

# THESIS

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## **Temperature-dependent sex determination in crocodylians**

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## **Abstract**

The gender of crocodilian eggs is determined by temperature during the incubation period. Temperature-dependent sex determination is a phenomenon occurring amongst all crocodilians and several other reptilian species. Thereinafter, high, and low temperatures produce female hatchlings, while intermediate temperatures engender males. Artificial incubation temperatures in various crocodile and alligator species identified the critical temperature ranges where sex ratios are skewed, the pivotal points at which males and females are produced in a 50:50 ratio and the time frame where gender of the hatchling can be altered.

With respect to the last six decades of research, this thesis will provide an insight into the molecular background of the sex determination mechanism in crocodilians, in special regard to the different patterns and mechanical hypotheses. Further, we will take an approach to post-hatching effects posed by the different incubation temperatures and the possible evolutionary advantages and risks for crocodilians exhibiting TSD.

## Abbreviations

SD	Sex determination
GSD	Genetic sex determination
ESD	Environmental sex determination
TSD	Temperature-dependent sex determination
SRY	Sex determining region Y
PT	Pivotal temperature
TRT	Transitional ranges of temperature
TSP	Thermosensitive period
PGC	Primordial germ cells
E <sub>2</sub>	Estradiol
E <sub>2</sub> -17b	Estradiol 17beta
T	Testosterone
DHT	Dihydrotestosterone
A	Androstenedione
E <sub>1</sub>	Estrone
AT	Aromatase
E <sub>1</sub>	Estrone
AI	Aromatase inhibitor
GAM	Gonado-Adrenal-Mesonephros complex
AMH	Anti müllerian hormone
SF-1	Steroidogenic factor 1
SOX9	Sry related HMG box gene 9
TRP	Transient receptor potential
TRPV4	Transient receptor potential vanilloid type 4
Ca <sup>2+</sup>	Calcium
Dmrt1	Doublesex and mab-3 related transcription factor 1
K-T Event	Cretaceous-tertiary event
IUCN	International Union for Conservation of Nature

## 1. Introduction

“Sex Determination is a synthesis of many molecular events that drives a community of cells towards a coordinated tissue fate (...). Classically considered to be the process by which an organism initiates the development of ovaries or testes from the embryonic gonad” [1].

This is just one of many scientific ways to explain the process of sex determination (SD).

The question of gender is a phenomenon that accompanies mankind constantly. Thus, for us humans, it is an exciting experience during pregnancy, and even in domestic- and wild animals, sex plays an essential role. Whether in breeding or in preservation of endangered species, gender is a factor that cannot be neglected in our nature. Over the millennia, different animal genera and species have adopted processes and mechanisms to further differentiate the early cell stages of an embryo into either male or female. Theories on SD and the mechanism behind it can be traced all the way back to popular philosophers such as Parmenides and Aristotle [2, 3]. Sure enough, their theories on sex being determined by the localization of the embryo in the uterus or from which testicle the sperm originates from, do not give any scientific explanation on how embryonic tissues are arranged into either testes or ovaries. However, it illustrates the importance of the matter and how centuries ago, mankind was already fascinated by the natural phenomenon [2].

The discovery of essential components of the reproductive system, such as Sertoli cells or Graafian follicles, and finally the detection of sex chromosomes by Nettie Stevens, gave a head start to the detailed examination of the SD mechanism in mammals, reptilians, and amphibians [2, 3]. Generally speaking, you can differentiate between two forms of SD occurring in vertebrates: Genetic sex determination (GSD) and environmental sex determination (ESD) [1, 4–6]. These mechanisms are utilized by various animal species.

When looking at reptilians, crocodilians are considered to be very remarkable. Not only because of their long history, ranging back all the way to the dinosaurs, or their unique anatomy and physiology, but also due to their SD mechanism. All species belonging to the class of crocodilians exhibit a special form of ESD, called Temperature-dependent sex determination (TSD). As opposed to GSD, no sex chromosomes determine the gender of the offspring, but certain temperatures during the incubation time of the egg.

This thesis will focus on presenting the most up-to-date data on TSD, ranging from the different patterns to changes during embryonic development, and will further give an overview of the current hypotheses surrounding the mechanism of TSD. The primary focus lies in the mechanism displayed by the crocodylians, referring therefore to exemplary studies on both crocodiles and alligators. However, due to the diversity of the SD mechanism and the broad popularity among reptiles, other species will be mentioned as well and differences between crocodylians and other fellow reptilians will be highlighted. Furthermore, we will take a critical look into the future, reviewing collected data to evaluate how climate warming in the upcoming years will pose an effect on species exhibiting TSD.

## 2. Sex determination mechanisms

GSD might be the most prominent SD mechanism and is exhibited by most mammals, birds, and certain reptilian species [7, 8]. Genotypic gender determination is characterized by the presence of heteromorphic sex chromosomes, which carry the relevant genetic information and are found in the karyotype of the respective species. The chromosomal arrangement can be classified as heterogametic and homogametic. When speaking about mammals, males are represented by the XY heterogamety and females by the homogametic XX. The distribution of sex chromosomes in other species differs compared to those of mammals. For instance, in birds, the alleles are switched, and the female is the heterogametic (ZW), and the male is the homogametic individual (ZZ) [8–10]. Few of the living reptiles have been examined for the presence of such heteromorphic sex chromosomes [4, 8]. Heterogametic sex chromosomes are present in snakes, (ZW female, ZZ male) and in lizards. Genotypic determination of sex seems to be scarce in chelonians [8, 11]. Quite a distinct variation of GSD can be seen in *Drosophila melanogaster* where the sex of progeny is determined via the chromosomal ratio of X chromosomes to that of autosomal [12].

SD itself is a complex mechanism consisting of various pathways and signals and thus cannot be pinpointed to one single factor [1]. Chronologically, SD in GSD begins with the setting of the genotypic sex at the time of fertilization, followed by the gonadal differentiation into either the ovary or testes and finally the shaping of external genitalia [13]. Generally speaking, the chromosomal sex is determined by the presence of either a XX or XY karyotype. Responsible for the determination of either male or female sex is most importantly a transcription factor, located on the short arm of the Y chromosome. The “sex-determining region Y” (SRY gene) is produced by precursor cells, that will develop into Sertoli cells and is responsible for initiating the development of primordial gonads into testes. No such gene has been identified on the X chromosome [1, 13]. Consequently, if the SRY, or mainly the Y chromosome, is absent, no male-inducing pathway can be initiated and instead a female pathway will be favored [1, 12, 13].

Varying greatly from those of mammals and birds, several reptiles and selected fish species evolved a system significantly differing from the genotypic SD [1, 4, 7]. ESD is predominantly based on the influence of extrinsic factors on the organism. Upon those, a sequence of processes is started in the embryonic tissue, which will ultimately determine the



future sex of the embryo. Here, the sex is not determined by the time of fertilization, but during the period of embryonic development [1, 14]. When examining species whose progeny is typically influenced by outer factors by means of laboratory diagnostic methods, it becomes clear that one of the major differences in animals exhibiting ESD is the absence of heteromorphic sex chromosomes [1, 4, 7]. The stimuli vary between species and are not at all universal. Especially applicable to the category of ESD are vertebrates such as reptiles. However, also invertebrates like nematodes and parasites are subject to certain factors determining their sex [15]. Namely, nutrition, temperature, water quality, or stress can serve as an external factor to switch on a number of mechanisms within the embryonic or neonatal body to determine the gender [1, 7, 15]. Among vertebrates, there is a strong tendency towards temperature being the predominant factor [15]. This distinct mechanism is the most prominent among reptilian species and was first discovered by Charnier (1966) in the lizard *Agama agama* [16]. Here, certain threshold temperatures during the incubation of the egg determine the sex of the embryo during development [7]. As of now many turtle species, some lizards, and all crocodylians examined were confirmed to exhibit TSD [4–8, 10, 11]. Note, that only a small portion of all reptilian species have been studied and evaluated so far, therefore the data on the occurrence of TSD is not entirely completed [4].

Studies around TSD have not only focused on the possible molecular background and possible genes involved in the process but also on determining crucial temperature points in development and the patterns expressed in the relevant species. Despite GSD and TSD working on different levels, it is valid to assume that both might be associated with one another. Evidence for that hypothesis lies in the co-existence of both mechanisms in certain species, such as lizards or geckos, where heterogamety, either male or female, and the sensitivity to environmental temperature play a specific part in SD [8, 10, 17, 18]. This suggests, that TSD and GSD might be connected in one way or another, meaning that one of the mechanisms can be viewed as an ancestral state [4, 8, 18, 19]. However, up to now, there is no clear answer as to why some reptilian species have committed to either one of the mechanisms and others have an ongoing transition between both. Bull et al. (1980) argued that a possible way for TSD and GSD to be present together would be if TSD was only put into action if outer environmental conditions changed drastically, and therefore altered the sex ratio towards the gender that could benefit the most from the change. The co-existence of GSD and TSD could in that case alter the heterogamety of the species, meaning XY females and XX males as a consequence of shifts in environmental stimuli. In the aftermath,

heterogametic females could produce offspring with heterogametic males, which were not subject to the shift. Finally, successors would be non-viable. Considering Bull's (1980) theory, a prevalence of GSD and TSD together seems unlikely and not advantageous for any species.

