) (Western et al., 2000). Although in the two latter species it was found that *Dax1* is expressed in embryonic gonads of both sexes, its levels were not determined.

On the other hand, *Dmrt1* encodes a transcription factor with a DM domain, a DNA-binding domain identified in the sexual regulators of *Drosophila melanogaster* (double sex) (Erdman and Burtis, 1993) and *Caenorhabditis elegans* (mab-3) (Raymond et al., 1998). DM domain genes have been implicated in sexual development in vertebrates as well. Expression of *Dmrt1* is sexually dimorphic in mouse, chicken, alligator, and red-eared slider turtle. In mouse embryos, *Dmrt1* is expressed in early genital ridges of both sexes and then increases in testis but decreases in ovaries (Raymond et al., 1999). In the chicken, the *Dmrt1* gene is found on the Z chromosome, suggesting that two doses of *Dmrt1* might be necessary for testis development, whereas a single copy may lead to female sexual differentiation (Nanda et al., 1999). In addition, the *Dmrt1* gene shows double gonad-specific expression in developing male gonads, compared with female gonads; this difference is evident before and during the time of gonadal sex differentiation (Raymond et al., 1999; Smith et al., 1999b). In the alligator, *Dmrt1* gene expression is initially detectable by RT-PCR in the urogenital systems of embryos incubated at both male- (MPT) and female-promoting (FPT) temperatures. However, gonadal expression subsequently became higher in male embryos than in female embryos (Smith et al., 1999b). In the fresh water turtle *Trachemys scripta*, *Dmrt1* expression is higher at MPT than at FPT during the thermosensitive period (TSP) (Kettlewell et al., 2000).

The *Sry*-related gene *Sox9* is upregulated in male gonads of mouse and chicken (Kent et al., 1996; Morais Da Silva et al., 1996). In reptiles lacking sexual chromosomes, the *Sox9* gene is also upregulated in male gonads. Three species with TSD have been studied: the red-eared slider turtle *T. scripta* (Spotila et al., 1998), the American alligator *A. mississippiensis* (Western et al., 1999), and the Olive ridley *L. olivacea* (Moreno-Mendoza et al., 1999).

In *L. olivacea* the MPT are 26–27 °C and the female promoting FPT are 32–33 °C, the TSP occurs between stages 21 and 26 (Merchant-Larios et al., 1997). In this species, *Sox9* expression in the gonads is regulated by temperature: whereas it is expressed in both MPT and FPT at stages 21–25, it is downregulated at FPT from stage 26 and onwards (Moreno-Mendoza et al., 1999). Shifting embryos or isolated gonads from MPT to FPT revealed that *Sox9* downregulation occurs at the onset of morphological differentiation of the ovaries in vivo (Torres–Maldonado et al., 2001) and in vitro (Moreno-Mendoza et al., 2001). Although shifting experiments

showed that downregulation of *Sox9* coincides with female sex determination, male determination occurs one or two stages earlier, when this gene is expressed in gonads of embryos incubated at both temperatures. Thus, it is reasonable to postulate that other gene(s) placed upstream of *Sox9* respond earlier to sex-determining temperature in *L. olivacea*.

In the present work, we used RT-PCR to make the mRNA expression profiles of *Dax1* and *Dmrt1* in gonads of *L. olivacea* during and after the TSP and compared these profiles with the expression profile of *Sox9*. The results were correlated with sex determination in this species.

2. Materials and methods

2.1. Embryos

Freshly laid eggs of *L. olivacea* (Olive ridley) were collected at La Escobilla beach on the Pacific Coast of Oaxaca, Mexico (96°27′16″W, 15°40′36″N). The eggs were transported to the laboratory in Mexico City and incubated at 26 °C (MPT) or at 33 °C (FPT). Embryos were sampled at stages 23–28 and at hatching according with Miller's criteria, 1985. All protocols described were approved by local animal rights committees of the Secretary for Environment and Natural Resources (SEMARNAT) and the National Autonomous University of Mexico.

2.2. Total RNA isolation and RT-PCR

Urogenital complexes were dissected out from embryos incubated at 26 °C (MPT) or at 33 °C (FPT). Gonads were separated from the adjacent mesonephros and frozen in an Eppendorf tube in variable numbers depending on the development stage: Four gonads at stage 23 (2 embryos); two gonads at stage 24, (1 embryo), and one gonad at stages 25–31, (1 embryo each). Experiments were repeated five times for each stage.

