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Mechanisms related to sexual determination by temperature in reptiles

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ABSTRACT

A number of strategies have emerged that appear to relate to the evolution of mechanisms for sexual determination in vertebrates, among which are genetic sex determination caused by sex chromosomes and environmental sex determination, where environmental factors influence the phenotype of the sex of an individual. Within the reptile group, some orders such as: Chelonia, Crocodylia, Squamata and Rhynchocephalia, manifest one of the most intriguing and exciting environmental sexual determination mechanisms that exists, comprising temperature-dependent sex determination (TSD), where the temperature of incubation that the embryo experiences during its development is fundamental to establishing the sex of the individual. This makes them an excellent model for the study of sexual determination at the molecular, cellular and physiological level, as well as in terms of their implications at an evolutionary and ecological level. There are different hypotheses concerning how this process is triggered and this review aims to describe any new contributions to particular TSD hypotheses, analyzing them from the "eco-evo-devo" perspective.

1. Introduction

Temperature-dependent sex determination (TSD) is a mechanism of environmental sex determination, where a physical factor, in this case the incubation temperature where the nest is located is responsible for determining the gonadal sex of an individual during development (Matsumoto and Crews, 2012; Mitchell et al., 2013). This phenomenon has been observed among various reptile orders (Warner, 2011; Miyagawa et al., 2018) such as: *Chelonia* (turtles) (Pieau, 1971, 1972; Yntema, 1976; McCoy et al., 1983), Squamata (lizards and geckos) (Charnier, 1966; Tokunaga, 1985; Viets et al., 1994; Flores et al., 1994; Warner, 2011), Crocodylidae (crocodiles and caimans) (Ferguson and Joanen, 1982; Deeming and Ferguson, 1989b; Lang and Andrews, 1993), Sphenodontidae (tuataras) (Mitchel et al., 2006). The first to describe the effect of temperature on sex ratio was Charnier in 1966, who when attempting to define the ideal temperature to incubate eggs from the *Agama agama* lizard, applied different incubation temperatures, discovering that each one produced a different proportion of sexes. Currently, out of the 7803 species of existing reptiles, 186 have been analyzed for TSD, and of these, only 121 species have been shown to manifest this (Warner, 2011). The others present some other mechanism such as genetic sex determination (GSD), where heterogametic males and females have ZZ/ZW or XX/XY chromosomal systems (Warner, 2011; Capel, 2017). It is also possible for both GSD and thermo-sensitivity to co-exist, the latter being observed mainly in a species of montane lizards *Bassiana duperreyi* (Shine et al., 2002) and turtles *Emys orbicularis* and Apalone mutica (Zaborski et al., 1988; Valenzuela, 2007). Since 1966 with Charnier's discovery, a series of studies and efforts have been added to elucidate the bases of this complex biological process defined as temperature-dependent sex determination (Warner, 2011).

During the 70's, various works described the importance of TSD, among which are those undertaken by Pieau in 1971 and 1972, where this mechanism was described for the freshwater turtle *Emys orbicularis* and for the Mediterranean turtle *Testudo graeca.* Likewise in 1976, Yntema discovered it in the *Chelydra serpentine* turtle (Pieau, 1971, 1972; Yntema, 1976; McCoy et al., 1983). These works focus on the incubation of eggs under laboratory conditions, from various species mentioned previously, in order to determine at what temperature males and females were obtained. Similarly, a fundamental element on which the research focused was how different patterns for TSD that exist among different species of reptiles, become established (Fig. 1).

In the 80s and 90s, research focused on the evolutionary and ecological implications of TSD (Bull, J., 1985; Janzen and Paukstis, 1991; Pieau et al., 1999), as well as on the molecular and physiological mechanisms involved in this phenomenon (Charnov and Bull, 1977; Pieau and Dorizzi., 1981; Merchant-Larios et al., 1989; Smith and Joss, 1993). From this, a number of hypotheses about TSD were formulated (which

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Abbreviations

5-3-β-HSD	5-3-β-hydroxysteroid dehydrogenase
Amh	Anti Müllerian hormone
AR	Androgen receptor
cAMP	Cyclic adenosine monophosphate cyclic
CamKIV	Calcium modulated protein KIV
Cirbp	Cold inducible RNA binding protein
CpG	Cytosine and guaine separated by only one phosphate 5'->3'
CREB	cAMP response element binding protein
Cyp191a	Cytochrome P450 191A aromatase
DHT	Dihydrotestosterone
Dmtr1	Doublesex and mab-3 related transcription factor 1
E2	17β-estradiol
E2SD	Estrogen-induced sex determination
Eco-Evo-Devo	Ecological – Evolutionary- Developmental
ER	Estrogen receptor
ERE elements	Estrogen response elements
ESD	Environmental sex determination
Foxl2	Forkhead box L2
FT	Feminizing temperature
G-A-M	Gonad-Adrenal-Mesonephros complex
GNRH	Gonadotrophin-Releasing Hormone
GSD	Genetic sex determination
HMH	Hypermethylated male region
H3K27	Histone H3
HSP	Heat shock proteins
KDM6B	Lysine Demethylase 6B
MT	Masculinizing temperature
ncEx1	non – coding Exon 1
PK	Protein kinase
PPT	(4,4',4"-(propyl-[1H]-pyrazole-1,3,5-tryl trisphenol)
PT	Pivotal temperature
Rspo1	R-spondin-1
Sf1	Splicing-factor-1
Sox9	Sex determining region-box9
Sox8	Sex determination region-box 8
Sry	Sex-determining region Y
TESCO	Testis-specific enhancer core element
TREK-1	Targeting two pore domain K+ channels
TRP	Transient receptor potential channel
TRPM5	Transient receptor potential channel melastatina
TRPV4	Transient receptor potential channel type 4
TSD	Temperature-dependent sex determination
TSP	Temperature sensitive period
TSS	Transcription start site
WNT	Wingless/integrated
Wt1	Wilms tumor-1

we will describe in the following) and likewise gonadal differentiation at a morphological, cellular, genetic and hormonal level of some reptiles such as *Emys orbicularis* (Pieau and Dorizzi, 1981), *Alligator mississippiensis* (Smith and Joss, 1993), *Trachemys scripta* (Wibbels et al., 1998) and *Lepidochelys olivacea* (McCoy et al., 1983; Merchant-Larios et al., 1989) were investigated, to mention a few examples. The signaling role of estrogen in TSD was explored in molecular terms (Jeyasuriya and Place, 1998). Correspondingly, the process of defining TSD patterns in reptiles and the temperature sensitive period (TSP, Fig. 2) continued, as did the search for the pivotal temperature (PT) among certain species (Pieau, 1996).

From 2000 onwards, research has focused on trying to identify more species of reptiles with TSD (Harlow and Taylor, 2000). There has also been an attempt to define the signal that triggers gonadal differentiation by temperature in classic reptile models (Lance, 2009), with the focus mainly on epigenetic (Matsumoto et al., 2013a, b), genetic (Matsumoto and Crews, 2012) and hormonal (Ramsey and Crews, 2009) studies. Apart from this, studies considering effects of climate change and environmental contaminants on species with TSD increased (Milnes et al., 2005). However, despite the efforts and excellent research carried out over all these years by several research groups, it has not yet been possible to fully ascertain how temperature triggers the differentiation of the bipotential gonad to become either an ovary or a testicle, during TSD (Kohni et al., 2014; Georges and Holleley, 2018).

2. About incubation temperature during TSD

The incubation temperature of a nest depends on multiple variables, related to the surrounding abiotic environment. Among these are the amount of light and shadow that the nest receives during the day, whether it is near or far from a body of water, size, the type of material from which it is built, whether it consists of decaying vegetation and the type of substrate. Other factors include its location and whether it is at the surface or buried (Mitchell et al., 2013). In turn, inside the nest the important variables that influence incubation temperature consist of: embryonic metabolism and the position of the egg within the nest (Deeming and Ferguson 1989a; Noble et al., 1990; Mitchell et al., 2013). Besides this, some nesting studies on reptiles have revealed that the embryo is able to move inside the egg, towards the direction where it can perceive stimuli related to external temperature, although it cannot thermoregulate as in adult organisms. This observation is interesting because Yntema (1968) proposed that the embryo was in fact more active during the TSP (Cordero et al., 2018).

Laboratory studies on species that are sexually determined by temperature, under highly controlled incubation conditions, have noted the direct influence of temperature on the sex ratio and apparently, in addition to feminizing temperature (FT, 100% females) and masculinizing temperature (MT, 100% males), there is a temperature at which the sexual ratio is 1:1, which has been defined as pivotal temperature (PT) (Deeming and Ferguson, 1989a, b; Warner, 2011). Determining this temperature is very complicated, because in ecological studies on sex ratio and nesting temperatures, PT often varies from one nest to another (Dodd et al., 2006) or depends on the geographic region (Ewert et al., 1994, 2005). This may be due to inherited maternal factors or to the fact that a nest is naturally exposed to different environmental conditions, where there are drastic oscillations in incubation temperature and therefore different effects are observed in terms of the sex ratio and PT (Rhen and Lang, 1995; Ewert et al., 1994, 2005; Dodd et al., 2006; Lopez-Luna, 2015). An example is the *Chelydra serpentine* turtle, for which different pivotal temperatures have been determined, one when it is incubated under laboratory conditions and another when the nests are incubated naturally, where the influence of the geographical region has been clearly observed (Ewert et al., 2005). Another example is the *T. scripta* turtle, where the PT varies from nest to nest (Dodd et al., 2006).

In some of the species that present a TSD pattern, the PT has been difficult to clarify (Fig. A1-D1), as is the case among all crocodile species, for which there is a suggestion that they have two temperatures (Pieau, 1996; Shoemaker-Daly and Crews, 2009; Fig. C1). However, in studies carried out on *A. mississippiensis* in which eggs have been incubated at 29–36 °C, the PT has not yet been clearly defined (Ferguson and Joanen, 1982; Deeming and Ferguson, 1989a, b; Lang and Andrews, 1993). Similarly, the PT, as well as the MT and FT can be seen to be affected by the presence of environmental pollutants or global warming, as there are nesting studies of *Crocodylus moreletii in situ* showing that temperatures during their incubation are close to what is