### 3. Temperature-dependent sex determination in crocodilians

SD mechanisms are remarkable in their complexity and strict organization. Crocodilians especially are quite unique by utilizing the scheme of TSD, most importantly regarding their different molecular foundation when compared to mammalian vertebrates. As mentioned in chapter 2 of this thesis, all crocodilian species are confirmed to have the gender of their offspring be determined through the external factor of temperature. Primary factor for this distinctive mechanism to be dominant includes the absence of heteromorphic sex chromosomes in those reptilians [1, 4–8, 10, 11].

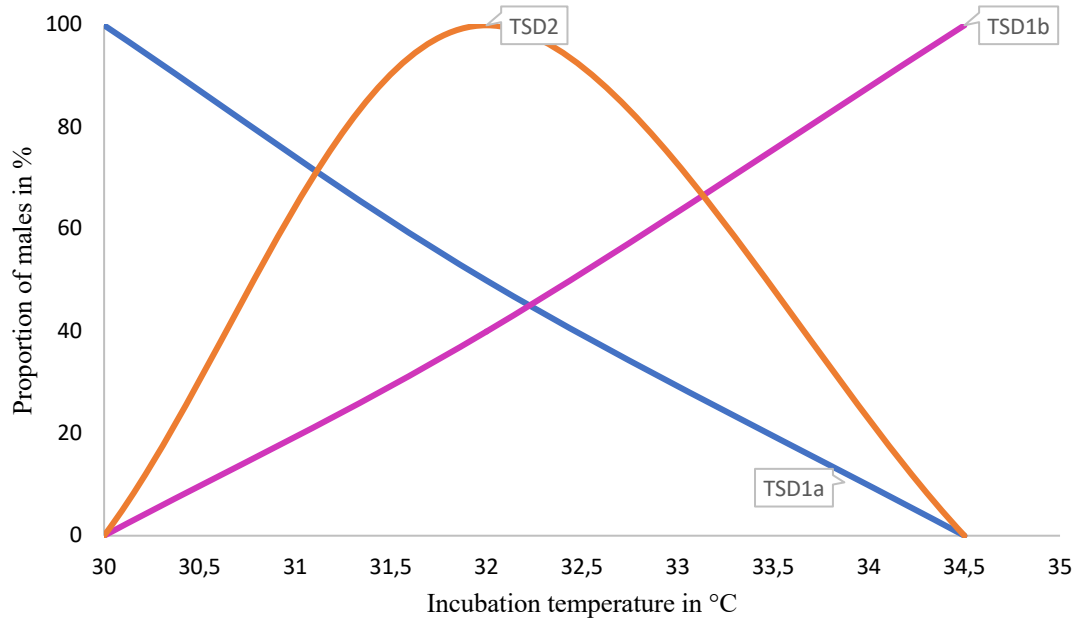
#### 3.1. Genome of crocodilians

The order crocodilians that we know today can phylogenetically be classified into the clade Archosaurs, the “ruling reptiles” [20], a monophyletic group that comprises of extinct and extant individuals. Nowadays, the only living orders belonging to the archosaurs are the crocodilians and birds. Within paleontology, crocodilians are considered to be the closest relatives alive to birds, based on many similarities found for example in anatomy and on molecular levels [10, 21–25]. To put them into a taxonomical categorization, the *Crocodylia* include a total of 27 species [26], further subdivided into the lineages *Alligatoridae*, *Crocodylidae*, and *Gavialoidea* [27, 28], with more subfamilies possible to be categorized [26]. Despite the close relationship of the “largest living reptiles” [27] to birds, their genome and karyotype show some major differences, thus also resulting in different physiologic, genetic, and sex determining variations between species [28, 29]. The total number of chromosomes in crocodilians varies in between the individual species, ranging from 30–42 [28, 29]. As opposed to the karyotype of birds and turtles, the genome of *Crocodylia* is characteristically represented by an absence of dot-shaped microchromosomes and a predomination of some larger-sized chromosomes [8, 28–30]. The presence of heteromorphic sex chromosomes, which are evidently responsible for the determination of the sex in mammals, has been denied in 21 tested species of crocodilians [4, 8, 28–30]. The karyotype of crocodiles appears to be highly conserved throughout evolution, with main changes occurring through fission- and fusion events, proposing that SD in crocodilians has been stable throughout evolution [14, 28–31].

### **3.2. Patterns of determination**

The basic rationale for TSD is that different incubation temperatures promote either male or female development. These temperatures can be classified according to specific patterns. Thus, TSD in crocodylians can be categorized and the relevant degrees during incubation can be determined. So far three patterns have been described [4, 8, 19, 31–34]. The patterns themselves have been universally defined throughout the research in TSD on reptiles, however, varying naming has been used by different experts. For simplicity reasons in the following parts of the thesis, we will refer to the patterns by TSD1a, TSD1b, and TSD2, which are also used by the authors González (2019) and Valenzuela and Lance (2004).

But what exactly do these patterns describe? They are defined by the different temperature influences during the incubation period of the embryo. Thus, as demonstrated in figure 1, low temperatures can form males and higher temperatures females, or vice versa. TSD1a suggests that male embryos emerge at cooler temperatures, and female embryos emerge at warmer temperatures. While this pattern is widespread in turtles, this sequence of temperatures has not yet been detected in crocodylians [23, 35]. On the contrary, TSD1b is the inverted image of TSD1a - Cooler temperatures produce females and warmer males. According to current knowledge, a third pronounced pattern, TSD2, occurs in all susceptible animals and is considered to be the most prominent one within crocodiles and alligators [23, 33, 35]. The authors Ewert et al. (1994) described the scheme as the “basal or ancestral” origin for TSD and further suggested that it serves as a template for the first two systems [4]. In this instance, female embryos are formed in high and low temperatures, with males occurring within. It is also of consideration that intersexes may develop in the intermediate temperature zone [11], however, this has not yet been proven in crocodylians [4, 8, 11, 22, 31–35]. As mentioned in Chapter 2, various species within the reptile taxa express TSD. Nevertheless, there are still major differences occurring between those species. We can see this in turtles, a species in which TSD is also widespread, where it is common to find the patterns TSD1a and TSD2, however, the TSD1b scheme was not yet detected in this order [23].



**Figure 1:** Schematic representation of TSD patterns by demonstrating the proportion of males (%) to the incubation temperature ( °C). The three colored lines represent the different patterns exhibited by TSD species. The blue line represents the TSD1a pattern, meaning that males have a higher proportion at lower temperatures, while the increase of females within a clutch with higher temperatures consequentially brings a low male proportion. TSD1b is represented by the pink graph, where females are produced at low temperatures and males increasingly at higher temperatures. The third pattern (TSD2), demonstrated by the orange graph, shows that males are only produced at higher proportions in intermediate temperatures, while their proportion is significantly less at low and high temperatures.

Source: Pieau (1996)

As a way of determining the significant temperatures and their effect on the embryo's gonads, several laboratory experiments have been carried out on different species. In the American alligator, *Alligator mississippiensis*, temperatures below 30 to 31 °C produce females exclusively. Intermediate climatic conditions, on the contrary, engender both male and female embryos, with, in the case of *Alligator mississippiensis*, a strong bias towards females [32, 34]. Higher temperatures above 33°C create favorable conditions for male development, with 100% phenotypic males occurring at 33 °C [31, 32, 34, 36, 37]. Female progeny hatching after an incubation temperature above 34°C presents with larger oviducts and a possible slower post-hatching growth rate [23, 31, 33]. One may argue about the expressed pattern within alligators, as researchers have provided opposing arguments and field data. While data obtained by Ferguson (1983), Deeming and Ferguson (1988, 1989),

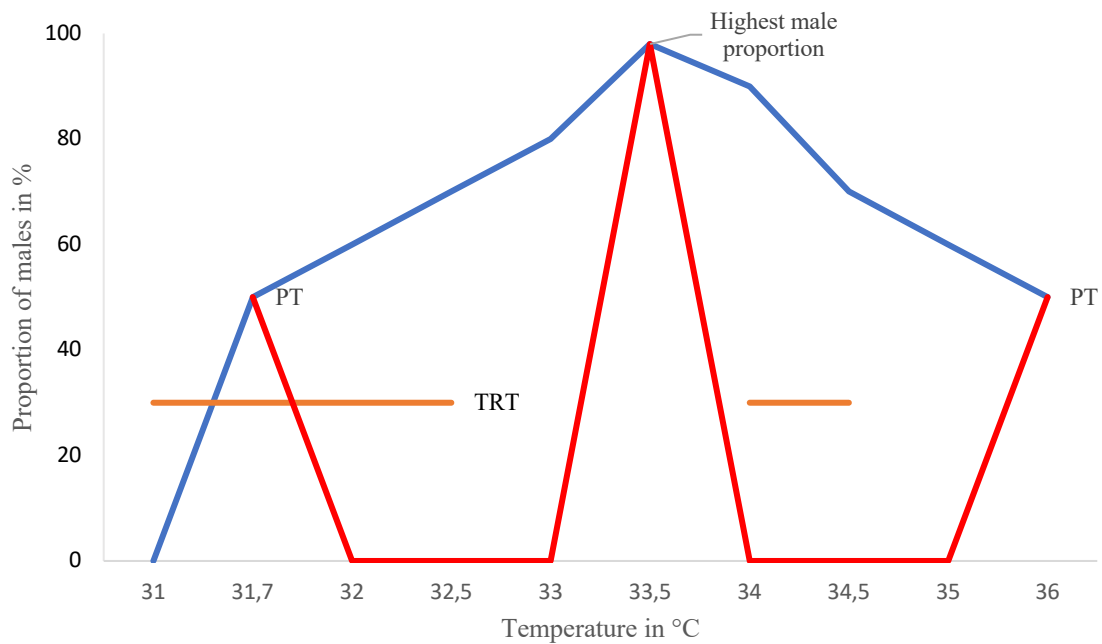
and Joanen (1982) indicate that alligators can be classified into the TSD1b pattern, meaning females produced at low temperatures and males at higher temperatures, newer published studies by Gonzalez (2019), Ewert (1994), and Lang and Andrews (1994) suggest the categorization for a TSD2 pattern. This can further be supported by incubation experiments that produced mixed sex ratios at 34 °C, despite the previous assumption of 34 °C being a 100% male-producing temperature [31, 32]. In the light of assumptions, that type 1b of TSD classification might be derived from pattern 2, as females commonly are determined in high temperatures, it can be implied that species expressing such pattern can ultimately still be categorized into the TSD2 scheme [4, 35].