Gonadal tissue was frozen and stored at −70 °C. Total RNA was prepared homogenizing the tissue in 100 μl Trizol reagent (Life Technologies Invitrogen) and 50 μl chloroform was added. The aqueous phase was precipitated in 150 μl isopropanol at 4 °C overnight. The RNA pellet was rinsed in 75% ethanol and resuspended in 12 μl DEPC-treated ddH$_2$O and immediately used for RT-PCR. Reverse transcriptase-polymerase chain reaction (RT-PCR) amplifications were performed using two degenerated primers designed from conserved amino acid sequences of DAX1 protein from *A. mississippiensis* (Western et al., 2000), *G. gallus* (Smith et al., 1999a), *M. musculus* (Swain et al., 1996), and *H. sapiens* (Guo et al., 1996). The sense primer was Dax1-1: 5′-ACC AAG

GAG TAC/T GCA/C/T/G TAC/T CT-3′, the antisense primer was Dax1-2: 5′-TCC AGA/C/T/G AGC ATA/G TCA/G TCC AT-3′, this primer amplifies a 271-bp fragment. For the *Dmrt1* gene we used the previously reported primers: JK41: 5′-CGC AGG TTG CAT TGA GAA GGC AGC-3′ (sense), JK42: 5′-TCT GCC ATT GGT TTC CTG ATT GGC-3′(antisense) (Kettlewell et al., 2000), these primers amplify a 473-bp fragment. For expression control, amplification of β-actin mRNA was performed using the following primers: Actin 1: 5′-TGG ATG ATG ATA TTG CTG C-3′ (sense), Actin 2: 5′-ATC TTC TCC ATA TCA TCC CA-3′(antisense) (Kost et al., 1983). The actin primers produce a 252-bp fragment.

RT-PCR reactions were carried out by means of the One Step RT-PCR Kit (Life Technologies Invitrogen), using 3 μl total RNA per reaction, 1× buffer reaction mix containing 0.2 mM of each dNTP, 1.2 mM MgSO$_4$, and 0.2 μM β-actin primers or 0.3 μM of *Dax1* or *Dmrt1* primers in separate tubes, in a total volume of 20 μl. All reactions for *Dmrt1* contained 6% DMSO. cDNA synthesis was performed at 50 °C for 30 min and amplification conditions were: 94 °C/5 min (once) for initial denaturation, 94 °C/30 s for denaturation, 56 °C (β-actin) 60 °C (*Dax1*) and 63 °C (*Dmrt1*)/60 s for annealing, 72 °C/60 s for extension (35 cycles), and 72 °C/10 min (once) for final extension. To confirm that the amplified products did not come from genomic DNA, we used a control sample with total RNA without reverse transcriptase and with only *Taq* DNA polymerase in the RT-PCR experiments. Ten microliters of each RT-PCR reaction were electrophoresed on a 2.5% agarose gel with 0.1 μg/ml ethidium bromide in TAE 1× buffer (Sambrook and Russell, 2001).

2.3. Sequences and analysis

The identity of the amplified fragments was confirmed by automatic sequencing. All sequences were bidirectionally determined with an ABI PRISM 310 sequencer, based on fluorescent PCR sequencing (Perkin–Elmer). All nucleotide sequences were submitted to GenBank and the protein sequence was deduced.

Homology analysis of the amino acid sequence alignments of *L. olivacea* and other vertebrates was performed with MEGA (Molecular Evolutionary Genetic Analysis) software (Kumar et al., 1993).

2.4. Densitometry

Gels were visualized by UV transillumination and photographed with a DS-34 Polaroid camera; the intensity of each band was quantified by densitometry using the Scion Image program (Beta 4.0.2. Scion Image. Copyright 1997–2000 Scion Corporation). The semiquantitative results are expressed as arbitrary units of the ratio between the mRNA levels of the studied gene (*Dax1* or *Dmrt1*) and β-actin. The ratios of the five samples analysed per stage were averaged and the means (±SD) is shown in the graphics. To compare the expression profile of the genes *Dax1* and *Dmrt1* with the expression profile of *Sox9*, the ratio between *Sox9* and β-actin was plotted, using the RT-PCR results of our previous paper (Torres–Maldonado et al., 2001).

