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REVIEW PAPER

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A review on temperature dependent sex determination (TSD) in freshwater turtles

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Abstract

Temperature plays a very crucial role in turtle's life starting from egg incubation to sex determination to day-to-day metabolism. Turtles exhibit two types of sex determination mechanism i.e. genetic sex determination (GSD) and environmental sex determination (ESD). ESD are the major mechanism observed in the turtle's species. TSD is the most observed ESD patterns in Turtles. Three different types of TSD patterns (TSD Ia, TSD Ib and TSD II) were observed in the turtles. Most freshwater turtles exhibit TSD Ia type of TSD in which females are developed at higher temperature and males are hatched at lower temperature. For the identification of sex on individuals in TSD study majorly two categories of methods are applied i.e. invasive and Non-invasive. Invasive methods involves laparoscopic and gonad histology examination of reproductive organ. While in non-invasive method various method like penal inversion, morphometric measurements and blood assay were used. The intensive literature review suggested that invasive methods are 100% reliable and accurate but are lethal while on the other hand all the non-invasive methods are easy to operate but not 100% reliable and accurate. Some they need to be validated by invasive methods. In fresh water turtles DMRT1, SOX9, AMH and WT-1 are the major gene identified responsible for the male and aromatase is responsible to induce the development of female reproductive organs depending upon the incubation period.

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Introduction

Turtles are oviparous and ectothermic reptiles right from egg development to daily metabolism they are dependent on the surrounding temperature. The most important stage of their life completely depends on the temperature in which they are developed i.e. embryonic development during incubation period. It is well studied in last two decades that sex determinations in most turtle (reptiles) are dependent on the environmental factors (majorly temperature). Sex determinations in turtles are done by two methods genetic sex determination (GSD) and environmental sex determination (ESD) (Valenzuela, 2004). Majority of vertebrate's classes (except fishes and reptiles) also possess GSD mechanism of sex determination. GSD are those in which an offspring's sex is purely dependent on the bases of sex chromosome fertilization and has no relation with the external environment. While ESD is the mechanism in which an embryo's sex formed after fertilization due to exposed to a specific environmental stimulus during the development (eg. Ambient/ incubation temperature). ESD is common in fishes and reptiles (Bull, 1980 & 1983). Environmental stimuli like temperature, moisture, salinity are the major factors responsible for the ESD of which incubation temperature is the most comely known (Ewert and Nelson, 1996). The most common form of ESD in reptile group (Crocodilians, Testudines' and Squamata) are temperature dependent sex (TSD) determination (Bull, 1983; Valenzuela *et al.*, 2003), a sensitive period during the middle-third of incubation which decided the sex of emerging offspring's (reviewed in Bull, 1983).

TSD is dependent on a very narrow range of temperature experienced (Bull, 1983) by the developing embryo for the limited window of time known as the thermosensetive period (TSP) (Mitchell *et al.*, 2006). Usually TSP is the middle-third duration of the incubation period during which gonads are developed and sex determined. After that period, sex of that particular cannot be altered or stimulated in any ways. Like GSD, species-exhibiting TSD does not have sex-specific chromosomes. TSD firstly reported in a lizard (Charnier, 1966) and as research progressed, discovered in turtles and crocodilians. With the advancement of methods and large scale study suggested that total nine families of freshwater, terrestrial and sea turtles exhibit TSD (Bull, 1980 & 1983; Ewert and Nelson, 1991; Miller, 1988; Standora and Spotila, 1985; Vogt and Bull, 1982; Vogt and Flores-Villela, 1986, for reviews). Until date TSD have been documented in the most turtles, some species of lizards, and all crocodilians studied (Augstenová *et al.*, 2018; Nagahama *et al.*, 2021; Rovatsos *et al.*, 2015).

This review presents an overview about the TSD in freshwater turtles (Table 1) explaining about the various aspect of the TSD includes various patterns of the TSD and different types of method used for sexing.

Patterns of TSD

In reptiles, three types of TSD patterns observed (Ewert and Nelson, 1991) depending upon the numbers of males and females born at different incubation temperature. Usually in most fresh water turtle species, eggs incubated at high temperature develop into males and those at low temperature into female (Raman, 2020) (Fig. 1).