TSD patterns of the Nile crocodile, *Crocodylus niloticus*, fall within the same ranges as *Alligator mississippiensis*. Furthermore, the sex ratio in this species weighs more in the direction of female individuals [23, 38]. Additionally, in the Estuarine crocodile, *Crocodylus porosus*, climatic conditions below the 30 °C mark and above 34 °C exclusively promote the production of female embryos [23, 39]. Varying results can be observed in different reptilian species. Alternating to crocodylians, turtles commonly express TSD1a. Here, males are produced at low temperatures, and females at high. In the European pond turtle, *Emys orbicularis*, males can be determined at 25 °C, while 100% of females are produced at 30 °C [40]. Another key thing to remember when talking about the patterns of determination are the transitional ranges between the female- and male-producing temperatures. These intervals can be defined as the transitional ranges of temperatures (TRT). Within those ranges, either female- or male embryos can develop, leading to mixed sex ratios [11, 23, 41]. Just by the different patterns of TSD, there can either be one or two TRTs. In each, TSD1a and TSD1b, one TRT is found as there is a single interval between the transition from male to female and vice versa. Regarding TSD2, two TRTs are determinable, one between the low-temperature female to an intermediate male and another between the intermediate male and high-temperature female [11, 23, 33, 35].

Concerning the width of the temperature transitions there are variations between the species. For instance, sometimes there are intervals of almost 2 °C (e.g., Mugger crocodile, *Crocodylus palustris*) in which both males and females can emerge. Nevertheless, the TRTs can be as narrow as 0.3 to 0.9 °C, as demonstrated in *Alligator mississippiensis* (Figure 2) and the Morelet's crocodile, *Crocodylus moreletii* [10, 31, 33]. Temperatures below and above TRTs may also be referred to as "feminizing" and "masculinizing" temperatures [11,

41]. In the case of sea turtles, all temperatures below the TRT are "masculinizing" and above "feminizing" [11]. Compared with crocodylians this may not be adaptable, as most species express the TSD2 scheme, with low-range temperatures commonly producing females [11].

Another important parameter in incubation experiments occurring at constant temperatures is the pivotal temperature (PT), also commonly referred to as the threshold temperature [41]. PT describes a temperature, that can produce a 1:1 sex ratio within a clutch, provided the incubation occurred at a constant temperature [11, 23, 35, 41]. While in genotypic SD species, a 1:1 ratio of males to females is applicable and favorable, it is not a realistic state in crocodylians [37]. Not only in laboratory conditions, but also in natural nests, sex ratios in crocodiles and alligators are skewed and prominently biased towards a female-dominated ratio [23, 31–34, 36–38, 42, 43]. Likewise to TRT, the number of PTs is linear with the patterns. Thus, one PT can be detected in each of TSD1a- and b, and two in TSD2 [11, 23, 35]. As seen in figure 2, two PTs are detectable in the case of *Alligator mississippiensis*, one at 31,7 °C, the other at 35,9 °C [33]. Variations in PTs are quite common amongst species and can be subject to various factors. As PTs do not apply to an individual egg and is regarded in the context of clutches, clutch effects can cause differences in PTs of the same species. Such alterations can be quite substantial, especially in free-roaming animals and their progeny, as possible shifts could drastically change the sex ratio of a clutch [41].



**Figure 2:** Diagram of the relation of Pivotal temperatures (PT) and transitional ranges of temperatures (TRT) to a temperature curve in *Alligator Mississippiensis*. The blue curve represents the pattern TSD2: Males have a low proportion at decreased and very high temperatures, while in the intermediate temperatures higher male ratios can be observed. The red line describes the development of the PTs of *Alligator mississippiensis*. 50% of male proportion were observed at 31.7 °C and 35.9 °C, while at 33.4 °C the highest male ratio was found. The orange lines represent the TRTs. Between 31.2 °C and 32.6 °C there is no clear tendency towards one sex, rather both have a chance of emerging. Similar ranges were observed at 34.3 °C, however, no upper limit was detectable.

Source: González (2019)

Laboratory experiments as such, coupled with a constant and favorable climatic environment and no external factors intervening with embryonic development, provide ideal conditions to determine the above-mentioned intervals. In the wild, this occurs to be more complex. In open nature, climatic weather conditions are less stable and usually fluctuate not only in celsius degrees but also in weather conditions, such as rain, humidity, and sunlight exposure. As opposed to an artificially created incubation, free nests are more exposed during their approximately 70 to 100 days of incubation [22, 32]. Depending on the environmental conditions, nest temperatures can fluctuate as much as 4 °C to 10 °C during the day [38, 43].

Alternating conditions during incubation may raise the question of whether TSD patterns behave differently in naturally occurring nests. Joanen et al. (1982) performed studies on

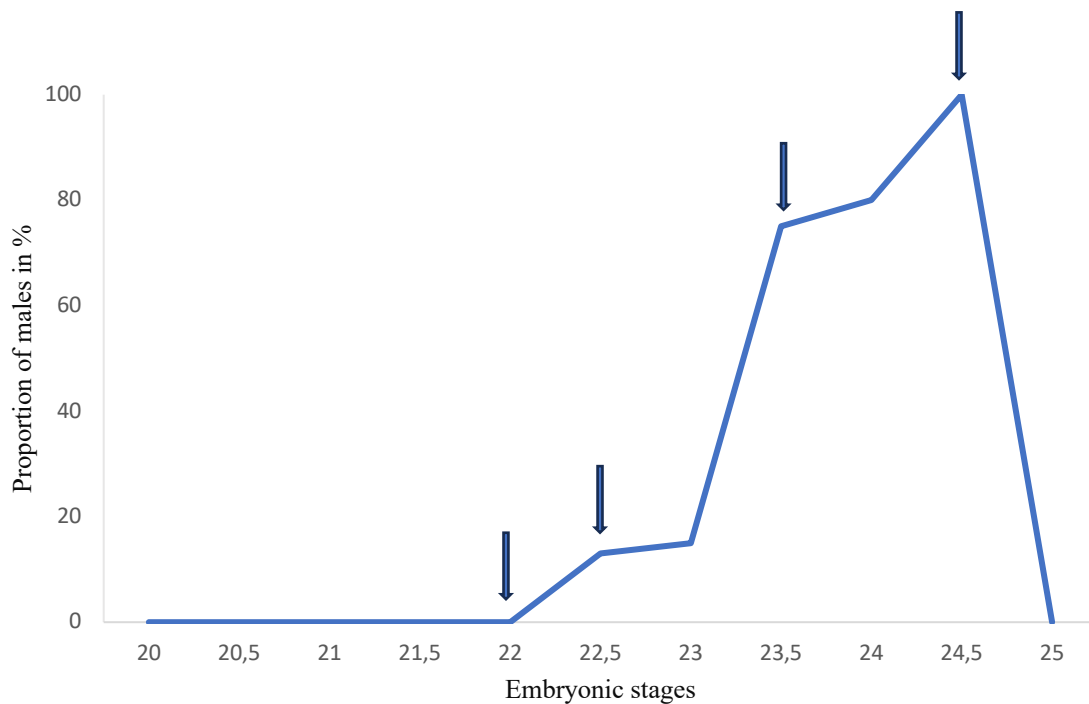


free clutches of *Alligator mississippiensis* to find answers for that obstacle and gave significant insight into TSD in wild roaming animals. Among the cooler degrees, between 29 °C and 31 °C, more female offspring hatched. Their eggs were mainly located in the periphery or the lower part of the clutch. Warmer temperatures around 34 °C produced mainly male individuals, which were located at the top of the nest [32, 37]. Not only was evidence found in *Alligator mississippiensis* for TSD in natural nests, but also in crocodiles, such as *Crocodylus porosus* [39] and *Crocodylus niloticus* [38]. Hot and cold temperatures ultimately produced females, while intermediate temperatures between 31 °C to 32 °C predominantly favored male embryos [39]. This data suggests, that TSD consequently is naturally occurring and not just inducible by constant incubation temperatures provided by artificial experimental conditions.

### **3.3. Thermosensitive period**

Gender determination in crocodylians does not occur at the time of fertilization but is set during a specific time of incubation, and is an irreversible state with no possibility of sex reversal [32, 37]. Such interval is referred to as the thermosensitive period (TSP) [32, 34, 41]. It is defined as that timeframe during embryonic development where gonadal differentiation begins and where variations in incubation temperature strike an effect on the sex ratio [11, 31, 41, 44].