3. Results

The *Dax1* fragment amplified with the pair of oligonucleotides designed for the present study was identified by sequencing (GenBank Accession No. AY077685). Table 1 shows the DAX1 amino acid partial sequence alignment with the sequence of other vertebrates and the comparative identity between them. Amino acid identity of the partial sequence of DAX1 of *L. olivacea* with other vertebrates is: human 73.07%, mouse 67.7%, chicken 90.32%, and alligator 90.13%. The highest identity of the Olive ridley was with chicken, and the lowest with alligator and mouse (Table 2).

The *Dmrt1* fragment amplified with the pair JK41 and JK42 oligonucleotides was identified by sequenc-

Table 1
Partial Sequence alignment of DAX1 in some vertebrates

Species	GenBank	Sequence[a]
Human	U31929	T**KEYAYLKGTVLFNPDVPGLQCVKYIQGLQWGTQQILSEHTRMTHQGPHDRFIE**
Mouse	U41568	T**KEYAYLKGTVLFNPDLPGLQCVKYIEGLQWRTQQILTEHIRMMQREYQIRSAE**
Chicken	AF202991	T**KEYAYLKGTVLFNPDLPGLQCTQYIEGLQKEAQEALNEHVRLIHRGDQARFAK**
Alligator	AF180295	P**KEYAYLKGAVLFNPDLPGLQCTQYIQGLQREAQQALNEHVRLIHRGDQARFAK**
Olive ridley	AY077685	T**KEYAYLKGTVLFNPDLPGLQCVQYIQGLQREAQQALNERVRLLHRGDQARFAK**
Human	U31929	**LNSTLFLLRFINANVIAELFFRPIIGTVSMDDMML**
Mouse	U41568	**LNSALFLLRFINSDVVTELFFRPIIGAVSMDDMML**
Chicken	AF202991	**LNVVLSLLRSINANVIAELFFRPIIGTVNMDDMLL**
Alligator	AF180295	**LNIALSLLRSINAN**- - - - - - - - - - - - - - -
Olive ridley	AY077685	**LNVVLSLLRSINANVIAELFFRPIIGTVNMQDMRD**

[a] Identical residues are in bold letters, dashes indicate unknown or absent residues.

Table 2
DAX1 amino acid sequence identities in some vertebrates

	Identity (%)				
	Human	Mouse	Chicken	Alligator	Olive ridley
Human	100				
Mouse	78.05	100			
Chicken	73.07	72.01	100		
Alligator	67.6	66.17	90.13	100	
Olive ridley	73.07	67.7	90.32	90.13	100

ing (GenBank Accession No. AF335421). The nucleotide sequence was translated into the amino acid sequence and compared with corresponding sequences of other vertebrates (Table 3). The identity of DMRT1 of *L olivacea* with other vertebrates is: human 65.26%, mouse 67.38%, chicken 82.27%, alligator 88.89%, and red-eared slider turtle 96.52%. The highest similarity of the Olive ridley was with the red-

eared slider turtle and the lowest between chicken and human (Table 4).

Fig. 1A shows the expression of *Dax1* detected by RT-PCR in gonads from embryos at MPT (26 °C) and FPT (33 °C). A 252-bp fragment of β-actin was used as internal expression control. Although the 271-bp fragment of *Dax1* was detected in all samples, its intensity differed. Whereas at stages 23–26 the band intensities were similar at both temperatures, at stage 27 the band intensity showed a clear decrement in gonads from embryos incubated at MPT. The expression profiles of *Dax1* at MPT and FPT showed no significant differences at stages 23–26. Only at stage 27 of FPT a significantly higher expression level of *Dax1* was found (Fig. 1B). The RT-PCR analysis of *Dmrt1* expression is shown in Figs. 2A and B. The 473-bp fragment of *Dmrt1* was detected in gonads of *L. olivacea* at all stages of MPT (Fig. 2A). At stages 23–25 the band intensities were