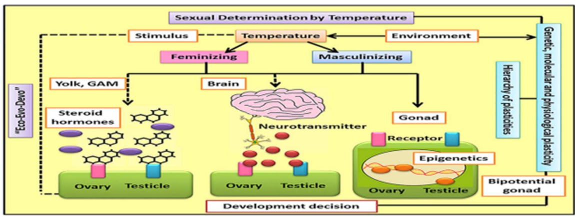

Fig. 1. A schematic diagram representing the TSD mechanism (Image - Martínez-Juárez and Moreno-Mendoza, 2019)

TSD Ia (Male-female or MF)

Conditions in which 100% male are hatched at low temperature and 100% female are hatched at high temperature considered for the incubation. It commonly is seen in species where adult females are larger than males (Ewert and Nelson, 1991). The perfect example of this is common snapping turtle in which males are hatched at 26°C, while females at 30°C (Janzen, 1995). In 2013, Vitt and Caldwell recorded that in *Testudo graeca* (Tortoises) males are hatched below 31-32°C while females hatched above it (Fig. 2).

Table 1. List of freshwater turtle species (with family) diagnosed with the TSD

SL	Species	Pattern
Carettochelyidae (n=1)		
1.	Pig-nosed turtle (*Carettochelys insculpta*), Georges, 1992	TSD Ia
	Dermatemydidae (n=1)	
1.	Central American River turtle - Hickatee (*Dermatemys mawii),* Thépot, 2021	
Chelydridae (n=2)		
1.	Common snapping turtle (*Chelydra serpentina*), St. Juliana *et al.*, 2004; Ewert *et al.*, 2005	TSD Ia
2.	Alligator snapping turtle (*Macrochelys temminckii*), Miller and Ligon, 2014	TSD II
Emydidae (n=13)		
1.	Spotted turtle (*Clemmys guttata*), Ewert & Nelson, 1991; Ewert *et al.*, 2004	TSD Ia
2.	Eastern Box Turtle (*Terrepene carolina IN*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD amb
3.	Eastern Box turtle (*Terrepene carolina FL*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD amb
4.	Painted Turtle (*Chrysemys picta IN*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD Ia
5.	Painted Turtle (*Chrysemys picta TN*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD Ia
6.	Chicken Turtle (*Deirochelys reticularia FL*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD Ia
7.	River Cooter (*Pseudemys concinna TN*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD Ia
8.	River Cooter (*Pseudemys concinna FL),* Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD Ia
9.	Coastal Plain Cooter (*Pseudemys floridana*), Ewert *et al.*, 2004	TSD Ia
10.	Florida Red-bellied Cooter (*Pseudemys nelsonia*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD Ia
11.	Texas River Cooter (*Pseudemys texana*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD Ia
12.	Hispaniolan Slider (*Trachemys decorata*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD Ia
13.	Red-eared Slider (*Trachemys scripta*), Crews and Ber geron, 1994; Ewert *et al.*, 2004	TSD Ia
Geoemydidae (n=7)		
1.	Japanese pond turtle (Mauremys japonica), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD Ia
2.	Vietnamese pond turtle or Annam leaf turtle (*Mauremys annamansis*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD Ia
3.	Indian black turtle (*Melanochelys trijuga*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD II
4.	Furrowed Wood Turtle (*Rhinoclemmys areolata*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD II
5.	Red-necked Pond Turtle (*Chinemys nigricansa*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD Ia
6.	Chinese Pond Turtle (*Mauremys reevesii*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD Ia
7.	Malayan snail-eating turtle (*Malayemys macrocephala*), Pewphong *et al.*, 2020	TSD Ia
Kinosternidae (n=14)		
1.	Alamos mud turtle (*Kinosternon alamosae*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD Ia
2.	Striped mud turtle (*Kinosternon baurii*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD amb
3.	Creaser's mud turtle (*Kinosternon creaseri*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD II
4.	Yellow Mud Turtle (*Kinosternon flavescens*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD amb
5.	Rough-footed mud turtle (*Kinosternon hirtipes*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD Ia
6.	White-lipped mud turtle (*Kinosternon leucostomum*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD amb
7.	Scorpion mud turtle (*Kinosternon scorpioides*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD amb
8.	Sonora mud turtle (*Kinosternon sonoriensea*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD Ia
9.	Eastern Mud Turtle (*kinosternon subrubrum hippocrepis*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD II
10.	Eastern Mud Turtle (*kinosternon subrubrum subrubruma*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD Ia
11.	Kinosternon Arizonese (*Kinosternon arizonense*)- *Extinct species of mud turtle,* Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD II
12.	Razor-backed musk turtle (*Sternotherus carinatus*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD II
13.	loggerhead musk turtle (*Sternotherus minor*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD II
14.	Common musk turtles (Sternotherus odoratus), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD II
Pelomedusidae (n=4)		
1.	African helmeted turtle (*Pelomedusa subrufa*), Ewert and Nelson, 1991; Ewert *et al.*, 2004	TSD II
2.	South American river turtle (*Podocnemis expansa*), Valenzuela, 2021	TSD Ia
3.	Magdalena river Turtle (*Podocnemis lewyana*), Gómez *et al.*, 2016	TSD Ia
4.	Six-tubercled Amazon River turtle (*Podocnemis sextuberculata*), Camillo *et al.*, 2022	TSD Ia