The timing of the TSP is determined via the induction of constant incubation temperatures at a one-sex producing temperature and switching to the opposite-sex-producing temperature after a certain amount of time. Single-shift temperature switches are permanent changes and eggs will be incubated at the second temperature until hatching [4, 8, 11, 32, 41]. To exemplify, to set the TSP in *Alligator mississippiensis*, eggs incubated at 30 °C, a female-producing temperature, are transitioned to a male-producing temperature, 34 °C, for the rest of the artificial incubation [32] (Figure 3). This procedure can be applied vice versa as well. For the results to be representative, eggs are separated into groups and switched to the different temperatures at alternating timestamps during their development [8, 32]. Besides the single-switches, “shift-twice” experiments are a common alternative [23, 31]. In other words, eggs are initially switched to the opposite-sex-inducing temperature and then eventually transitioned back to the starting point, to induce a reversed sex [23, 31].



**Figure 3:** Diagram of the effect of single-switch experiments from 33 °C to 31 °C during the TSP in *Alligator mississippiensis* eggs. Switches at different embryonic stages lead to different male proportions within the clutch. The later the switch occurs, the higher the proportion of males. The arrows demonstrate the timestamps during embryonic stages where switches from 33 °C to 31 °C were initiated.

Source: Lang and Andrews (1994)

Such experiments coupled with pulsating temperatures in the background of a sex-producing temperature have given contradictory results [31, 36, 41]. In Deeming and Ferguson (1989) the effects of pulses initiated for 7 days on the background were neglected as minor and not effective enough to ultimately alter the sex ratios in *Alligator mississippiensis*. Despite that, the follow-up study on TSP in *Alligator mississippiensis* carried out by Lang and Andrews (1994) concluded, that for the pulses to have a lasting effect on sex reversal, a cumulative effect of temperature and a significant magnitude were needed. Moreover, it was figured that the magnitude stands in correlation with the percentage of males produced during the TSP. For instance, a shift to 33 °C on day 15 and ultimately a shift back to 31 °C, consequently had a male proportion of 55% of embryos in *Alligator mississippiensis*. On the contrary, shifts from 31 °C to 34 °C (with a final shift back to 31 °C) produced 100% males on the same day [4, 31]. Nonetheless, shifting experiments cannot always contribute accurate results, as incubation periods and threshold temperatures between species vary. Namely, in *Alligator mississippiensis* temperatures at 33 °C can produce a 100% male ratio, and low

temperatures almost exclusively females [31, 32]. By contrast, crocodile species such as *Crocodylus palustris*, *Crocodylus porosus*, and the freshwater crocodile, *Crocodylus johnstoni*, do not show temperatures in their TSD pattern that elicit a ratio that only engenders males, suggesting a limitation within the determination of TSP in crocodiles [31]. Results therefore are hard to be considered as conclusive, as high temperatures may induce females and males [31, 41]. Note, that according to Lang and Andrews (1994) across crocodiles and alligators the temperature sensitive periods still present as quite similar, which subsequently was proven in further studies on species like *Crocodylus palustris*, *Crocodylus. johnstoni*, *Crocodylus porosus*, and *Crocodylus. niloticus*. [31, 39, 42].

Reptilian embryos are commonly staged based on their morphology. Ferguson (1985) concluded a staging system applicable to crocodilians, categorizing the embryonic development into stages 1 to 28. Each stage has characteristic morphological changes. Based on that information, the determination of TSP in its coherence to gonadal differentiation can be applied [31, 45]. For both, *Alligator mississippiensis* and the spectacled caiman, *Caiman crocodilus*, TSP was defined for the embryonic stages of 21 to 24, between days 30 and 45 during the middle third of embryonic development [31]. This stands opposed to the results presented by Joanen (1982) and Ferguson (1983) who determined the TSP in the American Alligator to be rather earlier, between days 20 to 35 days of incubation. A common finding, however, is that TSP in crocodilians has a rough span of approximately 15 days [31, 32, 36, 37]. Altering results between the two studies might be subject to different temperatures used in the switching experiments and additionally altering time frames. Temperature shifts prior to or later than the defined period are deemed to be ineffective regarding sex ratios. Mostly affected by temperature shifts are sex ratios between stages 21 and 23 [8, 31, 32]. However, alterations in the beginning of the TSP appear to have the biggest effect on either inducing maleness or femaleness [4, 8, 31, 41]. Incubation periods have varying lengths, notably controlled by the incubation temperature. Higher temperatures cause the length of incubation to decline, while lower temperatures extend the period [23, 31, 38, 39]. Accordingly, the average incubation period of crocodilians varies between 70 to 80 days [32, 42].

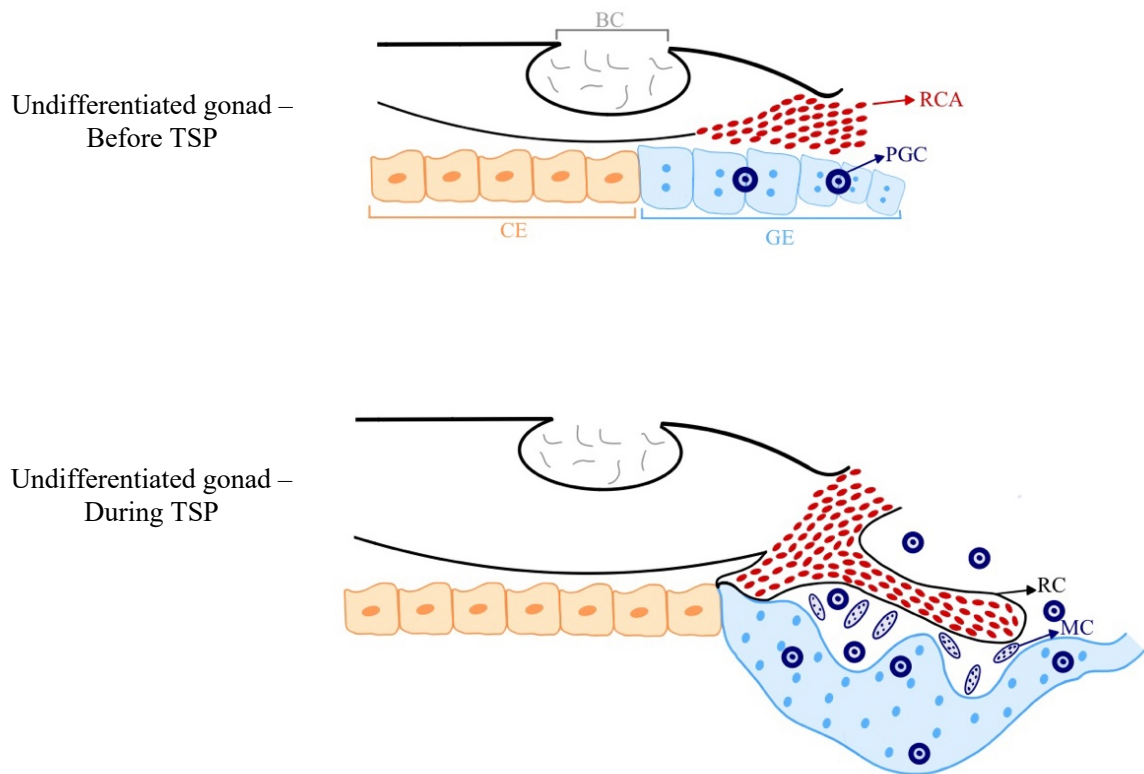
Generally, it has been established that TSP in crocodilians has its effect during the first 18% to 30% of embryonic development. In contrast, in turtles, it is usually limited to the middle third [8, 11, 18, 19, 31, 41]. Some opposing results occurred in *Crocodylus porosus* where it seems to be within the first half of development [39].

### 3.4. Gonadal differentiation

As mentioned above, TSP is the timeframe in embryonic development where the undifferentiated, bipotential gonads take a certain pathway into becoming either testes or ovaries, approximately between stages 21 to 24 [31, 46]. Unlike in species expressing SD through GSD, the sex will ultimately be set during that time and not the point of fertilization [10, 47]. Post TSP the sex of the embryo is not possible to be reversed [31, 36]. The differentiation of gonads arises initially from the coelomic epithelium, or surface epithelium, which provides the basis for the “genital ridges”, by thickening of the epithelium [10, 11, 46] (Figure 4). Moreover, it forms the epithelial compartment within the gonad, holding the epithelial and primordial germ cells (PGC). Those PGCs are primarily observable in the germinal epithelium and represent undifferentiated stem cells which can further develop into either oocytes or spermatozoa after traveling to the genital ridges [10, 48]. While the genital ridges undergo development, epithelial cells from various structures proliferate. The coelomic- and germinal epithelium give rise to such epithelial cells, which align to form the inner part of the gonad – the medulla. Epithelial cells arising from the coelomic epithelium further take part in the formation of the anlagen for the rete cords. These cords are a primary connection between the embryonic mesonephros and the developing gonad [11]. Another key component in gonadal differentiation are the sex cords, or medullary cords [11, 36, 46]. These are formed by the proliferation of cells from the germinal epithelium and commonly occur during the early stages of TSP. The presence of sex cords is indifferent to the gender of the embryo [11].

When it comes to the effect of temperature on the primordial gonads, there appears to be a slight difference in female- or male-producing temperatures. While in lower female-producing temperatures, and high as in the case of *Alligator mississippiensis*, the medullary cords appear to be thinner and less infiltrated by cells, the higher, male-initiating temperatures cause the cords to become thicker [11, 36]. It is, however, not entirely possible at this state to determine whether the embryo will ultimately become male or female. One of the few indicators of a certain sex might be the pre-Sertoli cells, which were demonstrated mainly in *Alligator mississippiensis*. A higher amount seems to be a pre-condition for testes to develop [11, 31]. Whether a gonad develops into an ovary or testes can initially be established by the state of the germinal epithelium. In the case of a male pathway, the epithelial compartment will show a flattened morphology and furthermore, germ cells will

regress. In contrast, ovarian differentiation is highlighted by a thickened epithelium and proliferation of not only epithelial but also germ cells. Additionally, the formation of an ovarian cortex is highly characteristic, with a distinct separation from the medulla by a basement membrane [11, 46]. Towards the end of TSP, most of the cells in a differentiated ovary will enter meiosis, while cells in determined testes differentiate distinctly as Sertoli cells by stage 23 [11, 31].