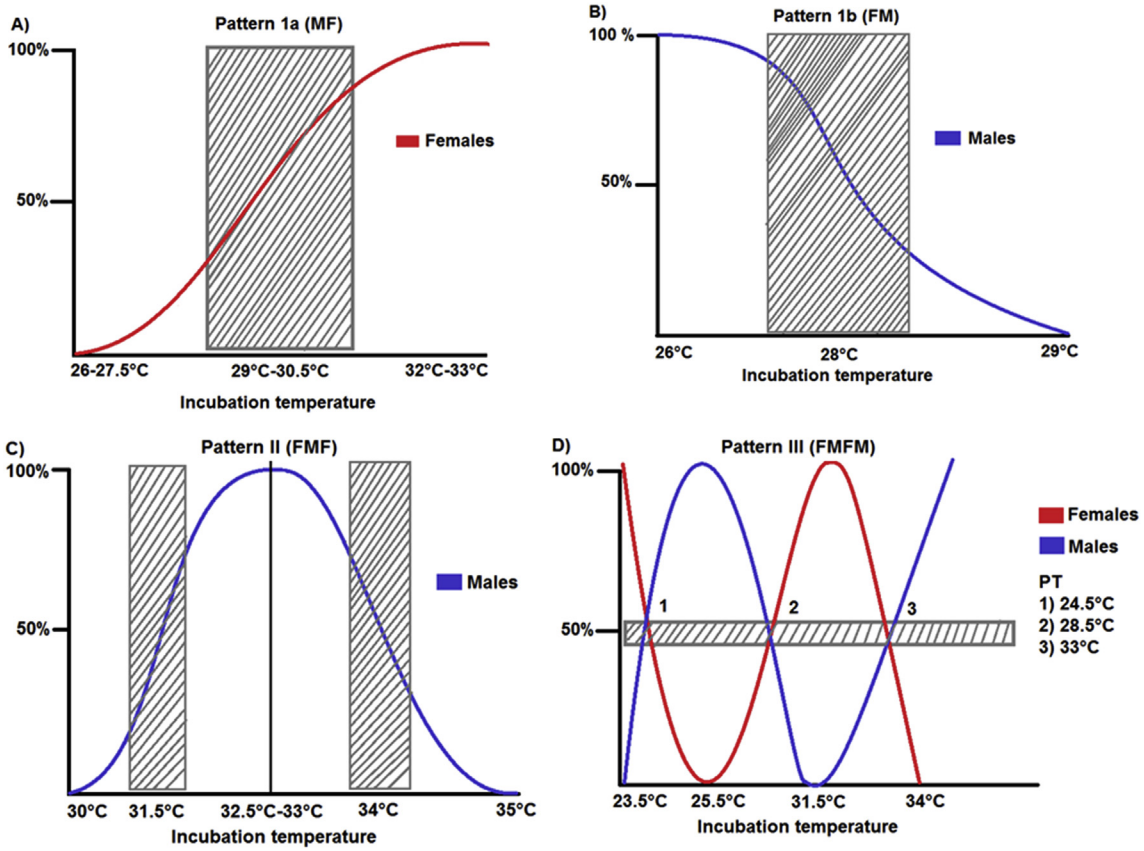

Fig. 1. Patterns of sexual determination by temperature in reptiles. Four patterns of sexual determination by temperature have been determined from egg incubation experiments under laboratory conditions. In pattern 1a (MF), 100% males are produced at a low temperature and at a high temperature 100% of females (in lizards and turtles, Fig. A1). In pattern 1b (HM), 100% of females are produced at a low temperature and 100% of males at a high temperature (in tuataras and lizards, Fig. B1). Both patterns have a PT (shaded rectangle) and possibly during evolution, only one allele that determines sex (monofactorial) was favored. In pattern II (FMF) produced females at low and high temperatures and males at a medium temperature. There are 2 PT (shaded rectangles). This seems to be the ancestral TSD pattern, and some authors suggest that the others may have emerged from it (Pieau et al., 1999). They partly refer to the fact that extreme temperatures during incubation cause greater mortality in some species, possibly favored by natural selection, forcing those temperatures to disappear among them. This occurs among turtles, crocodiles and lizards (Fig.C1). In pattern III (FMFM), 100% females and 100% males are obtained at high and low temperatures; and there are three PT (shaded rectangle, numbers 1, 2 and 3). This occurs among lizards and is evidence of great evolutionary plasticity in TSD and of great adaptive potential, when faced with climate change events (Fig.D1). Very possibly these last two patterns have developed from evolutionary events, where sexual systems are determined by several genes distributed in the genome (multifactorial) (Inamdar-Doddamani et al., 2012).

thought to be a PT in crocodiles, although possibly a greater number of males hatch due to hormonal disruptors or to the thermal effect of urbanized areas (Lopez-Luna 2015). The environment has also led to the selection and evolution of a wide variety of strategies in TSD among reptiles, because interestingly it has been found that the oviparous lizard *Calotes versicolor* lacks sex chromosomes but manifests a new pattern of TSD which has three PT (24.5 °C, 28.5 °C and 33 °C; Fig D1), for which the sex ratio is 1:1, two female temperatures (23.5 °C and 31.5 °C) and two male temperatures (25.5 °C and 34 °C) (Inamdar-Doddamani et al., 2012).

There is still much to understand concerning the fundamentals of TSD because it is a multidisciplinary problem that encompasses ecology-evolution-development (Gilbert, 2001; Matsumoto and Crews, 2012). This integrative approach is important and necessary in order to understand whether there is a PT, or whether this is only an effect related to nesting or time of incubation; in other words the environmental conditions (internal and external) to which the nest is exposed. As a particular embryo responds to different “stimuli” or “temperature fluctuations” during the course of the day, these stimuli have a stochastic influence on the genetic and physiological plasticity of the embryo (Fig. 3). This stochastic variation stabilizes the expression of genes that trigger the differentiation of the gonad, thus defining outcome in terms of developing either an ovary or a testicle (Matsumoto and Crews, 2012; Landry and Horth, 2014).

Therefore, the PT may well be the manifestation of an evolutionary throwback of great genomic plasticity, which existed in the past, comprising different forms or mechanisms for the determination of a sexual phenotype during TSD. This would explain why sex ratios during embryonic development, under natural nesting conditions, are different from observations made under conditions of constant temperature in a laboratory (Rhen and Lang, 1995; Ewert et al., 2005; Matsumoto and Crews, 2012; Lopez-Luna, 2015; Landry and Horth, 2014). In addition to explaining why different species with TSD have different PT, even within the same species, thus a phenotype (male or female) cannot be explained without also referring to an individual's internal and external environment (Gilbert, 2001; Sultan, 2007; Matsumoto and Crews, 2012; Landry and Horth, 2014).

3. About phenotypic plasticity and TSD

In genetic sex determination (GSD), the sex of an individual is determined exactly at the time of fertilization, as each sex has different genes, meaning that the sex chromosomes provide the genes that will be expressed during development by directing the differentiation and morphogenesis pathways of the gonad towards a particular sex (Wilhelm et al., 2013). Conversely, in TSD, individuals have an

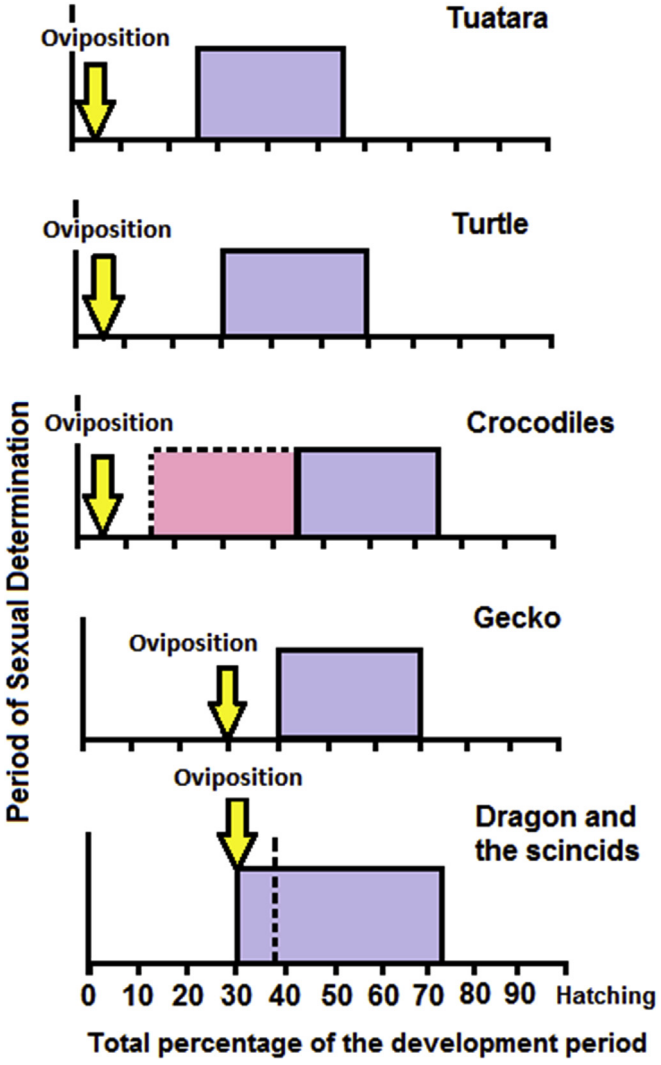

Fig. 2. Temperature sensitive period (TSP). The TSP is a moment during the development of the embryo, when it is able to monitor incubation temperature and irreversibly determine sex. These periods are relatively similar among reptile species, meaning they begin in the second quarter of embryonic development and extend until the third quarter of development, which coincides with the morphological differentiation of the gonad. In most turtle families, such as the species *E. orbicularis*, *Graptemys ouachitensis* and *Chrysemys picta* TSP initiates at stage 16 of embryonic development (Bull and Vogt, 1981; Wibbels et al., 1991; Pieau; 1998; Warner, 2011). In the case of the turtle *L. olivacea*, TSP occurs between stages 22 and 27 of embryonic development (Merchant-Larios et al., 2010). In crocodiles, the TSP is found in stages 21–24 (canonical TSP; Smith and Joss, 1993), although in a recent study by McCoy et al., (2015), they show that pulses at incubation temperature during early stages (stages 9–19) may influence sex ratio prior to the initiation of canonical TSP (pink box). Conversely, Ferguson et al 1982 proposed that the TSP starts during the early stages of 13–21 (Ferguson and Joanen, 1982; Lang and Andrews, 1993). In Gekos the TSP occurs. During stages 32–37 (Bull, 1987). In tuataras it occurs during 25% and 55% of the total incubation period (Mitchel et al., 2006). All these reptiles with TSD are oviposited at very early stages of development (before neurulation). However, the oviparous Squamata group retains an embryo during 30% of its incubation, so the TSP depends on whether it only takes oviposition or overall embryonic development into account (Andrews and Mathies, 2000; Andrews, 2004). Taken from Warner (2011) and modified by Shine, R.; Warner D.A.; Radder, R. 2007.

identical genetic makeup from the moment fertilization occurs, until the moment when the environmental stimulus (temperature) triggers the expression of genes that determine sex, and depending on whether or not this is FT or MT, the differentiation and gonadal morphogenesis

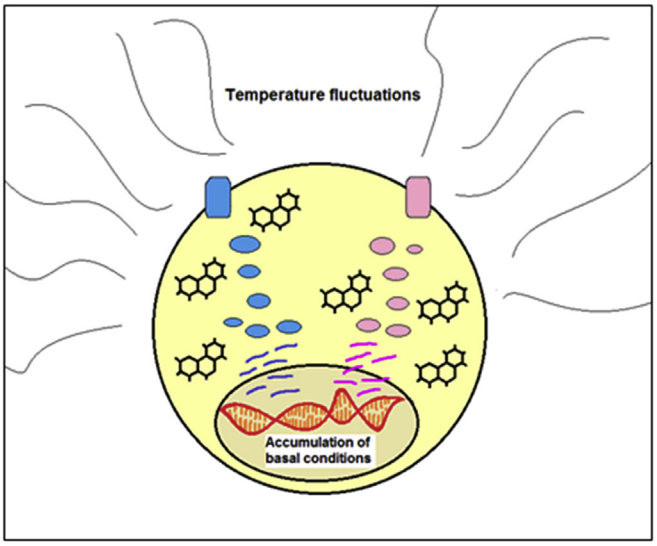

Fig. 3. Molecular plasticity in the pivot temperature and proportion 1:1. During incubation of the embryo in the nest, temperature fluctuations randomly stimulate the cells of the gonad, which produces the expression of both masculinizing and feminizing pathways at the basal level of elements. When the temperature sensitive period starts, these basal elements possibly facilitate the response of one of the ways sex can be determined (stochastic genetic stabilization). For this reason, if incubated at a PT the possibility of having the same basal expression of elements from the feminizing and masculinizing pathway is greater, so that the stimulus determined by sex would have a 50% probability of favoring and stabilizing the genetic expression of one of the pathways.