Table 3
Partial sequence alignment of DMRT1 in some vertebrates

Species	GenBank	Sequence[a]
Human	AF130728	QVALRRQQAQEEELGISHPIPLPSAAELLVKRENNGSNPCLMTECSG-TSQPPPASVPTT
Mouse	AF192561	QVALRRQQAQEEELGISHPIPLPSAAELLVKRENNASNPCLMAENSS-SAQPPPASTPTP
Chicken	AF123456	QVALRRQQAQEEELGISHPVPLPSAPEPVVKK-SSSSSSCLLQDSSSPAHSTSTVAAAAA
Alligator	AF192560	QVALRRQQAQEEELGISHPIPLPSATELFVKKENSGGSSCLLLESSSPTHSTSTVTTVST
Red-eared[b]	AF201387	QVALRRQQAQEEELGISHPIPLPSAPKLFVKKENNGGSSCLLLESSSPTHSTNTATTAST
Olive ridley[c]	AF335421	QVALRRQQAQEEELGISHPIPLPSAPELFVKKETNGGSSCLLLENSSPTHSTNTATTAST
Human	AF130728	AAS-EGRMVIQDIPAVTSRGHVENTPDLVSDSTYYSSFYQPSLFPYYNNLYNCPQYSMAL
Mouse	AF192561	AAS-EGRMVIQDIPAVTSRGHMENTSDLVSDPAYYSSFYQPSLFPYYNNLYNYPQYSMAL
Chicken	AF123456	SAPPEGRMLIQDIPSIPSRGHLESTSDLVVDSTYYSSFYQPSLYPYYNNLYNYSQYQMAV
Alligator	AF192560	SPS-EGRMLIQDVPSITSRGHLESTSDLVV- - - - - - - - - - - - - - - - - - -
Red-eared	AF201387	TPS-EGRMLIQDIPSITSRGHLESTSDLVVDSTYYSSFYQPSLYPYYNNLYNYSQYQMAV
Olive ridley	AF335421	TPS-EGRMLIQDIPSITSRGHLESTSDLVVDSTYYSSFYQPSLYPYYNNLYNYSQYQMAV
Human	AF130728	AADSASGEVGNPLGGSPVKNSLRGLPGPYVPGQTGNQWQ
Mouse	AF192561	SAESSSGEVGNSLGGSPVKNSLRSLPAPYVPAQTGNQWQ
Chicken	AF123456	ATESSSSETGGTFVGSAMKNSLRSLPATYMSSQSGKQWQ
Red-eared	AF201387	ASDSSSSDMGGTLAGSPVKNSLRSLPATYMSSQSGNQWQ
Olive ridley	AF335421	ASESSSSDMGGTLVGSPVKNSLRSLPATYMSSQSGNQWQ

[a] Identical residues are in bold letters, dashes indicate unknown or absent residues.
[b] *Trachemys scripta.*
[c] *Lepidochelys olivacea.*

Table 4
DMRT1 amino acid sequence identities in some vertebrates

	Identity (%)					
	Human	Mouse	Chicken	Alligator	Red-eared	Olive ridley
Human	100					
Mouse	85.98	100				
Chicken	59.32	62.17	100			
Alligator	62.94	62.94	79.56	100		
Red-eared[a]	65.96	65.96	80.86	90.12	100	
Olive ridley[b]	65.26	67.38	88.89	88.89	96.52	100

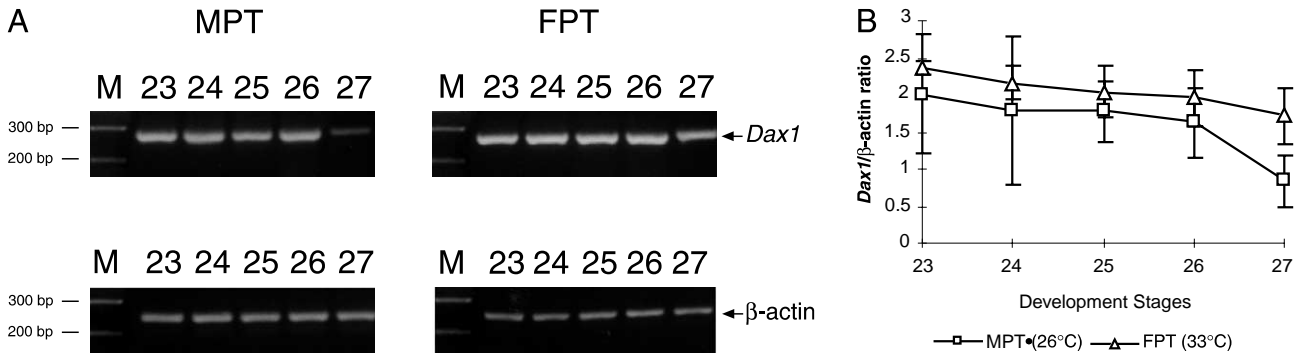[a] *Trachemys scripta.*
[b] *Lepidochelys olivacea.*