**Figure 4:** Comparative and schematic drawing of the development of the bipotential gonad before and during the TSP. During the undifferentiated state, the gonad is comprised of the coelomic- and germinal epithelium, and additionally the anlagen for the rete cord. Within the germinal epithelium, germ cells are present. These structures form the genital ridge, which is a predisposing component for testis or ovary differentiation. With the onset of the TSP, the cells within the rete cord start organizing. Germ cells will further proliferate, and the formation of medullary cords takes place.

BC: Bowman capsule; RCA: Rete cord anlagen; CE: Coelomic epithelium; GE: Germinal epithelium; PGC: Primordial germ cells; MC: Medullary cords

Source: Pieau (2001)

### 3.5. Mechanisms of temperature-dependent sex determination

TSD is a very complex system that is composed of many factors, eventually leading to the differentiation of an undifferentiated gonad into either testes or ovaries. While in GSD there are obvious components that we can pinpoint to SD, in TSD these remain elusive. The difficulty lies in finding a factor within the embryo that is sensitive to outer temperatures and reacts differently to either high or low climatic conditions. Stimulation of such factor would then eventually lead to a male- or female outcome, by either suppressing or activating a certain cascade of molecular events.

#### 3.5.1. Steroid hormones and aromatase

##### 3.5.1.1 Steroid hormones

Popular hypotheses regarding the molecular mechanism behind TSD include the involvement of steroid hormones, more specifically estradiol ( $E_2$ ), and testosterone (T). Even though numerous evidence has been gathered in research that confirm that steroid hormones are involved in gonadal differentiation, their specific role has not entirely been put into perspective yet [49, 50]. As an initial theory, Janzen and Paukstis (1991) proposed that their association might lie in the ratio of  $E_2$  to T, ultimately deciding the fate of the gonad to become either an ovary or testis. In light of that, application of exogenous steroids, such as estrogen (E) and T, was effective in altering the ovarian differentiation at female-producing temperatures or overriding the male-producing temperatures in the *Alligator mississippiensis* [51–53] and *Crocodylus porosus* [4, 54, 55]. Similar results were observed in turtles in experiments carried out by Wibbels (1991). The red-eared slider turtle, *Trachemys scripta*, expresses a TSD1a pattern, meaning that male embryos are produced at low temperatures, and females at high [56]. Injection of Estradiol  $17\beta$  (E- $17\beta$ ) at 28 °C causes the embryo to develop into a female, even though incubation temperatures are typical inducing testis differentiation [56]. In correlation, by applying E- $17\beta$  at temperatures that commonly produce a higher female ratio, less amount of the steroid hormone was needed to induce female differentiation. This goes to show, that also in turtles there is a correlation between  $E_2$  and the incubation temperatures [56].

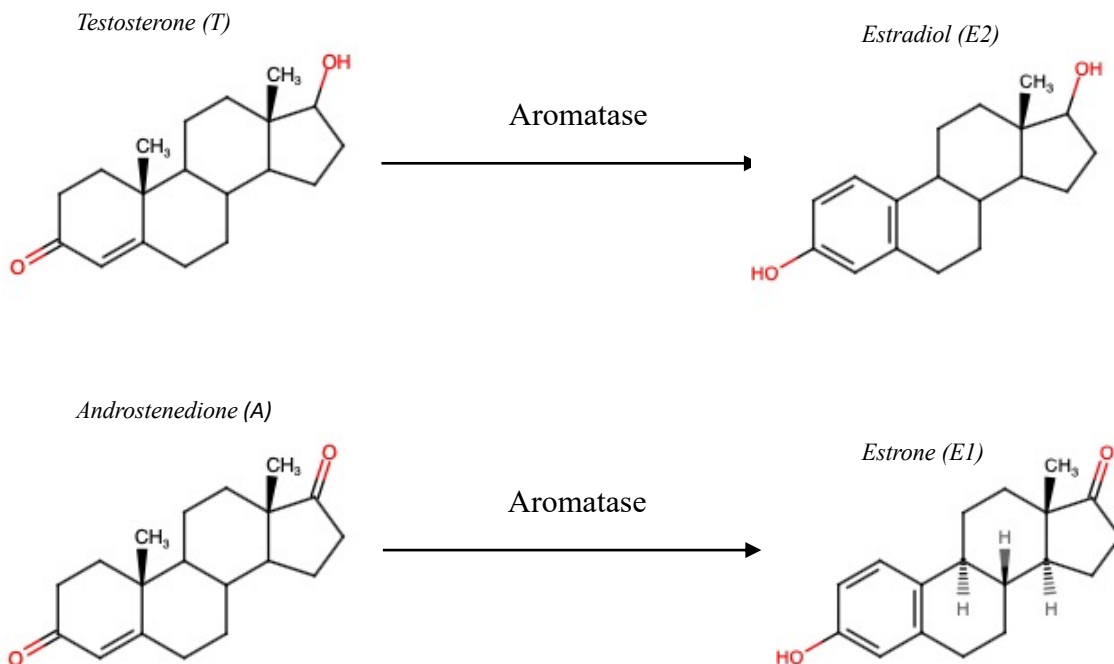
In addition to the results of Wibbels (1991), steroid hormone experiments carried out on the common snapping turtle, *Chelydra serpentina*, demonstrated, that injections of  $E_2$  and E agonists at their male-promoting temperatures initiated the differentiation of gonads into

ovaries. Interestingly enough, application of T and Dihydrotestosterone (DHT), a nonaromatizable steroid [11], at female incubation temperatures gave mixed and inconclusive results, in comparison to the E<sub>2</sub> injection at male-promoting temperatures. Masculinization through injection of DHT is therefore questionable [18, 52, 57]. Injection of E antagonists, such as Tamoxifen, gave contradicting results by occasionally acting as an E agonist. Such effects were observed in alligator embryos, where paradoxically the agent did not interfere with the ovarian development. When looking at turtles, treatment of *Emys orbicularis* with Tamoxifen at male-producing temperatures posed an agonistic effect and caused the development of ovotestes. Application of E<sub>2</sub> and Tamoxifen at the same time promoted the same outcome. In this instance, however, the agent acted as an antagonist. At female-producing temperatures, Tamoxifen was contrarily responsible for initiating sex reversal and the regression of the ovarian cortex [40, 58, 59]. Those findings in crocodylians and turtles implicate the significant role steroids play in the determination of the female pathway during the sensitive period of TSD.

Endogenously, steroids are provided by the egg yolk during embryonic development. In *Alligator mississippiensis*, E<sub>2</sub>, T, and Androstenedione (A), which can be converted to either E or T, are present even before the TSP begins, with A having the highest concentration. Higher concentrations of A in the beginning of the TSP are coherent with its conversion later on during the gonadal differentiation. Between the stages 21 to 23 the overall number of steroids declines, independently from the incubation temperature [50, 60].

### 3.5.1.2 Aromatase

The ratio of sex steroids is associated with the activity of the CYP450 enzyme Aromatase (AT) which is encoded by the CYP19 gene. The primary role of AT is the conversion of T to E<sub>2</sub> and A to estrone (E<sub>1</sub>) [11] (Figure 5). It is assumed that activity and/or synthesis of AT is correlated with the incubation temperature during the TSP. As a simple way of explaining the role of AT in SD: If the ratio of estrogenic to androgenic steroids is high, it determines the fate of the female pathway and vice versa [4, 46, 61, 62]. Injections of AT-inhibitors (AI), e.g., Fadrozole, in eggs of *Emys orbicularis* achieved the inhibition of ovarian development at female-producing temperatures. Furthermore, successful masculinization, characterized by a thickened germinal epithelium rich in Sertoli cells [47], was demonstrated in *Crocodylus porosus* and *Alligator mississippiensis* by using the AI 4-Hydroxyandrostenedione (4-OHA), by markedly reducing the activity of AT [52, 55].



**Figure 5:** Demonstration of the conversion of steroid hormones through the action of AT. T is converted to E<sub>2</sub> and A to E<sub>1</sub>.



Concentrations of AT are commonly measured within the Gonado-Adrenal-Mesonephros (GAM) complex and in brain tissues of reptilians [63]. *Alligator mississippiensis* embryos at stages in the beginning and during TSP only show a low activity of AT in their gonads, independently of the incubation temperature. Significant increases are primarily recognizable towards the very end of the TSP, and most typically at stage 24, post-TSP [58, 63, 64]. Similar results are observed in *Crocodylus porosus*, where AT reaches its peak concentration at stage 23 when the TSP has come to an end [54]. Regarding brain tissues, during the TSP concentration of AT is higher compared to that in the undifferentiated gonad, however, there is no notable difference between male- and female-producing temperatures. Based on that data, it is doubtful whether AT activity in the brain is a pivotal factor in the mechanism of TSD [63–65].