(Fig A4- 4F and Fig. 5) of an ovary or a testicle will occur (Warner, 2011; Matsumoto and Crews, 2012). This process of how the environment directs the expression of genes and cell signaling during development, producing a different range of phenotypes, in this case the differentiation of the bipotential gonad to the testis or ovary, is known as phenotypic plasticity (Sultan, 2007; Matsumoto and Crews, 2012; Landry and Horth, 2014). This process is very interesting due to the evolutionary and ecological implications in the development of reptiles with TSD, as temperature does not only influence the differentiation of the gonad, but also the size of the embryo and moment when it develops (Deeming and Ferguson; 1989b; Landry and Horth, 2014). This means that embryos that incubate at high temperatures hatch faster and are larger than those incubated at low temperatures (Deeming and Ferguson; 1989b; Landry and Horth, 2014). Several authors suggest that this has consequences for the adaptation of individuals to their environment during adulthood. For example, females geckos (*Eublepharis macularius*) produced by TSD are more attractive and less aggressive than those produced by exogenous hormonal action and incubated under a masculinizing temperature, meaning that TSD affects the social behavior of these geckos when they are adults during their reproduction (sexual selection), offering greater possibilities for reproduction and producing offspring; in other words for adaption (Flores et al., 1994). Interestingly, there are studies on lizards, showing that the adequacy of each sex is maximized due to the incubation temperature during embryonic development (Warner 2007). These observations, in addition to many more studies on reptiles and other vertebrates, have produced models that indicate the possible evolutionary transitions between environmental sex determination (ESD) and GSD (Fig. A6 and B6; Bull, 1985; Pezaro et al., 2017).

Therefore, we have attempted to investigate the possible evolutionary origin of TSD in reptiles, however there are many more unknown aspects related to this subject because it is not yet known how temperature can be transduced as a biological signal and produce a phenotype as a result. However, the expression of genes involved in sex determination during the embryonic development of reptiles is

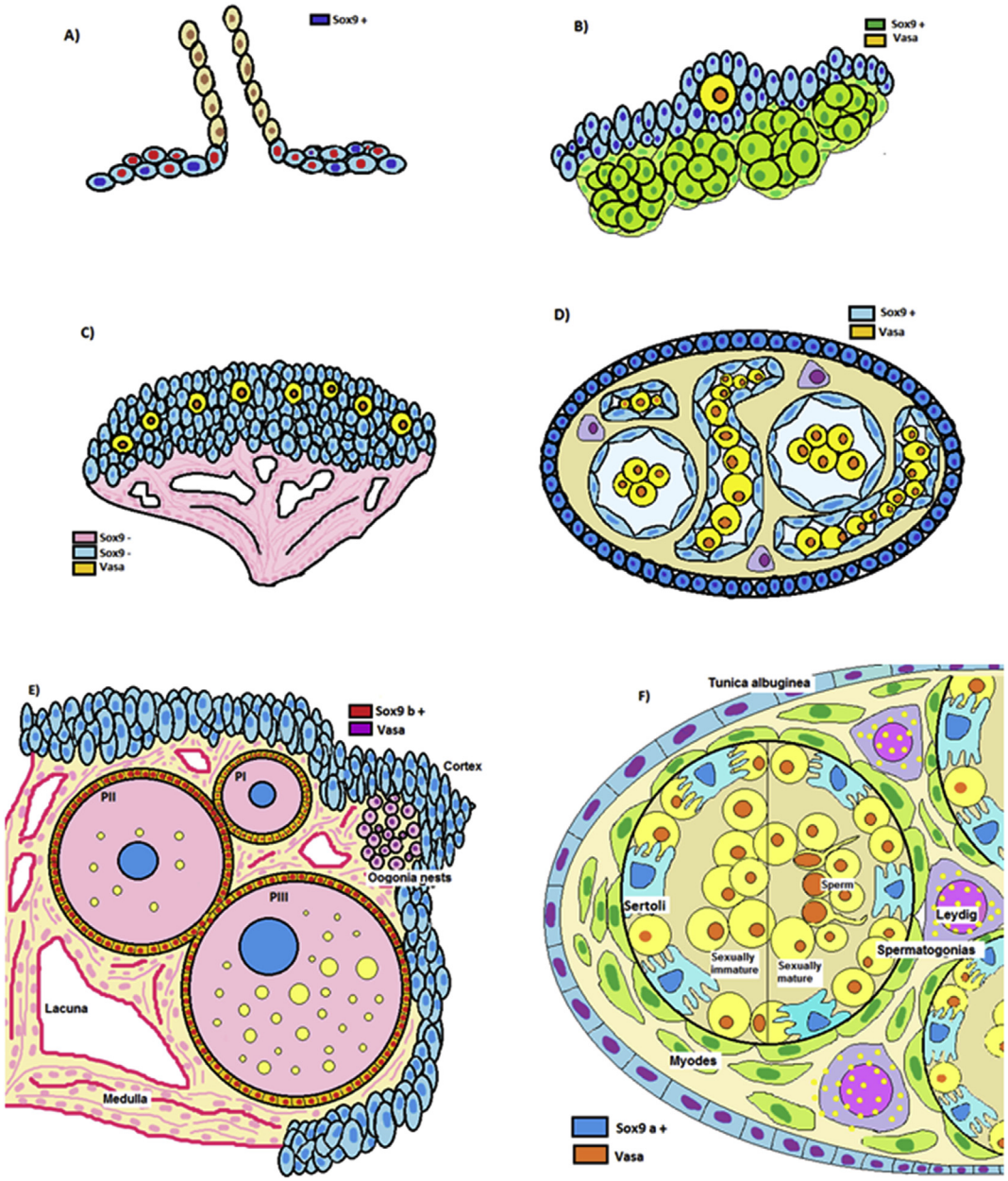

Fig. 4. Gonadal morphogenesis in reptiles. The bipotential gonad in reptiles is located in the ventromedial area of the mesonephros. Histologically it comprises two regions: the cortex and the medullary portion. The coelomic epithelium is of the pseudostratified type and germ cells are embedded within it. The marrow is formed out of somatic cells, such as epithelial cells, fibroblasts that form sexual cords, vessels, etc. When development initiates, the genital crest is formed (Fig. A4), in this case germs cells move towards a continuation of the celomic epithelium. Subsequently the gonadal blastema is formed by the coelomic epithelium and underlying it in the medullary portion are the sexual cords (Fig. B4). During the differentiation of the gonad into the ovary, the proliferation of somatic and germ cells in the cortex is observed and the marrow degenerates to form lacunas (Fig.C4). In the testicle, elongated cells (pre-Sertoli) and mesenchymal cells are increased in the medullary portion, and are subsequently reorganized to form testicular cords (Fig.D4). (Smith and Joss, 1993; Pieau et al., 1999). In the adult ovary in the cortex, oocytes in pre-vitellogenesis and nests of oogonia can be observed (Fig.E4). In the immature testicle, the seminiferous tubules lack light and spermatogonia, Sertoli cells, myod and Leydig cells are apparent. In the case of a sexually mature male during the reproductive season, there is light in the seminiferous tubule and sperm can be observed, together with other cell types (Fig.F4). The expression of genes in some reptiles has been characterized, from very early stages up to the formation of the ovary and the testicle (prior to hatching) in *L. olivacea*, *T. scripta, C. latirostris* (*Sox9*, *Vasa*). In the case of adults, it has been characterized in sexually immature young male and female adults of *C. moreletii* (Sox9, Vasa and Foxl2).

understood (Shoemaker et al., 2007), as these are highly conserved in the evolution of vertebrates and have been widely studied in mammals (She and Yang, 2014), as we briefly describe in the following.

4. Genes involved in sex determination by temperature in reptiles

4.1. Genetic network at masculinizing temperature

Of the genes involved in genetic networks of sex determination in mammals and other vertebrates, some have been found expressed in a number of reptiles. It is known that the *Sry* gene involved in the initiation of the determination of male sex in mammals (Berta et al.,

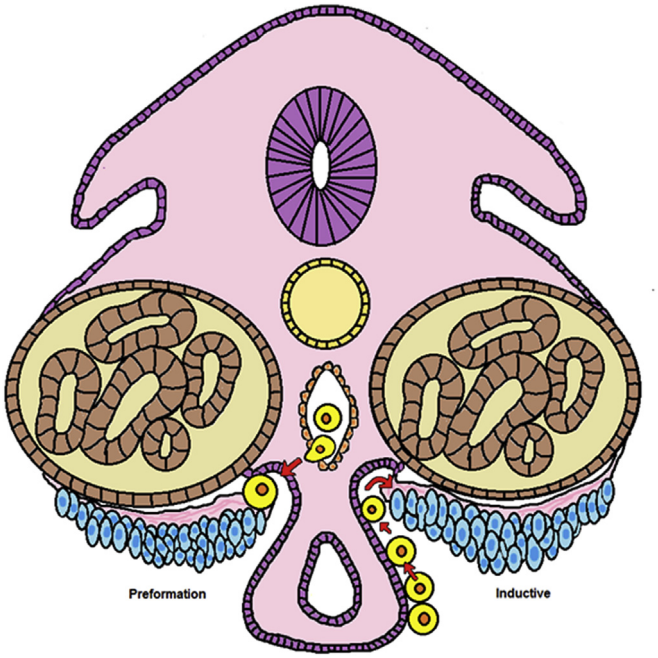

Fig. 5. Origin of germ cells in reptiles. This cell type is characterized histologically for its spherical shape, prominent nucleus, lipid droplets and size that is 4–5 times larger than somatic cells (Merchant-Larios et al., 1989). The specification of the germline in reptiles can occur in two ways. The first is preformation, where in early stages of somites, CPGs are attached to the endoderm, just outside the embryo, in the posterior or anterior lateral region, from where they migrate to the genital crest via the bloodstream (occurs in snakes, tuataras and is suggested for crocodiles), as occurs in birds. The second mode is inductive, which has been observed in lizards and turtles, where in early stages of somites, the germ line is induced by neighboring cells in the posterior part of the extraembryonic endoderm and subsequently migrates through the dorsal mesentery of the intestine to colonize the gonad, as occurs in mammals. There is still a lot to characterize regarding the germ line in reptiles (Kohni et al., 2014).