TSD Ib (Female-male or FM)

It is the reverse of MF, a condition in which 100% female are hatched at low temperature and 100% male are hatched at high temperature. This patters mostly observed in lizards. In 2017, Waters *et al.* recorded that *Pogona vitticeps* (lizard) females are hatched below 330 C and above it males (Fig. 2).

TSD II (Female-male-female or FMF)

Condition in which females are hatched at low as well as high temperatures and male are hatched at intermediate temperature. This type of patterns is seen in some species of alligators and crocodilians (Shoemaker and Crews, 2009). Miller and Ligon, 2014 recorded that in *Macrochelys temminckii* (turtle) females hatched below 22 and above 28, while males in-between. Lang and Andrews, 1994 mentioned that in *Alligator mississippiensis* females are hatched below 31 and above 34, while males in-between. Similarly, Mitchell *et al.*, 2006 stated that in *Sphenodon guntheri* (tuatara) females are hatched below 21.6 and above 24 and males in-between. Similarly, the reverse condition also observed in which males are hatched at extreme temperatures and females at intermediate temperatures and known as MFM (male–female–male) which is recorded in flatfish (Luckenbach *et al.,* 2009) (Fig. 2).

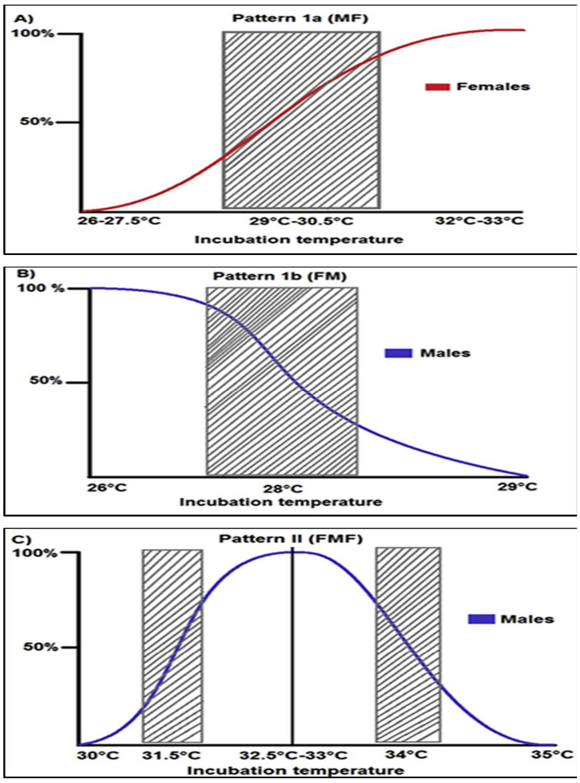

Fig. 2. Three patterns of TSD based on the incubation temperature. TSD 1a (MF) image A TSD 1b (HM) image B and TSD II (FMF) image C. Diagram source – Martínez and Moreno, 2019

Sexing methods

There are various methods are used to identify the sex of turtles depending upon the age of turtles and need of the study. Sexing of an adult turtle in most species is very easy as they are sexually dimorphic in adulthood but young turtles cannot be sexually differentiated in early life stages (Fig. 3). The literature review of various methods use for turtles sexing suggest that all the methods are broadly divided into two broad category i.e. Invasive and non-invasive.