Gonadal concentrations of AT during the TSP indicate that the enzyme might not ultimately be the determining factor for a male- or female pathway. AT increases only after TSP at female-promoting temperatures in *Alligator mississippiensis* [63, 64]. Opposite patterns have been identified in turtles. In *Emys orbicularis*, AT also had a low concentration at the beginning of their TSP, regardless of the temperature. However, in female-producing temperatures AT increased till the end of TSP. Like in crocodilians, the peak of AT is detectable after the TSP, but it is interesting to see that there seem to be different exhibiting patterns regarding the enzyme activity [66].

Taking all of this into account, it is questionable whether AT is the direct regulating factor of TSD. The scheme of AT activity during TSP proposes that temperature doesn't affect the activity of the enzyme, but rather the synthesis by acting on higher levels within the SD cascade [58]. A more logical conclusion is that increasing levels of AT are not a reason for gonadal differentiation, but rather a consequence of [58]. However, the upstream event leading to the increase is subject to discussion still. Joanen (1988) proposed in their study on TSP that for sex to be determined there should be a male-determining factor that reacts sensitively to the external temperature and consequentially initiates a cascade that will lead to either the activation or inhibition of the male-inducing-factor, resulting in female development. Since all crocodilians express the TSD2 pattern, the factor should therefore be active in intermediate temperatures and pose an inhibitory effect during high and low climatic conditions [64]. The fact that in crocodilians AT is present during embryonic

development, but only rises after gonads have developed into ovaries seems to support that hypothesis [58, 64].

AT is inhibited by the anti müllerian hormone (AMH), or anti müllerian inhibiting substance, which is exclusively produced by Sertoli and Leydig cells in the testis. The primary function of AMH is the regression of the Müllerian ducts, an essential part of the masculinization during gonadal development. Without the effect of AMH, the ducts develop into necessary parts of the female genital tract [13, 59, 67–69]. Aligning with the pattern presented in AT, AMH peaks in its concentration after TSP and seems to be undetectable at stages of a bipotential gonad. First detectable values occur from stage 22 onwards [69, 70]. Considering that no AMH is detectable while the gonad is still in its undifferentiated bipotential state before TSP, it is undeniable that it is specific to the male sex-differentiation process in the gonads. Nonetheless, regardless of the effects exhibited by the hormone, it is possibly not the initiating factor for male determination. Western et al. (1999) examined the correlation of testis differentiation to the activity of AMH, with special regard to the gonadal development in *Alligator mississippiensis* embryos. They detected that, even though AMH was active during the early stages of TSP, gonadal tissue arrangement towards a male pathway was initiated earlier, during stage 21. It is therefore possible to assume, just as with AT, that AMH itself is not in the front seat of male determination but is rather the consequence of a previously occurring event. Additionally, Western et al. (1999) failed to detect AMH at female-promoting temperatures, neither at high nor low. This suggests that the occurrence of AMH is fully limited to a testis-determining gonad and entirely relies on the presence of premature- and mature Sertoli cells. Concerning the timing and concentrations of AMH there is a strong suggestion that it in fact can override the effect of AT in the gonad, which is not only limited to reptilians. Similar results were obtained in ovine embryos injected with bovine AMH. The activity of AT in the gonad was fully inhibited [71]. Apart from AMH, a possible regulatory origin on AT lies in genes involved in SD, which either suppress or activate the responsible gene of the enzyme [11].

### 3.5.1. Genes involved in temperature-dependent sex determination

AT and steroid hormones play a significant role, as previous experiments have shown. However, they can't be accounted for the pivotal factors that determine whether a gonad develops into a testis or an ovary. AT only reaches peak concentrations after the TSP has ended, meaning the gonad has already committed to the female pathway. This indicates that upstream events must be responsible and thermosensitive. Therefore, regulatory genes might be in the background and therefore contribute to the determination of sex in crocodylians [64]. In mammals, several genes are involved in the process of SD. The most important factors include SRY, the steroidogenic factor-1 (SF-1), and Sry related HMG box gene 9 (SOX9). Through cloning and isolation, it has been established that in embryonic alligators, several analogs to those mammalian SD genes can be found. SRY, or as determined in mice as Sry, is the testes determining factor in the mammalian genome, which is located on the Y-chromosome, but no homolog was found in crocodylians [1, 11, 13].

#### 3.5.1.1. SOX9

In mammalian vertebrates, SRY is considered to be the driving and crucial determining gene for male SD [1, 12, 13, 72]. Crocodylians, however, do not seem to express any homolog to SRY [11, 69]. Instead of SRY, another member within the family, more specifically belonging to the HMG box gene, was successfully isolated and cloned in reptiles, including crocodiles and alligators. SOX9 is widespread between mammals and other species of vertebrates and has primary functions in the male-sex determining pathway. Furthermore, in adult animals, SOX9 is quite important regarding the maintenance of normal reproductive function [73]. Due to its highly conserved nature, the organization of alligator SOX9 and that of *Crocodylus palustris* shows very similar patterns to SOX9 of birds and humans, and therefore comparable purposes can be demonstrated [69, 72, 74].

In mammalian vertebrates, SOX9 is upregulated by SRY and further influenced by SF-1, a testis-differentiation specific gene. Further down the SD cascade, the box gene acts as an upstream regulator of AMH, by positively affecting the expression through Sertoli cells in the gonad. SF-1 and SOX9 can be viewed as co-workers in the upregulation of AMH [11, 68, 75, 76]. Male sex determination and testis differentiation are highly dependent on Sertoli cells which usually are present first in an immature state and then further differentiate into mature and full functioning Sertoli cells [11, 31, 69]. Just like AMH, SOX9 expression is

Sertoli cell specific, and was detectable in immature pre-Sertoli cells and the differentiated mature ones. The specific location of expression travels from the cytoplasm into the nucleus of the cells [68, 73, 75].

Despite the gene being highly conserved, it is noticeable that the timing of expression is not similar between mammals and non-mammalian vertebrates. Gonads of mice, for instance, produce a detectable amount of Sox9 shortly before gonadal differentiation. With further organization into testicular structures, the expression increases significantly and is absolutely limited to differentiated Sertoli cells. In neither of the species is any evidence given that suggests, that Sox9 is also expressed in gonads that are committed to the female pathway, confirming once more the male-specificity of the gene [70, 75]. *Trachemys scripta* shows similar patterns regarding the SOX9 expression. It is upregulated when incubated at male-producing temperatures, reaches its peak when differentiated Sertoli cells organize into testicular structures, and moreover initiates the expression of AMH towards the end of the TSP [70].

According to the presented results, SOX9 is an upregulating factor of AMH and due to the conserved nature of the gene, it is logical to assume that SOX9 behaves similarly in crocodylians [77]. In the light of this hypothesis, Western and Graves (1999) mapped the activity of SOX9 and AMH through the timeframe of the TSP, from stage 21 through stage 24, in embryos of *Alligator mississippiensis*, both at male- and female-producing temperatures [11, 31, 35, 69]. Strikingly, opposite results were observed, compared to mice or *Trachemys scripta*. Right before the onset of TSP, the gonad is undifferentiated and has the potential to commit to either the male- or female differentiation pathway. At those stages of embryonic development, mice and chicken gonads already expressed basal amounts of SOX9. No such observation was made in *Alligator mississippiensis* [75, 77]. Instead, it became apparent, that AMH was primarily expressed by pre-Sertoli cells at stage 22, as opposed to SOX9, which was first detectable at stage 23. Neither of them was identifiable at high- or low female-producing temperatures. Both genes peaked in their expression, at stages right after the TSP came to an end and were primarily observed in fully differentiated Sertoli cells which were finely organized into testis cords [77]. SOX9 being a testis-specific factor is further highlighted by the application of exogenous E<sub>2</sub> into eggs incubated at male-promoting temperatures. In response to the steroid hormone injection, SOX9 and AMH are significantly downregulated, while ovarian stimulants like AT are noticeably increased [78].

Overall, SOX9 in *Alligator mississippiensis* does not seem to have any upregulating function on AMH, therefore doesn't passively initiate the regression of Müllerian ducts and consequently is possibly not the crucial factor in male determination. If anything, SOX9 might be viewed as a differentiating factor, but not a determining one, in regards to TSD [69, 74, 77].

### 3.5.1.2. SF-1

Despite the evident involvement of AMH and SOX9 in the male- determining- and AT in the female-determining pathway, it is unlikely for them to be the crucial thermosensitive factors regarding SD. Each of those components seems to be mainly responsible for sex differentiation and must consequently be regulated from a higher level. Apart from SOX9 and AMH, there is one more major regulatory gene, which is also involved in the mammalian SD process. SF-1 belongs to the family of orphan nuclear receptors and is connected to the embryogenesis of mammals and non-mammalian vertebrates. It is present in the genital ridge of mammalian embryos and expresses a sexually dimorphic pattern, meaning it is present in both male and female gonads [11, 79, 80].

Primary function of SF-1 within SD can be viewed from the male-determining or the female-determining pathway, as activity is detectable in ovaries and testes. Generally, SF-1 is produced in supporting cell lineages, but their expression is mainly detectable in Leydig cells of the differentiating testis [68]. The role of the gene is primarily limited to the direct regulation of proteins included in steroidogenesis and the upregulation of AMH along with SOX9, by acting on the Sertoli cells [11, 79–82].