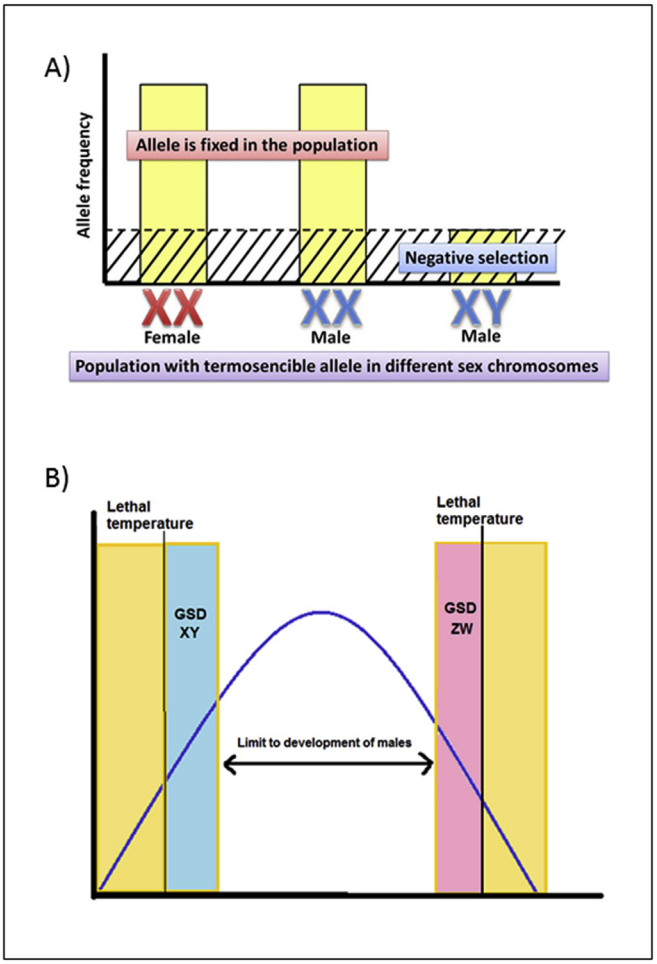

Fig. 6. Models for the hypothesis concerning evolution of environmental sexual determination. In A) Evolution of GSD based on environmental sexual determination (GSD - > ESD). This model proposes that ESD could have derived from GSD, which is possible by means of an increase in the allele that determines sex in XX males selected with a high frequency in a population with XX females and XX, XY males; because the adaptive value depended on the environment (temperature) and the XY zygotes were negatively selected (Bull, 1985). In B) Evolution of environmental sexual determination based on genetics (ESD - > GSD). This model proposes the emergence of males and heterogamous females, because of the failure to maintain certain viable sex ratios, due to combinations of climatic patterns and the population's reaction. Thus GSD became established in XY or ZW depending on which sex could not be produced Fig. 6. For example, the inability to produce males at lethal temperatures (low temperatures) caused a proto-chromosome Y to spread among the population (Pezaro et al., 2017). Both models have been proposed, based on observations among certain reptiles; it is also thought that sexual determination in vertebrates manifests greater genetic plasticity, because the allele of the gene that determines sex is possibly able to increase its frequency in a population quickly and change the way in which it is sexually determined.

1990; She and Yang, 2014) is not found in any reptile studied to date (Spotila et al., 1994). However, despite this absence, the *Sox9* gene is expressed during sex determination by temperature in several reptile models (Kent et al., 1996; Moreno-Mendoza et al., 1999; Western et al., 1999a; Valleley et al., 2001; Shoemaker et al., 2010). This gene is involved in the differentiation of Sertoli cells, which are fundamental to testicular differentiation and for maintaining a masculinizing environment during adulthood (Kent et al., 1996; Martínez-Juárez et al., 2018). *Dmrt1* is a gene found upstream of *Sox9* in models such as: *Oryzias latipes* (Lutfalla et al., 2003), *Xenopus tropicalis* (Yoshimoto et al., 2006), *Gallus gallus* (Smith et al., 2009) and *Mus musculus* (Matson et al., 2011) and also reptiles as *T. scripta, Pelodiscus sinensis* (Murdock and Wibbels, 2003; Picard et al., 2015). In the testicular development of the mouse, *Dmtr1* directly represses the *Foxl2* gene, restricting the activity of the receptor for α retinoic acid, thus inhibiting the formation of an ovary and also as the *Sox9/Sox8* complex, *Dmtr1* participates in preventing the transdifferentiation of Sertoli cells to granulosa in postnatal testes and adults (Matson et al., 2011; Minkina et al., 2014; Barrionuevo et al., 2016). In *G. gallus*, its participation in the differentiation of the testis during embryonic development has also been described (Smith et al., 2009) and in *T. scripta* it is known to manifest dimorphic expression during the temperature sensitive period, expressed at MT (Murdock and Wibbels, 2003). Additionally in the same species of turtle, in 2017 the Chutian Ge group showed that *in ovo* and *in vitro, Dmtr1* participate in regulating TSD, as it is found upstream of the *Sox9* gene and *Amh* (anti-Müllerian hormone) during the onset of testicular differentiation. It is expressed in the development of the testicle, specifically in the nuclei of Sertoli cells together with the *Sox9* gene, when the gonad or the embryo is incubated at MT (Ge et al., 2017).

Interestingly in vertebrates, *Dmtr1* has two functions that depend on regulation at the transcriptional level of mRNA. One of these is the masculinization of somatic cells during testicular development and in postnatal testes, where two isoforms for type 2 *Dmrt1* and S*Dmtr1* are expressed in this cell type, exclusively in males and their mRNA promoters, known as CNS2 or CNS3 contain consensus sequences for *Foxl2* and *Sox9* transcription factors to which this regulates negatively and positively, respectively. The other function has to do with the development of germ cells, where the type 1*Dmtr1*L isoform is expressed in the germ cells of gonads of both females and males, and its promoter

known as CN1 contains consensus sequences for the Nanog transcription factor, which is required in the pluripotency of germ cells (Mawaribuchi et al., 2017).

This is due to the fact that evolutionarily, the *Dmtr1* promoter lost a non-coding exon (ncEx1), which is present in lampreys (*Lethenteron reissnen*), amphibians (*Xenopus laevis* and *Silurana tropicalis*) and absent in reptiles, geckos (*Eublepharis macularius)*, chickens (*G. gallus*) and mammals (*M. musculus*). This resulted in different varieties of *mRNA* messenger that acquired different functions during vertebrate evolution (Mawaribuchi et al., 2017).

Recently, it has been proposed that the Sox9 gene also possesses two isoforms in reptiles, as its expression was found in males and in sexually immature *Crocodylus moreletii* females. This transcription factor is expressed in the nucleus of the Sertoli cells of the testis and in the nucleus of the granulosa cells of oocytes in previtellogenesis, where apparently it may play an important role during folliculogenesis. In embryonic development, the *Sox9* gene is only expressed in gonads of embryos incubated at MT, but not when incubated at FT, possibly due to the fact that in the development of a testicle, one isoform of *Sox9 a* type and another of isoform *Sox9b* type are expressed, meaning it participates in folliculogenesis, the latter would not be observed during sex determination and gonadal morphogenesis, because the formation of the primary follicles in an ovary of a young crocodile is post-hatching, which would temporarily separate the expression of these isoforms during development (Martínez-Juárez et al., 2018). Therefore, these genes are pleiotropic because they play a role in TSD and in other developmental processes (Landry and Horth, 2014).

Another important gene in testicular differentiation is the anti-Müllerian hormone (*Amh*), which is a key regulator in the differentiation of Sertoli cells, the production of testosterone and in the development of the Wolfian duct (Oreal et al., 1998; She and Yang, 2014). The expression of *Amh* in mammals precedes that of *Sox9* (She and Yang, 2014), however in reptiles this pattern of expression is not conserved (Kohni et al., 2014). Thus in *A. mississippiensis*, *Amh* is expressed first and then the expression of the *Sox9* gene occurs, an expression pattern that is also found in *G. gallus* (Oreal et al., 1998; Western et al., 1999b; Shoemaker et al., 2007; Urushitani et al., 2011). However, in *T. scripta Amh* expression is similar to that occurring in mammals (Shoemaker et al., 2007; Shoemaker-Daly and Crews, 2009). *Wt1* and *Sf1* genes are very important for testicular differentiation, as they are responsible for maintaining gene expression involved in masculinization during embryonic development. They are also responsible for maintaining the morphology of the testicle in terms of the constitution of seminiferous tubules, Sertoli cells and germ cells, as *Wt1* regulates the expression of *Sf1;* the latter participates in the maintenance of *Sox9* expression and in the proliferation of the somatic cells in the testicle, likewise in *M. musculus* it also modulates the expression of *Amh* (Spotila et al., 1998; She and Yang, 2014). Similarly in *T. scripta, Sf1* presents an exclusive expression during the TSP at MT (Ramseys et al., 2007a), and also regulates the expression of the *Cyp191a* enzyme (aromatase), as the promoter of this enzyme contains sequences that bind positively to the *Sf1* transcription factor, which has been purported to putatively favor the methylation of its promoter (Pannetier et al., 2006; Wang et al., 2007).

This leads us to suppose that the master gene for testicular differentiation is *Sox9*, as it is highly conserved in the evolution of all vertebrates and that this gene is activated by different signals (*Dmtr1* or *Sry*) (She and Yang, 2014; Ge et al., 2017). The other genes, although important for initiating and maintaining differentiation in the development of the testicle, have been diversified in the different groups of vertebrates, resulting in different contexts of genetic networks for sexual determination, which aim to either initiate or repress expression of *Sox9* (Kohni et al., 2014; She and Yang, 2014; Capel, 2017).

Evolutionarily, these mechanisms that have been selected to activate *Sox9*, are the result of the genetic plasticity of embryonic development, provided by each necessary component that facilitates the sensitivity with which this gene is expressed in response to incubation temperature, meaning that variability in the transcripts, proteins, lipids, hormones and epigenetic modifications generates the molecular plasticity which orders the genotype to trigger the phenotype, which in this case is a testicle. This is why adaptively different mechanisms or genetic networks are favored in the TSD of reptiles, resulting in the different patterns of TSD that have been described. Likewise, it would explain why *Amh* is expressed in turtles in a way similar to mammals, but not in crocodiles and birds. Thus, very possibly we are looking for more than one form of signaling that triggers TSD in reptiles. All of the above relates to Bull-Charnovs' idea in 1985, in which they mentioned that there might be a polyfactorial genetic effect caused by the combination of several genes distributed in the genome, which when favored evolutively, rapidly increase their alleles in a population, altering the way sex is determined in species (Bull, 1985; Oreal et al., 1998; Moreno-Mendoza et al., 1999; Sultan, 2007; Landry and Horth, 2014; Ge et al., 2017).