Fig. 1. Expression of *Dax1* mRNA analysed by RT-PCR and densitometry. (A) Representative agarose gel electrophoresis of gonads from embryos incubated at MPT or FPT. Total RNA from gonads at stages 23–27 (lines 23–27) was used. β-actin served as reference of the loading amount of total RNA for each stage. (B) Expression profiles showing the *Dax1*/β-actin ratio in gonads incubated at MPT (□) or FPT (△) sampled at stages 23–27.
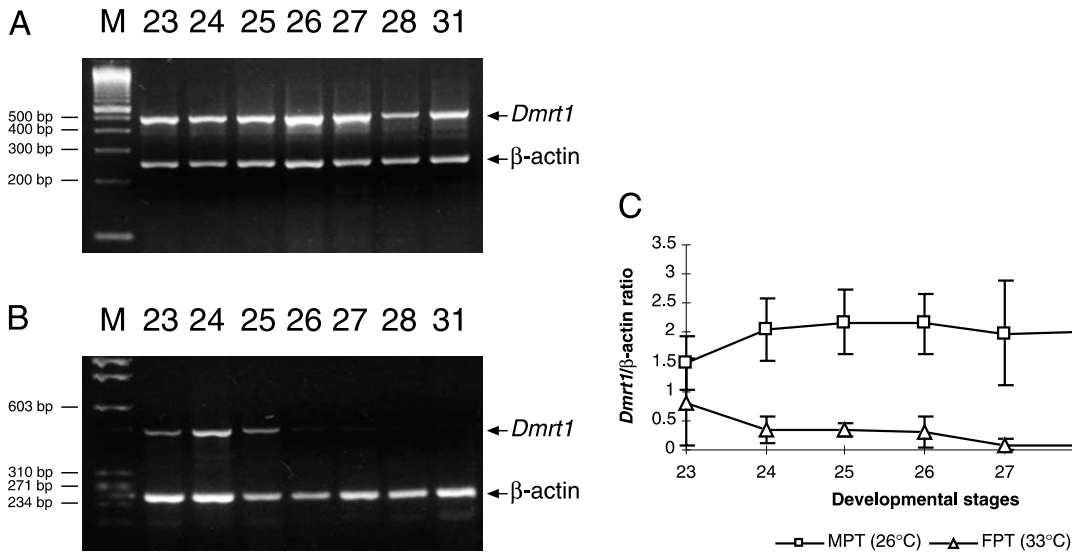

Fig. 2. Expression of *Dmrt1* mRNA analysed by RT-PCR and densitometry. Total RNA from gonads at stages 23–31 (lanes 23–31) of development at MPT (A) or FPT (B). β-actin was used as reference. (C) The expression profiles of transcript levels quantified by densitometry are expressed as *Dmrt1*/β-actin ratio per stage of development (23–31) at MPT (□) or FPT (△).

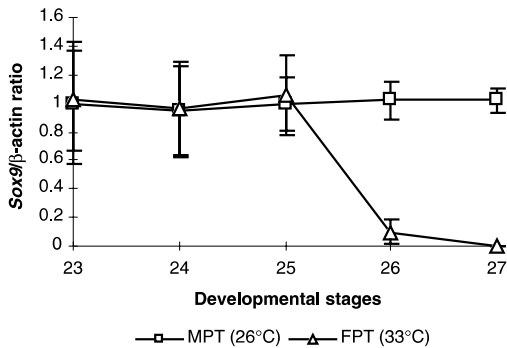Fig. 3. Profile levels of expression of *Sox9* analysed by densitometry. Results are shown as *Sox9*/β-actin ratio analysed in gonads incubated at MPT (□) or FPT (△) at stages 23–27.

similar, they increased at stages 26 and 27 and at stages 28 and 31 they were similar to the intensities shown at stages 23–25. In contrast, with FPT the *Dmrt1* fragment was detected only at stages 23–25.

The expression profiles of *Sox9* showed that the levels of expression are similar at both temperatures for stages 23–25. From stage 26 onwards a dramatic fall of expression occurs with FPT (Fig. 3).

The levels of expression of *Dmrt1* showed a profile quite different to the *Dax1* profiles. Although at stage 23 the *Dmrt1* expression levels were not significantly different at MPT and FPT, the levels were significantly higher at MPT than at FPT from stage 24 onwards. At FPT, the low level of mRNA expression of *Dmrt1* decreased at stage 27 and disappeared at stage 31 (Fig. 2C).