In what way exactly SF-1 belongs to the SD mechanism in crocodylians, was demonstrated by Western and Graves (2000). Although the author focused primarily on the role of SF-1 in the gonadogenesis in embryos of *Alligator mississippiensis*, there is a strong indication to assume that expression and function of SF-1 in crocodiles would deliver similar results, as the gene has been strongly maintained and conserved amongst vertebrates. Adding to that, cloning of SF-1 homologues in *Alligator mississippiensis* revealed a strong similarity towards the human orthologue [82]. During the early stages of embryonic development, where the gonad is yet to differentiate, SF-1 shows a clear sexual dimorphism and is

detectable around stage 20, with the onset of TSP in *Alligator mississippiensis* being set to stage 21. At both female- and male promoting temperatures SF-1 is active in the gonad, all throughout stage 27 post gonadal-differentiation. The detectable mRNA in ovary and testis during the TSP implies that SF-1 is not an exclusively male or female differentiating factor and thus most likely is involved in feminizing and masculinizing processes [82].

There are multiple ways on how to connect SF-1 to ovarian- or testicular differentiation. First, SF-1 is noticeably downregulated in the embryonic gonad at male-producing temperatures during the temperature-sensitive, critical period of the *Alligator mississippiensis*. It is not until after the gonad has committed to a fully organized testis, that SF-1 is significantly upshifted within the testicular structures. Moreover, the low concentration of SF-1 in the beginning stages of gonadal differentiation are quite similar to the expressional pattern of SOX9, as described in chapter 3.5.1.1. . Both, SOX9 and SF-1, are thought to have regulatory functions on AMH, as it was demonstrated in mice [70, 75]. The preceding upregulation of AMH prior to SOX9 and SF-1 in *Alligator mississippiensis*, does raise the question on the regulatory relationship between the genes, but it is possible that low amounts of SF-1 might be sufficient enough to significantly upregulate AMH [68, 82]. Additionally, SF-1 positively influences the synthesis of androgens, which are essential in the male-differentiating pathway [79–81].

Even though SF-1 is sexually dimorphic in alligators and mammals, there are quite a few deviations concerning the expressional patterns through embryogenesis. Male-producing temperatures in *Alligator mississippiensis* did not provide a constant basal level of SF-1 in the differentiating gonad, instead, the levels continuously fluctuated. In contrast, in putative ovaries, the mRNA was expressed constantly and in a higher amount as opposed to testes [82]. In mammalian vertebrates, however, this pattern is typically expressed vice versa, and SF-1 is strongly associated with testis differentiation. Compared with ovaries in alligators, the activity in mammals notably decreases once the differentiation process is more or less complete [79].

As of now, there is no clear explanation as to why regulatory patterns of homolog genes differ from one species to another. In *Trachemys scripta*, SF-1 behaves likewise to the characteristic pattern described in human and mouse gonadal differentiation. mRNA is increasingly detectable at male-determining temperatures and downregulated at female-

producing temperatures. Shift experiments further demonstrated the increasing activity of SF-1 when switching eggs from female- to male incubation temperatures [80, 81, 83]. Nevertheless, none of these results provided the conclusion, that SF-1 is a one-sex-related factor and is solely involved in the determination of either the testis or ovary [11, 80]. Even if male- or female-expression levels are higher, this does not deny the presence of mRNA in the opposite gonad.

AT, as one of the major ovary-determining factors, is also upregulated by SF-1, due to the promoting effect on steroidogenesis. Despite that, exogenous applications of AI consequently produced high levels of SF-1 and successfully hatched 100% males [83].

### 3.5.2. TRVP4

TSD in crocodylians is a cumulative, complex system and a concrete determination of a factor remains elusive. Possible factors involved range from steroid hormones to genetic components. Sure enough, they are all part of the cascade, but the triggering candidate is still yet to be determined (Figure 6).

The newest approaches in research turned to the transient receptor potential (TRP) family. This group of ion channels has become of significant interest in TSD mechanisms due to their sensitivity to heat stimuli and the elicited Calcium ( $Ca^{2+}$ ) increase in the body [84, 85]. Several channels belong to this group; however, temperature reactivity has only been observed in a few. Five of those, called transient receptor potential vanilloid type (TRPV) channels, were identified in the *Alligator mississippiensis* by Yatsu et al. (2015). Out of TRPV2, TRPV4, TRPM3, TRPA1, and TRPM8, the second listed channel proved to have the most temperature sensitivity in the American alligator and additionally in *Crocodylus moreletti* [73, 84]. While in the female gonad, TRPV4 is commonly expressed within the steroidogenic follicular cells, in the testes Sertoli cells and localized cells around the testicular cords are responsible for the synthesis [73]. Since TRPV4 is argued as a possible thermosensitive triggering factor in TSD it is of interest to determine how exactly it behaves during embryonic development and if major differences are occurring between male- and female-differentiating gonads.

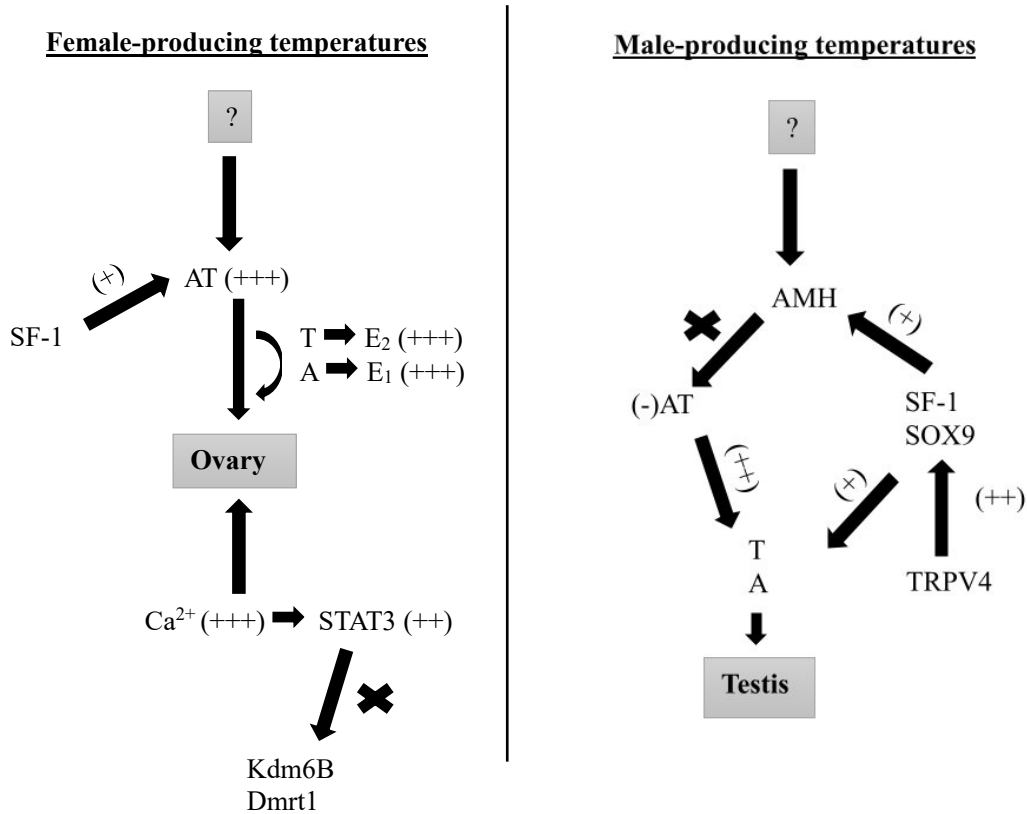
Concerning the male-determining pathway, TRPV4 has a suspected up-regulatory function on SOX9 in crocodylians. Evidence, based on TRPV4 antagonist injections into *Alligator mississippiensis* embryos at stage 19, suggests that downregulation of the ion channel negatively affects the expression of SOX9 and AMH – Both genes majorly involved in testicular differentiation. Application of agonists into a bipotential gonad did not result in significant alterations, only a mild elevation of the SOX9 gene. This suggests, again, that there is a direct connection between the heat-sensitive ion channel and genes involved in the testis differentiation, but also indicates that female differentiation is affected minorly to none [73, 84].

Upon thermal stimuli TRP channels commonly cause an increased intracellular influx of  $\text{Ca}^{2+}$  [85]. This specific reaction might be in interesting relation to a factor named Signal Transducer and Activator of Transcription 3, short STAT3 [86]. Yet to be studied in crocodylians, but already examined in turtles, STAT3 acts as an inhibiting factor for an epigenetic regulator called Kdm6B, which is considered to be in connection with male-determination. STAT3 itself is activated by phosphorylation at warmer, female-incubating temperatures through the influx of  $\text{Ca}^{2+}$  [86, 87]. Kdm6B, a histone demethylase, actively promotes testicular differentiation in *Trachemys scripta* by binding to a promotor region on the Doublesex and Mab3-related Transcription Factor 1 (Dmrt1) downstream of the cascade [86]. One as well as the other are significant in the male pathway and the deficit of either of them leads to a notably high decrease in AMH and SOX9. While Dmrt1 is a thermosensitive factor, it still needs to be upregulated by factors higher up in the process. On the contrary, there is no detectable sensitivity of Kdm6B towards outer temperature and switches to either male- or female-producing climate [86]. The inhibiting function of STAT3 on Kdm6B was properly demonstrated in laboratory experiments in *Trachemys scripta*. Application of STAT3 inhibitors successfully leads to a high expression of Dmrt1 and Kdm6B, even at female-producing temperatures.

The correlation between the  $\text{Ca}^{2+}$  influx and a consequential upregulation of a sex-determining factor raises the attention to TRPV4, which indeed causes an intracellular increase of the mineral in response to higher temperatures [73, 84, 86]. Such an involvement would indicate that TRPV4 is also somewhat included in a female-determining pathway, as higher calcium means more upregulation of STAT3. Overall, the final role of TRPV4 in TSD is subject to speculation and cannot entirely be defined. While it is true that male-



determining genes are directly affected by the absence or presence of TRPV4, there is no substantial evidence for the channel to be actively involved in the process of the gonad committing to an ovary [73, 84].