4.2. Genetic network at the feminizing temperature

Contrastingly, ovarian differentiation in mammals has been purported to occur because of the expression of genes such as *Foxl2* (She and Yang, 2014; Capel, 2017). In the case of reptiles with TSD such as *T. scripta*, expression of *Foxl2* has been observed during the TSP, when the gonad of the embryo is incubated at FT (Shoemaker-Daly and Crews, 2009; Shoemaker et al., 2010), as well as in crocodiles that express this transcription factor in the nuclei of the follicular cells of young females (Martínez-Juárez et al., 2018). This gene participates in the balance of ovarian steroidogenesis, as well as in the differentiation of granulosa cells and primary follicles (Uda et al., 2004; Ottolenghi et al., 2005; Pannetier et al., 2006; Bertho et al., 2016; Nicol et al., 2018). In mice, the *Foxl2* transcription factor binds directly to the estrogen receptor 2 or β (ER2 or ERβ), regulating estrogen production in granulosa cells; in addition this transcription factor together with ER1 or ERα synergizes repression of *Sox9* by negatively regulating the testis-specific enhancer core element (TESCO) unit of the promoter (Kim et al., 2009; Jakob, 2010; She and Yang, 2014; Georges et al., 2014). Its expression also coincides with that of the enzyme aromatase, which is responsible for the production of estrogens through the irreversible catalysis of androgens to estrogens, as well as affecting the balance in the production of steroid hormones (Yamaguchi et al., 2007). In the *Chelydra serpentina* turtle, it is known that this enzyme increases its activity during development at stage 16 and continues until stage 24, with the most dramatic increase at stage 20, when regression of the medullary cords occurs (Place et al., 2001; Rhen et al., 2007; Ramsey and Crews, 2009). In alligators, its activity begins after sex determination and continues until after hatching (Smith et al., 1995; Ramsey and Crews, 2009). The transcriptional plasticity of this gene has also been investigated, considering that the polymorphisms of this enzyme may be a fundamental factor in regulating the levels of steroid hormones and promoting an environment for sex differentiation in the bipotential gonad. It is apparent that the *Cyp191a* enzyme has 22 exonic and 1268 intronic polymorphisms, of which 12 were exclusive to the *T. scripta* species, 10 were synonymous changes and 2 were not synonymous. However, in this study, no evidence was found to indicate a difference between males and females as a consequence of polymorphisms, incubated at a temperature that restricts gonadal differentiation (Matsumoto and Crews, 2017). *Rspo1* is another gene that is expressed in reptiles during TSD, and in mammals it is known to fulfill an important function in ovarian differentiation during early embryonic development, regulating the signaling of the WNT/β-catenin pathway, as it is involved in the determination and differentiation of the progenitors of follicular cells and also regulates the meiosis of germ cells (Vainio et al., 1999; Smith et al., 2008; Chassot et al., 2008, 2014; Liu et al., 2010; Tang et al., 2017).

The genes that have been described so far play an important role in

the genetic architecture of TSD in the formation of a phenotype (a testicle or an ovary) (Rice et al., 2017), however various aspects of the signals that trigger the expression of this entire genetic network are still unknown. In spite of this, several mechanisms have been proposed, which will be described in the following.

5. Steroid hormones in sex determination by temperature

These types of hormones such as estrogens, progesterone and androgens are small molecules, which are biochemically characterized as lipophilic. They are synthesized from cholesterol fatty acid during the course of steroidogenic biosynthesis and in mammals they are known to be widely distributed throughout various body tissues. Target cells that recognize this type of signal express the estrogen receptor (ERα, β) or androgen (AR). These receptors are found mainly in the cytoplasm of the cell, although recently in mammals their expression has been described in cell membrane (Matsumoto et al., 2013b; Levin, 2018; Wilkenfeld et al., 2018). Briefly, the canonical signaling pathway of steroid hormones causes the receptor to be stimulated by the hormone and once the ligand binds to its receptor, a conformational change occurs, resulting in the dissociation of the interaction between the receptor and a Heat shock protein (HSP), which keeps the receptor in an inactive state in the cytoplasm until it is activated. Subsequently, the active nuclear receptor acts as a transcription factor that translocate to the nucleus and binds to the DNA in the steroid hormone response element (HRE), thus regulating the expression of the promoter in its target gene (Matsumoto et al., 2013b; Saczko et al., 2017; Wilkenfeld et al., 2018). Besides this, the receptors of these hormones can participate in non-genetic signaling pathways, that is, they regulate downstream protein kinases, so they may not act directly as a transcription factor; however, several aspects of this mechanism are still not understood (Wilkenfeld et al., 2018). Steroid hormones participate in several important processes that occur in organisms, as they can regulate several morphological and physiological features of development. The fact that these participate in so many mechanisms is due to the great diversity of hormonal axes, which are the result of the evolution of genetic variation in endocrine signaling, which has had significant influence on the development of evolutionarily favored phenotypes (Landry and Horth, 2014; Wilkenfeld et al., 2018). Because of this, from the point of view of the “eco-evo-devo”, this endocrine signaling mechanism is a very important process indecision making during development that responds to the environment; as steroid hormones together with their receptors and the enzymes that produce them are particularly crucial for regulating the expression of the genetic network in TSD and for directing differentiation towards an ovary or a testicle (White and Thomas et al., 1992a; Ramsey and Crews, 2007b and 2009; Landry and Horth, 2014).

The first studies concerning participation of these hormones in TSD mainly concerned an attempt to reveal the physiological role of this mechanism, while also defining their concentration in blood before, after and during the TPS in different reptile models; mainly in turtles (White and Thomas et al., 1992b). In the case of *T. scripta*, it was found that the body and serum of embryos incubated at a masculinizing temperature contain more testosterone and estrogen during the early to intermediate phase of PST and more progesterone half way through it, compared to embryos incubated at a feminizing temperature. However, it has been difficult to analyze the correlation of steroid hormones to DST, due to the large amount of evidence that exists about its synthesis, as this can occur in the gonad, in the gonadal-adrenal-mesonephros (GAM) complex or in the yolk, as well as concerning the great variety of its effects during the DST of different reptile species. Although, an interesting aspect of this great heterogeneity, is that it results from the way different endocrine signaling pathways were favored during the evolution of DST, as these surely function as development strategies in order to affect the sex ratio and thus prevent the offspring being biased towards one sex (White and Thomas et al., 1992a,b; Ramsey and Crews, 2009; Landry and Horth, 2014).

Given the above, a very important part of the research concerning the role of these hormones in DST, has been to clarify the expression and activity of the enzymes that produce them. The most studied enzyme is aromatase which participates in the production of estrogens during the DST of different reptile models (Jeyasuriya and Place, 1998), as it is known that this enzyme is extremely important in sex determination and even in the sex reversal of vertebrates, for example in the fish group (Guiguen et al., 2010).

The synthesis of estrogens in embryonic development plays an important role in sex determination (Jeyasuriya and Place, 1998; Ramsey and Crews, 2009). This was demonstrated by experiments using steroid hormones (Crews et al., 1991; Tousignant and Crews, 1994). In the particular case of reptiles, it is known that if they are incubated under a MT and an estrogen treatment is applied, sex is reversed, as only females hatch. Conversely, if eggs are incubated under a FT and inhibitors of the aromatase are applied, a masculinizing effect occurs (Crews et al., 1991; Ramsey and Crews, 2009). Similarly, eggs from *T. scripta* incubated at PT, with a dose of a non-aromatizable androgen dihydrotestosterone (5α-dihidrotestosterona: DHT), caused 100% males to hatch. Likewise, the inhibition of the enzyme 5α-reductase, the enzyme that converts testosterone to DHT, causes predominant hatching of females (Wibbles, 1992; Wibbels and Crews, 1995; Crews and Bergeron, 1994; Miyagawa et al., 2018). From all these observations, the first hypotheses concerning possible mechanisms of TSD were established, where estrogens, through the enzyme that synthesizes them, play a fundamental role (White et al., 1992c; Jeyasuriya and Place, 1998; Ramsey and Crews, 2009).

Among mechanisms indicating how estrogens work in TSD, it was proposed that the balance of their concentration in the gonad was essential for directing differentiation towards either male or female (Jeyasuriya and Place, 1998). Thus, high concentration of estrogen in the bipotential gonad results in degeneration of the medulla and differentiation of the cortex towards an ovary is ordered. Contrarily, a high concentration of androgens designates the differentiation of sexual cords in the medullar region. However, it is not yet clear whether testicular differentiation of reptiles with DST requires signaling by androgens, as in *in ovo* exposure treatments with DHT, they have no effect on sex determination. There is also evidence that although there is variation in the enzymatic activity of the steroidogenic enzymes, in a number of tissues in the embryo during the stages of sex determination (undifferentiated, differentiated and differentiated gonad), apparently this activity manifests no temperature related pattern. Thus, the inhibition of the enzyme 5α-Reductase is the clearest evidence of the possible role of androgens in the sex determination of males. Thus balanced concentration of steroid hormones is an important factor in their DST (Thomas et al., 1992; Jeyasuriya and Place, 1998; Lance, 2009; Ramsey and Crews, 2009; Miyagawa et al., 2018).

However, what is it that regulates the concentration of these hormones in the gonad that has to do with temperature? Initially, two molecules were proposed that were observed to be modified by temperature: the aromatase and 5α-reductase, which are important enzymes for the production of estrogen and for the conversion of testosterone to DHT, respectively (Jeyasuriya and Place, 1998). Several studies on the activity of these enzymes during the TSP, as well as on regulatory mechanisms have been carried out. Although as previously mentioned, the role of 5α-reductase and androgens in DST has not been fully characterized, (Miyagawa et al., 2018). In the case of the enzyme aromatase, this is known to be expressed in various tissues (ovary, placenta, bone and brain), which is a consequence of its promoter region, which as mentioned previously, in turn causes the gene to present several polymorphisms (Jeyasuriya and Place, 1998; Matsumoto and Crews, 2017). The cDNA that codes for this enzyme has also been isolated and sequenced for several species of vertebrates, including reptiles (Matsumoto and Crews, 2017). This enzyme is positively regulated by the FOXL2 transcription factor in vertebrates (Pannetier et al., 2006;

Wang et al., 2007). It has also been observed in *Oreochromis niloticus* that the aromatase enzyme promoter contains sequences to which the transcription factor Sf1 can bind favoring its transcription (Wang et al., 2007). However, in reptiles Sf1 plays an important role in the masculinization of the gonad, so it could probably interact with some other transcription factor and thereby favor the methylation of the aromatase enzyme promoter by inhibiting its expression (Ramsey et al., 2007a; Parrott et al., 2014; Takasawa et al., 2014).