Invasive

Methods are the lethal ways of sexing in which either individual is sacrificed or gonads are surgically observed in live individual. There are majorly two method are widely used under the invasive category of sexing. The first one is dissection of sacrificed individuals to see or histologically studied the gonads of turtles. This is the only standard method the sexing the neonates because there is no other way to determine the sex of a neonates (Wibbels *et al.*, 1991; Wibbels *et al.*, 1998; Lazar *et al.*, 2008; LeBlanc *et al.*, 2012; King *et al.*, 2013). The another method involve the visual inspection of gonads of a live individuals using the laparoscopy using surgical procedure (Rostal *et al.*, 1994; Spotila *et al.*, 1994; Wyneken *et al.*, 2007). This method is requires the special surgical skills and related equipment's and facility as individuals need to be kept under observation post procedures. Thought this method not involves the sacrifice of individuals but there is some risk of mortality during the procedures or after wards. This is entirely based on the skills of the observer and most importantly on the health of targeted individual. These both methods are lethal but typically result in high accuracy in sex determination. The other drawbacks of both these methods are that you a LAB setup to perform these methods.

A third but not much widely used method is also available, it's the derivative of laparoscopy methods in which laparoscope inserted in the body through cloaca and this method is referred as cloacoscopic (Divers, 2019; Divers *et al.*, 2022; Perpinan *et al.*, 2016). Penis in turtles are situated on the wall of cloaca, which is observed by inserting the laparoscope in cloaca and depending upon the present and absence of penis sex is decided. This method have high level of accurate result but at the same time possess many complications like bladder perforation, moderate hemorrhage at the incisional site, less visibility due to pre-existing coelomitis, unable to identify non-differentiated gonads and temporary bladder prolapse through the surgical incision. All this complication unnecessarily increases the surgery time.

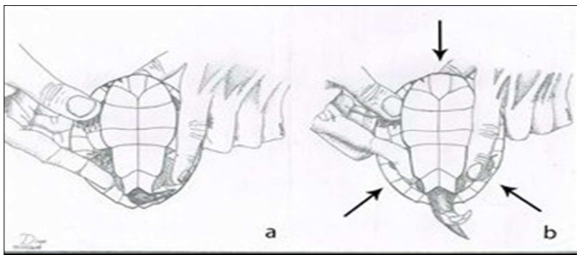

Fig. 3. Illustration of a male turtle held for the sexing using the method explained by Rodrigues *et al.*, 2014. a) Male turtle vertically immobilized by holding head and limbs, b) Male everting the penis after applying little pressure in the hind limbs and neck. Arrows indicate the pressure applying points. Image source: Rodrigues *et al.*, 2014

Non-invasive

Methods involve the various non-lethal procedures to determine the sex of an individual externally. These methods are typically helpful to identify the sex of sub-adult and adult individuals because morphometric or secondary sexual characteristics are mostly started to develop in sub-adult or adult only. These methods includes the assay of blood, hormone or amniotic fluid (Wibbels *et al.*, 1987; Gross *et al.*, 1995; Xia *et al.*, 2011), sexual dichromatism in head spots (Moll *et al.*, 1981; Bulté *et al.*, 2013) and morphometry (Gibbons and Lovich, 1990; Valenzuela *et al.*, 2004; Casale *et al.*, 2005; Rueda-Almonacid *et al.*, 2007; Readel *et al.*, 2008). The most advance methods included manual penile eversion to identify male individuals.

Penile eversion

Is the most advance method of sexing used in fresh water turtles? Penis in turtles is located on the cloaca walls ventrally and inflates during the copulation (Zug, 1966; Carvalho *et al.*, 2010; Cabral *et al.*, 2011). In large-size reptiles like mugger and Gharial penis is find out by inserting finger into the cloaca (Rueda-Almonacid *et al.*, 2007) and a common method used to hemipenile eversion in snakes, lizards, and amphibians (Reed and Tucker, 2012) however this method is lethal and useless in small individual. The literature reviews on the penial erection till date in freshwater turtles suggested there are two methods basically used. Initially to perform the penial erection, individuals are vertically held by immobilizing the head and limbs (Rodrigues *et al.*, 2014). Usually holding the individual in upright and shaking in vertically and horizontally trigger the penal eversion in active individuals (Solla *et al.,* 2001; Dustman, 2013) but a gentle pressure was applied on the head and hind limbs to accelerate the penal eversion in less active individuals. In 2001, De Solla *et al.* reported that when *Chelydra serpentine* much stressed they started to evert the penis. In 2011, Famelli *et al.* mentioned that when *Hydromedusa maximiliani* are captured and hold in hand they start everting the penis as a defensive display. The reporting of these observations are the basis of the development of above-mentioned sexing method. Later in 2017, McKnight *et al.* described second method of penal everting using the vibrations on body shell and tail.