The expression profiles of *Dax1*, *Dmrt1*, and *Sox9* in gonads of embryos incubated at MPT or at FPT are shown in Fig. 1B, Fig. 2C and Fig. 3, respectively. At stages 23–25 the levels of expression of *Dax1* and *Sox9* did not show differences significant in embryonic gonads incubated at both temperatures. At stage 26, however,

the expression level of *Sox9* decreased dramatically whereas the level of *Dax1* showed a significant decrease only at stage 27 (Fig. 1B and Fig. 3).

4. Discussion

The similarity of the DAX1 amino acid partial sequence of the Olive ridley with human and mouse was lower than the similarity with chicken and alligator. The greatest similarity when all vertebrates were compared was between *L. olivacea* and chicken with 90.32%, the lowest between alligator and mouse with 66.17%. The *L. olivacea* DAX1 appeared to be closer to chicken and alligator DAX1. However, current results suggest that the similarity of DAX1 between different groups of vertebrates is low. *Dax1* is X-linked in human and mouse (Zanaria et al., 1994; Swain et al., 1996), but is autosomic in marsupials (Pask et al., 1997). Its chromosomal localization in chicken, alligator, and Olive ridley are still unknown.

The role of *Dax1* during gonadal development appears to be diverse in different groups of vertebrates. In mouse, *Dax1* expression is detected early in male and female gonads and is downregulated in males (Swain et al., 1996). Strains of transgenic mice carrying weak alleles of *Sry* together with extra copies of *Dax1* can show male to female sex reversal suggesting that *Dax1* acts as an "antitestis" factor antagonizing *Sry* function (Swain et al., 1998). In the chicken, however, *Dax1* is not downregulated at the onset of testis differentiation as it is in the mouse, suggesting that its role is different among vertebrate species (Smith and Sinclair, 2001).

The present data of *Dax1* expression in *L. olivacea* confirm results obtained in the alligator *A. mississippiensis*, a species with a different pattern of temperature sex determination (Western et al., 2000). *Dax1* expression was detected during the thermosensitive period (TSP) with both, MPT and FPT. In *L. olivacea* current results show that *Dax1* is expressed similarly with both temperatures at stages 23–26. Significant differences between samples at FPT and MPT were detected up to stage 27, one or two stages after female or male sex determination, respectively (Merchant-Larios et al., 1997). Assuming that sex-specific levels of gene expression during the TSP are directly related to sex determination, the fact that significant differences of *Dax1* expression were found after the TSP, suggests that this gene is not involved in TSD in this species. One may speculate that *Dax1* in *L. olivacea* may be related to functional differences between ovaries and testes as suggested in other species (Yu et al., 1998).

The similarity between DMRT1 amino acid partial sequence of *L. olivacea* with human and mouse is lower than with chicken, alligator, and the red-eared slider turtle. The highest similarity comparing the studied vertebrates is between the Olive ridley and the chicken with 90.32%, the lower is found between the alligator and the mouse with 66.17%.

In mammals, birds, and reptiles, early sex-specific expression of *Dmrt1* in gonads suggests its possible role in sex determination (Kettlewell et al., 2000; Nanda et al., 1999; Raymond et al., 1998; Raymond et al., 1999; Raymond et al., 2000). In two species with TSD, the American alligator and the red-eared slider turtle, sex-specific *Dmrt1* expression precedes *Sox9* expression in males suggesting that *Dmrt1* may act upstream of *Sox9*.

Current results in *L. olivacea* show that the levels of *Dmrt1* expression remain lower at FPT than at MPT as in *A. mississipiensis* and *T. scripta*. Since this occurs during and after the TSP, it is clear that *Dmrt1* expression is regulated by temperature in the three species. As was previously found, *Sox9* downregulation at stages 25–26 is related to female sex determination in *L. olivacea* (Moreno-Mendoza et al., 1999; Torres–Maldonado et al., 2001). In this species however, testis are committed at stage 24, when *Sox9* is expressed similarly at both temperatures. Thus, the higher level of expression of *Dmrt1* at MPT than at FPT during the TSP supports the idea that *Dmrt1* may act upstream of *Sox9* maintaining its expression in male gonads. Furthermore, the finding that there is a correlation between female sex determination and downregulation of *Sox9* and low levels of *Dmrt1* suggest that these two genes are actively regulated at FPT to allow ovarian commitment and female sex determination in *L. olivacea*.

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