Subsequently, the estrogen signaling pathway was further investigated, which is how they began to discover how the ERs α and β, participated in TSD during the temperature-sensitive period in *T. scripta* (Crews et al., 1991; White and Thomas et al., 1992c; Ramsey and Crews, 2009; Barske and Capel, 2010), *Eublepharis macularius* (Rhen and Crews, 2001), *Pseudemys nelsoni* (Katsu et al., 2008) and *A. mississippiensis* (Katsu et al., 2004; Kohno et al., 2015; Miyagawa et al., 2015). These receptors are expressed throughout the differentiation of the gonad and in the sensitive period, however they do not exhibit a sexually dimorphic pattern related to TM versus TF (Katsu et al., 2004, 2006; Ramsey and Crews, 2009). It is known that the AR receptor is sensitive to the availability of estrogen in gonad development, because if this decreases, the expression of this receptor increases (Ramsey and Crews, 2007b and 2009; Barske and Capel, 2010; Miyagawa et al., 2015). This receptor was cloned in the crocodile species *A. mississippiensis*; its structure is conserved in evolution, in keeping with that in other vertebrates, specifically in the amino acid residues that interact with DHT and the DNA binding domain (Miyagawa et al., 2015). Likewise, the *ERα* and *β* receptors are expressed in the bipotential gonad, during the TSP, which in turn coincides with an increase in the production of estrogens by means of the aromatase enzyme. However, *ERα* is only expressed at the beginning of ovarian differentiation (regression of the marrow) and subsequently the expression of *ERβ* is maintained during late differentiation of the ovary, playing a fundamental role in cortical proliferation (Ramsey and Crews, 2009). The *ERα* receptor is also known to play an important role in the development of the oviduct in *A. mississippiensis*, where by stimulating *in ovo* with PPT (4,4',4"-(propyl-[1H]-pyrazole-1,3,5-tryl trisphenol), which is an agonist for this receptor, they were able to observe the development of the Müllerian duct towards structures similar to those found in an adult ovary (Doheny et al., 2016). ERα has also been cloned in *A. mississippiensis* and *Crocodylus niloticus*, which maintain highly conserved characteristics principally between crocodile and bird species, providing evidence that genes that code for the steroidogenic receptors of crocodiles are conserved and evolve slowly (Katsu et al., 2004, 2006). In *T. scripta* gonads incubated at FT, it is known that in the medullar region, estrogens negatively regulate the *Sox9* gene (Ramsey and Crews, 2009; Barske and Capel, 2010). It is also interesting that in the promoter region of the *Sox9* gene in humans, binding sites have been found for ERs and in mice, ER binds to these sequences and inhibits the *Sox9* gene (She and Yang, 2014). However, a question arises from this: how is it that steroid hormones are increased during gonad differentiation? Three mechanisms are proposed: 1) that steroidogenic hormones are found in the yolk (Janzen et al., 1998; Elf, 2003), 2) that they are synthesized in the adrenal-mesonephros-gonad complex (White 1992c; Ramseys and Crews 2007c) and/or 3) that they are synthesized in the gonad (Warner 2011) (Fig. 7).

Firstly, it is known that steroid hormone concentrations during egg incubation may come from estrogens and androgens, which due to their lipophilic properties were deposited in the egg yolk during oocyte vitellogenesis (Elf, 2003; Lance, 2009). In this case, the aromatase enzyme is activated or inhibited during the synthesis of estrogens during the TSP; possibly because of maternal epigenetic influence, which occurs in this hormonal environment (Ramsey and Crews, 2009; Matsumoto et al., 2013a, b). The latter is derived from the observation that on some occasions when incubating at FT, MT or PT, anticipated sex ratios are not obtained (Dodd et al., 2006). Some recent studies suggest that the presence of these estrogens in the yolk alters the proportion of sexes as a result of interaction with environmental factors, as mentioned by Warner et al., in 2017, who carried out a study on the *Chrysemys picta* tortoise. They observed two clutches from different years under different environmental conditions. The eggs were incubated at a constant temperature and were treated with fadrozole, an inhibitor of aromatase or 17β-estradiol (E2). They showed that when the natural nesting temperature is temperate, nest temperature and E2 play a fundamental role in the sex ratio, but under conditions of extremely high natural temperature, they respond exclusively to E2 chemical treatment. They thus suggest that the amount of estrogen in yolk can vary between nesting seasons and have a direct influence on the physiological plasticity of development, meaning that the mother can also influence by buffering the effects of TSD (Warner et al., 2017).

Secondly, in the gonad-adrenal-mesonephros complex (G-A-M), it has been observed that estrogens are synthesized during the TSP, coinciding with the activity of the aromatase and 3-β-hydroxysteroidenzyme, dehydrogenase. During ovary differentiation, there is also an increase in the activity of the aromatase in the G-A-M complex

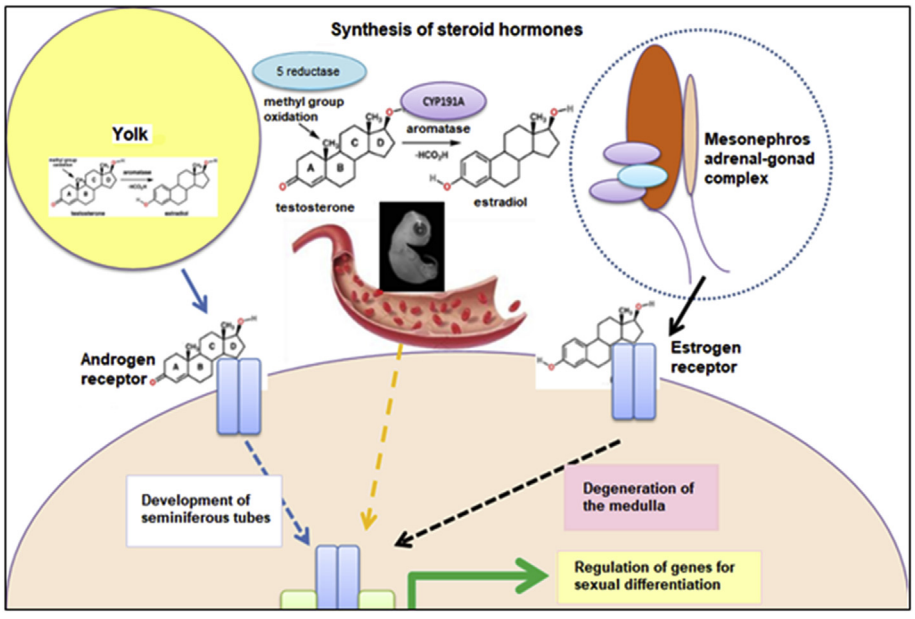

Fig. 7. The participation of estrogens in the differentiation of the reptile gonad is presented. This hypothesis suggests three types of synthesis or ways of obtaining estrogens. The first is derived from the egg yolk, where steroid hormones are deposited during vitellogenesis, which must arrive through the bloodstream to the gonad and initiate signaling for differentiation into a testicle or an ovary. The other route is through the G-A-M complex, where synthesis of estrogens may initiate and the other option is that the gonad alone carries out the synthesis of estrogens. Notably, in this hypothesis the balance in the concentration of estrogens and androgens, as well as in their respective receptors is responsible for triggering the signals for development of semi-sperm tubes in the morphogenesis of the testicle, or even the degeneration of the marrow and proliferation of the cortex for ovarian differentiation. Turtle embryo figure taken from: Greenbaum, 2002 and the image of the blood capillary was taken from: https://ocularis.es/retinopatia-diabetica-i-conceptos/.

(Thomas et al., 1992; Lance, 2009; Ramsey and Crews, 2007b, c).

In the two previous cases, the steroid hormones travel throughout the embryo via bloodstream and chorioallantoic fluid and studies on the *Carreta carreta* turtle, suggest that this type of hormone (estrogens and testosterone) can accumulate in the shell during TSP. The quantity differs between males and females, so this could be used as a way of obtaining a method of quantification and sexing using steroid hormones. This is interesting to use as a tool for ecological studies on nesting under natural conditions of species with TSD (Kobayashi et al., 2015).

In the third case, the synthesis of estrogens initiates in the gonad by the aromatase during the TSP. The previous idea arises from several studies in which it has been observed that its expression and the amount of its metabolites in the gonad are increased, when embryos are incubated at FT. This means that ovarian differentiation must initiate as a result of endogenous estrogen signaling (Jeyasuriya and Place, 1998; Pieau et al., 1998; Ramsey and Crews, 2007c; Lance, 2009).

Concerning estrogen signaling during TSD, the aromatase has been extensively studied, with several studies indicating its importance in this process. In 1999, Pieau proposed three models to show how this enzyme can be regulated during TSD. The first mechanism consists of a thermo-sensitive factor via cAMP, it may directly or indirectly regulate the transcription genes for sex determination, mainly in the gene for the enzyme, aromatase, so in this case it was proposed that a GPCR-type receptor must play an important role, however to date it is not known whether any member of this family of receptors plays a role in DST, but what is known is that this type of receptors can sensitize the ion channel TRPV4 via cAMP by means of PKA, and that this ion channel, which will be discussed in detail later, has been shown to play a role during DST (Darby et al., 2016). The second mechanism proposes that a thermo-sensitive factor directly or indirectly activates the aromatase; in this case the Amh was proposed. The last proposal refers to the existence of a heat shock protein involved in the binding or dissociation of estrogen receptors (Pieau et al., 1999). This mechanism has been studied and demonstrated in mammals and specifically in the analysis of the genome for the *A. mississippiensis*, where sexual dimorphism was discovered in the expression of the mRNA of the heat shock proteins HSP27 and HSP70A, during DST (Kohno et al., 2010). It still has not been demonstrated that aromatase is the main factor affecting DST. But it certainly turns out to be a very interesting aspect related to this type of signaling, as apparently it provides great plasticity in this process, as it does not participate exclusively in the feminization of the bipotential gonad, but is also a factor whose regulation is predominant in the modification of the sex ratio, partly demonstrated by the effect of exogenous estrogens *in ovo*.

From all the previous studies, an interesting question arises: is there a physiological difference during gonad development between individuals who have been sexually determined by exogenous hormonal treatments and individuals who have been sexually determined by incubation temperature? The answer is yes, because in 2018, Canesini et al. showed that *C. latirostris* females produced by exogenous estrogens (E2SD) present alterations when compared to females produced by incubation at FT. These alterations mean that among females, E2SD increase the expression of ERα, aromatase, the PR and the proliferation/apoptosis of the germ cells increases, suggesting that damage to their DNA has occurred. In contrast, the females produced at the FT of TSD do not show any variation in the expression of ERα during development, have less aromatase enzyme, PR expression is lower, proliferation is lower during development and they manifest low apoptosis, even though these increase at the end of ovarian development and are found exclusively in oocytes where meiosis failed (Canesini et al., 2018). These effects may have to do with the fact that exogenous estrogens are not at a normal physiological concentration for the developing embryo and synergize their effect on the bipotential gonad.

It is also important to mention that these studies on hormones and TSD are important for several reasons; one is that they include studies on environmental pollution and climate change, where many alterations in the sex ratio and even the development of hermaphrodites have been detected. For example; the chemical compound 17α-methyl-testosterone, acts as a potent androgen in crocodiles and hatches males despite being incubated in at FT (Murray et al., 2016). 17β-Estradiol can generate polyovular oocytes and polynuclear oocytes, while also reducing the activity of the aromatase enzyme in the G-A-M (Canesini et al., 2018). Similarly, the increase in ambient temperature decreases the activity of the aromatase, therefore increasing the number of males as a result of climate change (Warner et al., 2017).

It is thus important to understand that environmental compounds with estrogenic or androgenic activity that pollute the environment, may have serious consequences by altering the reproductive health of offspring exposed to these hormonal disruptors, as well as their evolution and ecology. As hormones at TSD regulate many aspects of the trajectory in the morphogenesis of the developing ovary and testicle, thus the effects of temperature on the sex determination of the embryo relate to the quantity of circulating hormone, the sensitivity of the receptors in their cellular targets and downstream signaling pathways that activate (the amplification of the signal), indicating the great plasticity of physiology that exists in this process, as mentioned in the hypotheses described in this section (Ramsey and Crews, 2009; Landry and Horth, 2014).

6. The hypothalamic-pituitary axis in sex determination by temperature

When talking about TSD, the question arises of how the gonad can assess temperature, in order to initiate sex differentiation. To date, little is known about this signal. It has been proposed that the brain may act as a sensor for temperature (Merchant-Larios et al., 1989). The first evidence about this hypothesis came from studies on *Lepidochelys olivacea*, where innervations of the nervous system were observed in the parenchyma of the undifferentiated gonad, which are positive to acetylcholinerase at different stages of development (24–27) and during TSP (Merchant-Larios et al., 1989; Gutiérrez-Ospina et al., 1999).