Morphological measurements

This is the most commonly used method of fresh water turtles sexing in field conditions. Morphometric features like length of claws, tail size and shape, cloaca position, plastron morphology, body size comparison among both sexes and other secondary sexual features (Gibbons and Lovich, 1990; Valenzuela *et al.*, 2004; Casale *et al.*, 2005; Rueda-Almonacid *et al.*, 2007; Readel *et al.*, 2008; Reed and Tucker, 2012). The only difficulty with this method is experience. An inexperienced person will defiantly face difficulty, as he /she do not have the reference features.

Blood assay

This assay includes the detection of various sex specific hormones like estragon, testosterone, androgen and anti-mullerian hormone (AMH) to know the sex of any individuals. AMH is responsible for the development of Mullerian duct in male embryos (de Santa Barbara *et al.*, 1998; Arango *et al.*, 1999). In 2020, Boris Tezak *et al.* studied the AMH on the freshwater turtle neonates - Red-eared slider (*Trachemys scripta*). They initially perform the western blot analysis to detect the presence of AMH in blood samples and later verify by laparoscopic and got 100% result. Similarly, radioimmunoassay analysis done by Xia et.al, 2011 and Gross et.al, 1995 on green and loggerhead respectively to determine the estradiol-17β and testosterone ratios from chorioallantoic/amniotic fluid (CAF) used to identify sex of neonates. This method is very tedious, as it requires extensive handlings of eggs prior to hatchling to obtain sufficient amount of amniotic fluid and prevent contaminations. The other issue with this method is that in radioimmunoassay required blood samples of about >100 uL that cannot be removed from neonates (Owens, 1978; Wibbels, 1999; Wibbels *et al.*, 2000; Mader and Rudlof, 2006; Boris *et al.*, 2020) which makes this method difficult and practically impossible to implement in large scale study.

Species exhibiting TSD

The literature reviewed suggested that total 69 species of freshwater turtle species were studied under the controlled temperature to diagnose the TSD until date. Out of 69 species, 42 have TSD (listed in Table 1) been representing 7 of 12 freshwater turtle family. The Asian freshwater turtle family geoemydids included large species like *Cuora* and *Batagurs* are untested. Ewert and Nelson, 1991 and Pewphong *et al.*, 2020 have already proved that seven species of *geomy.did* already exhibit the TSD.

Genes responsible for the TSD in freshwater turtles

DMRT1, SOX9, AMH and WT-1 are the gene identified for responsible for the expression or induce the development of male reproductive parts (testis) in freshwater turtles during the gonadoenesis (Kettlewell *et al.*, 2000; Spotila *et al.,* 1998), while aromatase is the primarily responsible for the development of ovary (Giannoukos and Callard, 1996). Aromatase gene helps in the production of enzyme aromatase (CYP19 P450) which converts C 19 steroids such as testosterone and androstenedione to estrogen.

Discussion

An overview of TSD in freshwater turtles has been included in this review article allowing accumulating the information on diverse methods of sexing use in freshwater turtles. In this article, each section summarized the overall leaning drawn from the various studies done in the field of TSD. The additional conclusion cum own perspective on these topics are as below. In the beginning, we are have tried to explain the concept of TSD and clear the various terminology associated with the TSD like ESD, GSD, TSP and TSD with proper definition cum explanation. Later explained the various types of patterns explained observed in vertebrates especially in reptiles. In continuation to various patterns of TSD observed, a detailed review of the various methods of sexing in freshwater turtles mentioned. Current research or study use the various types of method for sexing depending upon the size, age and need of the study. The invasive method (laparoscopic and gonad histology) are the most accurate and correct methods while all the non-invasive methods are less accurate. In some cases validity of non-invasive methods (penal inversion and morphological examination of neonates) are dependents on the invasive methods. A complete list of freshwater turtle studied and recorded to exhibiting the TSD was also mentioned. The reviewed data suggested that four big family of freshwater turtle species (*Geoemydidae, Pelomedusidae, Trionychidae* and *Chelidae*) comprising more than 135 species are still untested for the TSD. In the end major gene responsible to induce the development of male or female organs are mentioned. The expression of these genes are directly dependent on the incubation temperature and other stimulus like salinity, moisture etc.. For example at female inducing temperature, activity of aromatase increases and in the end leads to the formation of ovary that's why females are hatched at higher temperature (Pieau and Dorizzi, 2004; Manolakou *et al.*, 2006).

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