In the work carried out by Gutiérrez-Ospina et al., in 1999, they reconstructed serial sections of slices from the gonad, by marking the nerve fibers with acetylcholinerase. The authors observed that the nerve fibers first enter the gonad from the subcortical region, up to the division between the gonad and the mesonephros. Likewise, the afferent innervations in the medulla radiate out from the hilum of the gonad and grow radially towards the cortical region (Gutiérrez-Ospina et al., 1999).

Due to the presence of neurons in the gonad, the question arises: does the central nervous system participate in sex differentiation? And how does this process occur? In answer to the first question, it has been proposed that the brain of reptiles with TSD presents a sexual dimorphism. A possible reason for this is that proposed by Jeyasuria et al., in 1998, where this would be determined by factors that cause a change in the activity of the aromatase and the 5α-reductase (Jeyasuriya and Place, 1998). In this way, the activity of the aromatase in the brain increases during differentiation of the ovary, but this increase is greater in males than in females, besides this, the presence of the mRNA of the aromatase was found in the development of the brain before it was found in the ovary, possibly due to its participation in other processes of neuronal differentiation and metabolism (Jeyasuriya and Place, 1998; Pelletier, 2010). The activity of the 5α-reductase in turtles is also lower in the brain than in the gonad. Bogart in 1987 used this data to hypothesize that the balance between estrogens and androgens plays an important role in sex determination, as a high concentration of estrogen in the brain initiates the signaling path to develop males and a low concentration initiates signaling to obtain females (Fig. 8A). This happens in an opposite way in the gonad (Jeyasuriya and Place, 1998).

Currently, the expression of ER and AR in the brains of turtle

Chrysemys picta (Mak et al., 1982), lizard *Anolis carolinensis* (Cohen et al., 2012) and crocodiles *Caiman latirostris* and *A. mississippiensis* has been characterized (Gabriel et al., 2001; Varayoud et al., 2012). Other animal models also manifest sexual dimorphism, which has been found in different regions of the brain, and α-fetoprotein have even been found that can protect the female brain from the masculinizing effects of aromatization (Jeyasuriya and Place, 1998).

Related to the above, it was also found that in *A. mississippiensis* the amount of GnRH and aromatase in the brain of individuals incubated at FT and MT are similarly expressed (Gabriel et al., 2001). An interesting thing about this research is that they studied the sensitivity of the reproductive system, when encountering different thermal signals during development, because they wanted to know if there were differences between the transcripts and hormones of the gonad and brain, related to the different temperatures that generate the same sex of individual (see Fig. 2). No differences were found that can be explained by the intensity of embryonic incubation temperatures, except for the magnitude of dimorphic expression in *Amh* and *Sox9* genes, which seems to be exclusively related to the incubation temperature in the gonad, but not in the brain (Gabriel et al., 2001; McCoy et al., 2016).

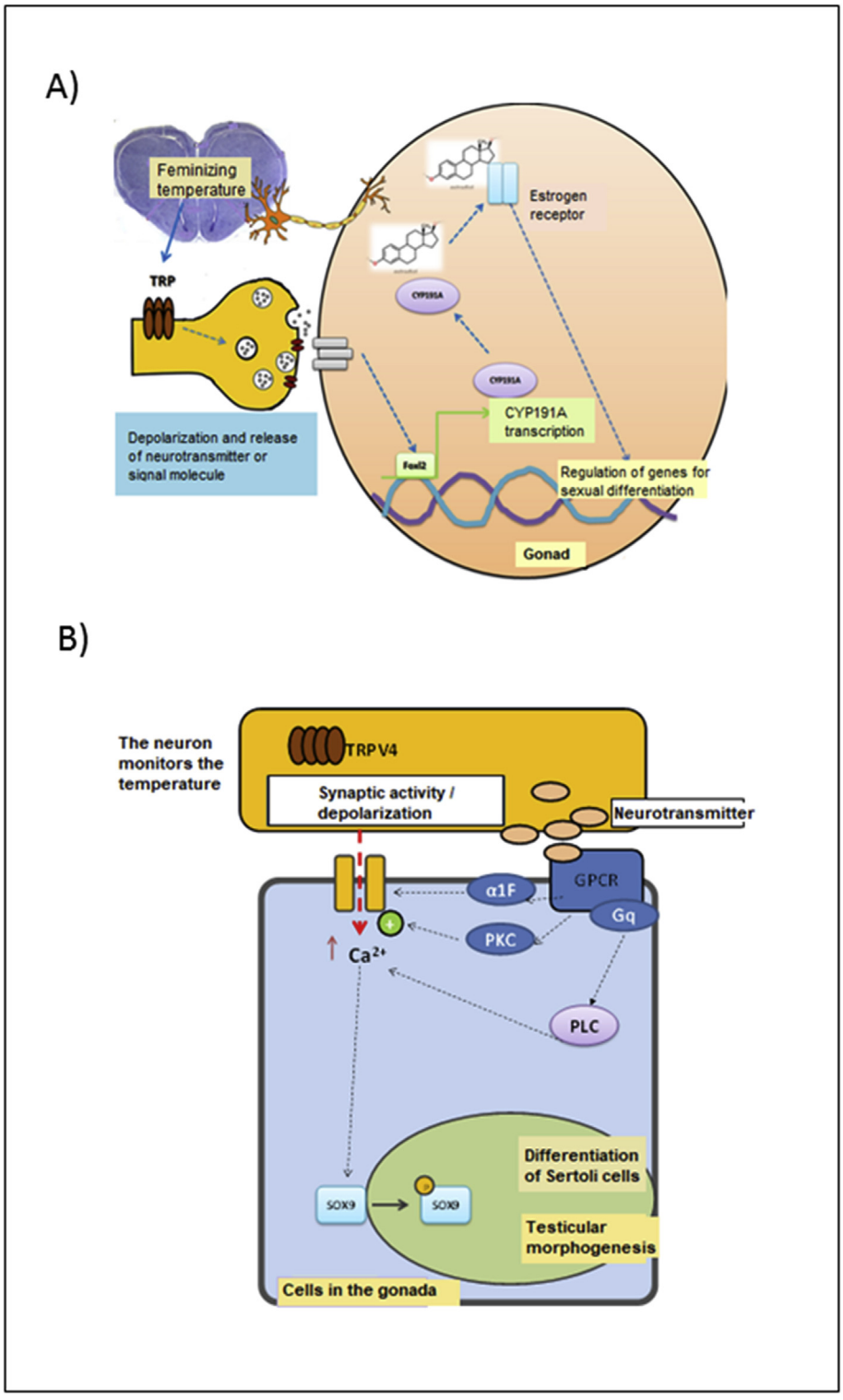

Fig. 8. Possible role played by the nervous system in the differentiation of the ovary and testicle. In A) the undifferentiated gonad has nerves in the parenchyma and in the cortex (Varayoud et al., 2012). In reptiles, ionic receptors from the TPR family have recently been characterized (Nagai et al., 2012; Yatsu et al., 2015). It has been suggested that temperature may be monitored through a TRP receptor, which is expressed in the neuron and when stimulated with the feminizing temperature may release a chemical messenger that induces the expression of the transcription factor Foxl2, which is known to directly regulate the expression of the aromatase P450 enzyme in the differentiation of the ovary in mammals. Estrogen production and the expression of its receptor may combine to regulate the expression of genes for sexual differentiation and inhibit the Sox9 factor, which is expressed at the moment a gonadal blastema is formed. In masculinization B) the temperature can be monitored by TRPV4 that is expressed in a neuron, producing the depolarization of it, thus releasing a neurotransmitter that stimulates a GPCR receptor such as acetylcholine, in order to positively activate pathways that increase calcium intracellular by sensitizing calcium channels via the α1f subunit of G proteins, or via the Gq/PLC protein pathway. It may also be the case that the neurotransmitter acetylcholine, rather than activating a calcium channel, in fact activates TRPV4 via PKC in order to increase positively intracellular Ca + 2.

In contrast, it is thought that the nerve endings within the gonad are able to perceive the incubation temperature spatially and temporally (during the TSP) and release some chemical messenger that influences the sexual determination of the individual (Gutiérrez-Ospina et al., 1999; Seebacher and Craig, 2005; Seebacher and Murray, 2007). In reptiles, little is known about thermo-reception processes. The most studied systems are those for detecting the heat of prey by snakes, which occurs through nerve endings, which via protein kinase C (PKC), are able to detect infrared frequency or other stimuli (Seebacher and Craig, 2005; Seebacher and Murray, 2007). In *M. musculus*, specialized peripheral nerve endings, are able to respond to changes in temperature, due to the presence of thermal ion channels known as TRPs. This type of ion channel triggers the depolarization of the neuron, when faced with a thermal stimulus, converting this signal into action potentials, which are transmitted through the nervous system and the information is processed by excitatory or inhibitory inter-neurons, as far as the thalamus region in the brain and then the somato sensory and insular cortex facilitate the perception and discrimination of thermosensitivity (Liedtke, 2008; Li and Toyoda., 2015; Shibasaki et al., 2015).

The TRPs are characterized by 6 trans-membrane domains, which differ from cations in terms of homology and permeability and are divided into 6 subfamilies (Luo and Hu et al., 2014; Garcia-Elias et al., 2014; Liman, 2014; Li and Toyoda et al., 2015). Of these, the TRPV3 subfamily is expressed in a variety of mammalian tissues such as: kidney, liver, brain, spinal cord, pituitary (an organ that in reptiles monitors heat), vascular smooth muscle, ovary and testis (Luo and Hu et al., 2014). However, it participates in the perception of harmful heat, but there are others that can register different temperature ranges such as: TREK-1, which is activated in the ranges of 22–32 °C (Kang et al., 2005; Li and Toyoda et al. 2015). TRPM5 is activated in the ranges of 15–25 °C (Liman, 2014). In reptiles, TRPs have been characterized in crocodiles and lizards (Seebacher and Murray, 2007; Nagai et al., 2012). Recently the TRPV4 channel has been investigated in *M. musculus*; this ion channel is expressed in the central nervous system in neurons of the hippocampus where they are constitutively active with stimuli from physiological temperatures and it is also found to regulate several physiological processes in the peripheral nervous system (Shibasaki et al., 2015; White et al., 2016). However, what is its relationship with TSD? It is known that this receptor is expressed in the embryonic gonad of *A. mississippiensis* during the temperature sensitive period, so it may monitor the temperature that determines sex, specifically promoting the masculinizing one, thus regulating the release of calcium by activating signals downstream for the differentiation of a testicle (Yatsu et al., 2015). The expression of this TRPV4 ion channel in ovaries and adult *C. moreletii* testis has also been demonstrated. The expression of TRPV4 was found in Sertoli and follicular cells (Martínez-Juárez et al., 2018). But how is it that this ion channel participates in TSD? The answer has to do with observations made in studies of chondrocytes, where it has been well documented that the ion channel TRPV4 can positively regulate the Sox9 transcription factor through calmodulin, although other elements of the signaling pathway may be involved in this process (Muramatsu et al., 2007; Hurd, L et al., 2015). It is known that in mammals those participating in the regulation of the *Sox9* gene, include: the protein kinase A (PKA), the enzyme adenylate cyclase from cAMP, the transcription factor CREB mainly in a possible signaling pathway such as that described in Fig. 8 (Juhász et al., 2014), and elements that positively regulate the activity of TRPV4 include acetylcholinergic receptors and PKC and PKA proteins, Fig. 8B (Piera-Velazquez et al., 2007; Adapala et al., 2011; Martínez-Juárez et al., 2018) (see Fig. 9).

7. The gonad monitors temperature during sexual determination by temperature

In this last mechanism, it is thought that the gonad possesses a factor that intrinsically controls temperature. This is derived from experiments, where turtle embryos are be headed (Lance, 2009), and their sexual differentiation observed, without intervention of the nervous system, as well as *in vitro* cultures of the gonad, where the gonads were carefully isolated in the early stages of development and the expression of the *Sox9* gene was observed, in *L. olivacea*. Based on these studies, it was suggested that the gonad possesses a capacity for perceiving temperature during the TSP and depending on whether the incubation temperature is FT or MT, it differentiates into an ovary or a testicle (Moreno-Mendoza et al., 2001; Yatsu et al., 2015). More recently, Shoemaker et al., in 2010 also observed the expression of genes such as *Dmtr1*, *Sox9, Amh* and *Foxl2* in *T. scripta* gonads kept *in vitro* (Shoemaker et al., 2007; Shoemaker and Crews, 2010).

In the search for this intrinsic factor of the gonad that regulates the signaling pathways for the TSP, molecular studies have been carried out on the expression of genes, heat shock proteins and epigenetic regulation, involved in sexual differentiation (Moreno-Mendoza et al., 2001; Li et al., 2012; Geroges and Holleley, 2018).

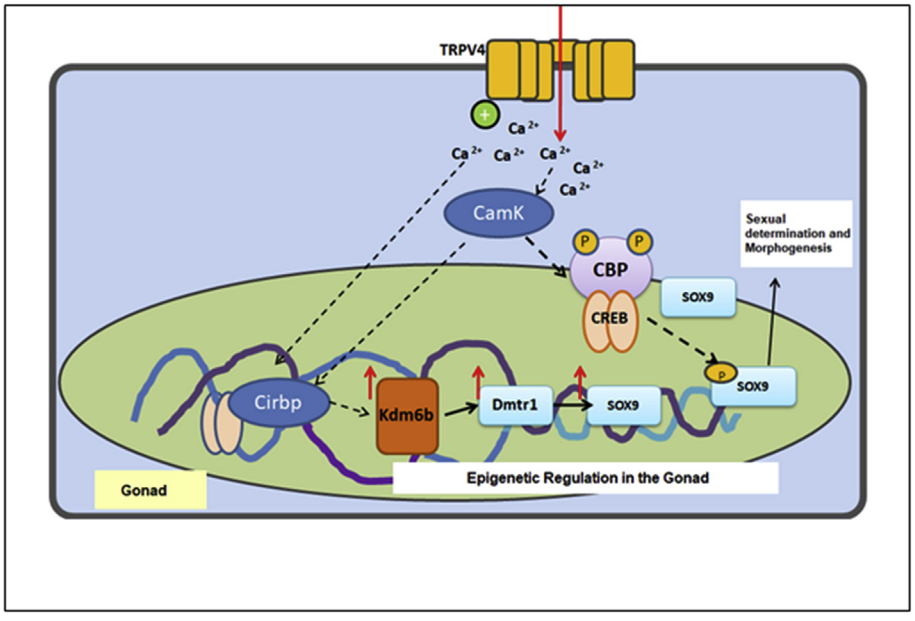

Fig. 9. Epigenetic signaling pathway in the sexual determination of the testicle. In this case, TRPV4 may monitor temperature, producing depolarization in the membrane of a cell in the gonad, thus increasing the amount of intracellular Ca2+ allowing the enzyme calmodulin to regulate histone demethylase enzymes such as Kdm6b upstream, which in turn positively regulates the Dmtr1/Sox9 pathway. It has also been recognized that calmodulin in chondrogenesis and in processes of transcriptional neuronal response, where epigenetic marks are regulated by CBP/CREB transcription factor, must be important for maintaining the phosphorylated Sox9 signal in the nucleus. Finally, there is also the possibility that the Cirbp protein is thermo-sensitive and triggers the upstream pathway of Kdm6b/Dmtr1/Sox9.

7.1. Epigenetics in the TSD

During the interaction between the environment and genome, modifications in DNA such as: methylations, acetylations and histone modification stake place. Likewise, there is expression of non-coding RNAs (Piferrer, 2013; Kuroki and Tachibana, 2018), which are of interest to the TSD, as they may be involved in translating the temperature signal into different genetic pathways for sex determination (Kuroki and Tachibana, 2018; Geroges and Holleley, 2018). In reptiles, this type of modification has been found in the promoter methylation of the aromatase in the *T. scripta* turtle, where it was observed that when embryos were incubated at FT, the promoter of aromatase is demethylated in CpG regions, enabling its transcription (Matsumoto et al., 2013a, b). In the *A. mississippiensis*, it was apparent that the promoter region of aromatase is hypermethylated when embryos are incubated at MT, repressing their transcription. Evidently, the *Sox9* gene can also be negatively regulated, as it is hypermethylated in embryos incubated at MT. Therefore, it is apparent that methylations of these genes are dimorphic (Kohni et al., 2014; Parrott et al., 2014).

Epigenetic regulation during sex determination has been observed in several vertebrate species, such as: fish *Dicentrarchus labrax* (Navarro-Martín et al., 2011), *G. gallus* (Kohni et al., 2014) and *M. musculus* (Nishino et al., 2004; Tachibana, 2015). Particularly in the fish *D. labrax*, it has been observed that the methylation of the promoter region of the aromatase gene is involved in masculinization, as it causes a decline in the expression of this enzyme (Navarro-Martín et al., 2011). In the case of *G. gallus*, the hypermethylated male region (HMH) is a region that is not methylated in the female and produces non-coding RNAs that down-regulate the *Dmrt1* gene. Kohno et al. proposed that this region might be important during the TSD, due to the evolutionary relationship between crocodiles and birds (Kohni et al., 2014). *Sry* has been shown to be epigenetically regulated in mammals, as although not expressed, its promoter remains hypermethylated. Moreover, its expression is associated with low methylation in this promoter region (Nishino et al., 2004).

A very important contribution made by Ge et al. (2018) is that they discovered the direct relationship between epigenetic regulation and sex determination by temperature. Thus, they demonstrate that the histone demethylase KDM6B is expressed dimorphically and at the masculinizing temperature (26 °C) in *T. scripta*, this enzyme regulates the expression of H3K27 (trimethylated lysine 27 on histone 3) demethylating it, as it regulates negatively to the *Dmtr1* gene. Therefore, high amounts of KDM68 at MT stimulate activity in the *Dmtr1* gene and low amounts of KDM68 inhibit the expression of *Dmtr1* and lead to the development of females, even when the embryo or gonad is incubated at a MT (Ge et al., 2018). It is not known whether KDM68 responds directly to temperature or is regulated upstream by temperature sensitive elements; an RNA binding protein, Cirbp (cold-inducible RNA binding protein) has been proposed, as this is expressed early in the development of the gonad and plays an important role in the temperature sensitivity of the embryo, as some mutation of this protein is sufficient to eliminate it (Georges and Holleley, 2018) (Fig. 9).

Although the factors that determine a male by temperature have already been identified, many elements of the physiology of this process are unknown. Notably enzymes that modify histones can be activated by various stimuli. For example; in epigenetic modifications dependent on neuronal synaptic activity, the intracellular calcium flow (Ca^{2+}) and the activation of the kinase IV-dependent calmodulin protein (CamKIV) are important for initiating the signaling pathway that will modify epigenetic marks associated with the function of enhancers, regulating gene expression. An important factor related to these last proposals is that the ion channel TRPV4 is a channel that regulates the flow of Ca^{2+} into the interior of the cell, but in turn, as previously mentioned, regulates the transcription factor Sox9 during chondrogenesis via Calmodulina. We therefore wonder whether this might be the factor that monitors the temperature and up-regulates the epigenetic marks during TSD, specifically at MT by means of Ca^{2+} flow. This TRPV4 ion channel would thus be upstream of the demethylase enzymes and the Cirbp protein (Fig. 9) (Muramatsu et al., 2007; Riccio, 2010; Ge et al., 2018; Georges and Holleley, 2018).

The study of the epigenome in TSD is interesting, because the modifications of this, in response to temperature in an individual enable the development of two alternative phenotypes, in this case the development of an ovary or a testicle, in the absence of sex chromosomes. Epigenetics thus encompass all these processes, which apart from the DNA sequence are involved in phenotypic plasticity, revealing the complexity of the sex determination mechanism from an “eco-evo-devo” point of view (Riccio, 2010; Landry and Horth, 2014; Capel, 2017; Ge et al., 2018; Georges and Holleley, 2018).

7.2. Heat shock proteins

In 1999, Pieau proposed heat shock proteins as possible molecules that regulate all signaling downstream for TSD. Recently in 2010 Kohno et al., in a study carried out on the *A. missisippiensis*, analyzed the dimorphic patterns of the mRNA of these proteins during the TSP. To this end, genes similar to heat shock proteins were cloned from other vertebrates such as mice and humans. They found that HSP27, HSP70A and HSP90α are expressed dimorphically. The HSP27 type proteins have greater expression in the testicle, compared to ovarian tissue at one month of age. It seems that it can negatively regulate estrogen signaling, competing for the transcription of the estrogen receptor in ERE elements of the promoter. Likewise, the expression of HSP70A is dimorphic and is known to participate in the synthesis of vitamin D, an important molecule for the synthesis of estrogens; in fact it can induce the activation of the aromatase via the vitamin D receptor. HSP90α is a chaperone protein, which participates in signaling during the degradation of extracellular matrix and during migration of cancer cells. It has been proposed that this may participate during the migration of germ cells from the cortex to the marrow, during testicular differentiation (Pieau et al., 1999; Li et al., 2012; Kohno et al., 2010).

8. Conclusion

Several hypotheses have been proposed about how TSD occurs in reptiles, which are based on 52 years of research. Despite the efforts of several groups, many aspects concerning the basis of TSD in reptiles are still unknown. Currently, it has been demonstrated that the most favored hypothesis explaining the factor that triggers TSD, are the epigenetic modifications prior to stimulus by temperature, proposed by Ge et al. (2018), because they are very close to describing the canonical pathway in TSD (1a pattern). However, as mentioned throughout this paper, TSD is a process with great genomic and phenotypic plasticity from the “eco-evo-devo” perspective, as there are possibly at least 4 signaling pathways or 4 ways to regulate TSD. This is the same amount of patterns for sexual determination by temperature that exist, as these depend on the transcriptome, proteome, metabolome, genome and epigenome, which together provide the physiological, morphological or developmental plasticity necessary for the formation of a phenotype (hierarchy of plasticities). Similarly, in future projects, the study of TRPs is important, as these may be the factors that monitor temperature and trigger the signaling pathways for sex determination by temperature upstream, by means of an increase in Ca^{2+}.

Conflicts of interest

The authors declare that they have no conflicts of interest associated with the contents of this manuscript.

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