**Figure 6:** Schematic figure summarizing the involvement of steroid hormones, enzymes, and genes in the TSD mechanisms.

(+): low increase; (++): moderate increase; (+++): high increase; (-): decreased activity; ✖ : Inhibitory action; AT (Aromatase); T (Testosterone); A (Androstenedione); E2 (Estradiol); E1 (Estrogen); Ca<sup>2+</sup> (Calcium); AMH (Anti müllerian hormone); STAT3 (Signal Transducer and Activator of Transcription 3); Kdm6B; Dmrt1 (Doublesex and Mab3-related Transcription Factor 1); SF-1 (Steroidogenic Factor 1); SOX9 (SRY box like gene 9); Question mark: represents the unidentified thermosensitive factor within the TSD cascade.

### 3.5. Selective advantage of TSD

It is still a topic of discussion whether the presence of a TSD mechanism is of a higher evolutionary advantage for reptiles, as opposed to the occurrence of GSD. The *Charnov-Bull model*, first described in 1977, suggests that reproductive fitness in the same environmental conditions is differently expressed in males vs. females and further highlights the significance of ESD evolution [35, 88]. Bull and Charnov (1977) proposed that natural selection processes should benefit the hatchling for its most advantageous outcome. These advantages can be related to weight, length, and most importantly in maximizing reproductive fitness in relation to its gender. To give a more precise understanding of the Charnov-Bull Model, here is an illustrative example: Imagine a piece of land, with many different vegetative characteristics. On the far left of that land you find a big river with surrounding pastures, extensively exposed to broad sunlight. On the contrary, the right side is mainly foresting territory, offering shadow and cool spaces and lots of predator animals. These differences in the flora and fauna turn our fictional one piece of land into a lot of little islands – Islands that can favor either the reproduction of female individuals or that of males. Hence, the environmental conditions occurring in those little spots will favor the best outcome for the hatchling – Something which in GSD would not be a possibility, as it is not driven by the influence of the embryos surrounding [14, 15, 36]. Despite the difficulty of finding confounding evidence for the model, it poses the most realistic and applicable explanation as to why TSD has prevailed over GSD in certain species [8, 10, 89].

There are two ways to categorize the effects of incubation temperature on the embryo. On the one hand, it directly influences the outcome of the gender, and on the other hand, it affects the hatchling in various non-sexual ways. *Crocodylus niloticus* embryos that were incubated at warmer temperatures, at 34 °C, usually had a smaller body size compared to those incubated in cold and intermediate temperatures [38]. Hutton's (1987) study was confirmed through similar observations made by Deeming and Ferguson (1989) on *Alligator mississippiensis* hatchlings. Offspring incubated at intermediate temperatures had the largest body size after hatching. As intermediate temperatures in crocodylians produce predominantly male progeny, the larger body size poses the biggest selective advantage for that gender. Larger-sized male crocodylians are usually at an advantage when it comes to territory fights against predators or achieving better reproductive fitness [23, 36]. The increasing temperature stands in correlation with the accelerating growth rate of the embryos

during incubation and post-hatching development [36, 39]. However, if the temperatures border an extreme limit, the growth and survival chances decline significantly [32, 36]. Such observations in post-hatching effects by temperature act in favor of the proposed model by Bull and Charnov (1977) [6, 89].

Despite size being an important factor regarding selective significance, body weight is less subject to effect. One outstanding factor regarding body weight includes the egg yolk which forms throughout embryonic development. Significant amounts are detectable after the end of the TSP, at stage 25, where the yolk surrounds the embryo remarkably. Absorption of the yolk is a noteworthy factor in the development of the hatchling. It is predominantly absorbed into the abdominal cavity between stages 26 to 28, whereas stage 28 marks the time of hatching. How much of the yolk is absorbed and how it affects the hatchling is highly dependent on temperature [45]. Colder temperatures, which determine female sex (see Chapter 3.3.), typically produce heavier embryos, as they absorb more egg yolk. Less yolk is absorbed at higher temperatures. Additionally, growth rate might be positively correlated to the amount absorbed, as well as reaching sexual maturity [36, 37, 39]. Thus, a higher body weight due to a larger absorption of yolk is an advantageous precondition for female individuals to grow faster and improve their reproductive fitness. A higher number of sexually mature females, compared to that of males, creates an essential benefit in maintaining and expanding a population [32]. For TSD to be of evolutionary and selective significance, there need to be positive long-term effects on individual hatchlings and the population. Species with a longer lifespan, such as crocodylians, can balance their sex ratios over a long period of time, by means of TSD. Consequently, they are able to survive changes in their environmental conditions, even if alterations can be drastic disasters, such as climate warming. Individuals with short lifespans will benefit more from a genetic-driven SD system, as they can't adapt to changes over an extended period of time [4, 6, 8].

Concluding results on the significance of TSD and the correlation between the two major SD mechanisms are still missing and no definite answer can yet be elicited to either of the questions. The Charnov-Bull Model (1977) provides the most substantial explanation as to why TSD has been established in the reptilian order and how it favors individuals that lack heteromorphic sex chromosomes.

#### 4. The role of climate change

The earliest traces of crocodylian existence by the analysis of fossils can be traced back to the late-cretaceous period, around 95 million years ago [24, 27]. With the catastrophic cretaceous-tertiary event (K-T event), a major cleft was left in the ecosystem across the globe, leaving animal species the choice to either adapt, or to inevitably face extinction. Despite the closely associated relationship between crocodylians and dinosaurs, both belonging to the archosaurs, it is curious that only one of the two managed to prevail into modern centuries. Additionally, the phylogenetic similarities leave one to wonder, whether dinosaurs also used TSD and were, however, not capable of adjusting their adaptiveness to a new world, post-K-T event [21, 22, 24].

One way or another, history proved that crocodylians could adapt their population to significant changes in their environment. With global warming and climate change becoming a more significant issue at our present times, there is no denial in the fact that species using TSD are at a high risk of facing extinction. According to the World Meteorological Organization, global surface climate will be around 1.1 °C to 1.8 °C higher when compared to the detected temperatures in the 1900s [90]. This is without taking the effect of human-produced greenhouse gases into consideration. Natural catastrophes caused by climate change are detrimental to our ecosystem. Flooding and bushfires cause unthinkable damage to the vegetation and natural habitats of animal species. Rising sea levels additionally force land species to expand to alternative geographical locations.

Taking all of this into consideration, how is SD a crucial factor in the face of climate change? Species that determine their sex via chromosomes are at no risk of their sex ratios within a population being altered by environmental stimuli, such as increasing temperature. The sex of the offspring is set at fertilization and cannot be reversed or overridden [24, 25]. TSD species, on the other hand, depend on the outer temperature during egg incubation. Any changes to PT or TRT of even 1 °C, could drastically change the sex ratio of the population. A tendency towards warmer climates usually results in a ratio that is biased towards more females, or possibly also an overproduction of males, if temperature shifts fall within the male-producing temperatures. Such predictions were made for the population of *Alligator mississippiensis*.

Bock et al. (2020) forecast a temperature increase in nests within the geographical contribution, along the East Coast of America, around 1.1 °C to 1.4 °C by 2050. Mean temperatures currently range between 32 °C, favoring the production of male offspring. As the American alligator is known to use the TSD2 pattern, increases of such temperatures would not drastically change the principle of progeny. Males would still be produced, as they commonly still hatch at 100% around 33 °C [31, 32, 35]. However, due to the occurrence of less low-temperature females, the male:female ratio is expected to bias significantly towards males, leaning around 90% [91]. The lack of females within the population could pose a significant problem, as a high number of reproductive cycles of the few remaining female individuals would be needed to restore a balanced sex ratio [24, 25, 91].

Considering that global warming is not a stagnant process, and under the precondition that the alligator population would be able to overcome such initial skew in ratios, climatic conditions and the effects on the population will become even more drastic. A further prognostic by Bock et al. (2020) states, that by 2100 nests temperature will be 4.6 °C higher compared to nowadays. With this being true, males at intermediate temperatures would only seldom be produced and the sex ratio of *Alligator mississippiensis* would shift drastically, from 90% males to not more than 2% [91, 92].

In what way it is realistic that crocodylians are at the edge of extinction, however, is hard to forecast. As of right now, 8 crocodylian species are regarded as critically endangered, as listed on the red list by the International Union for Conservation of Nature (IUCN). Further, climate change and the consequential relocation to different areas are listed as an explicit reason by the IUCN for the categorization to critically endangered, apart from hunting and invasion of wildlife by humans. Although their SD mechanism lacks certain variety, due to the absence of sex chromosomes, knowing that crocodylians already survived a global catastrophe once, there is hope and confidence in assuming that they could do it again, even with using TSD as a SD mechanism [8, 24, 25, 91].

A possible way for them to adapt to the change our globe is facing is by the simple tool of geographical migration. Just like the Bull and Charnov (1977) model suggested, the primary evolutionary advantage of TSD lies in the ability of animals to choose which patches in our environment are most favorable for their outcome. If natural habitats are destroyed by

bushfires or floods, those original suitable patches might not be available anymore, hence crocodilians need to find new areas to live in. Such relocation and migration have been documented by fossil records over crocodilian history, showing that they are able to adapt to changing circumstances [24, 93]. At the moment, several natural habitats by crocodilians are affected and critically put at risk. *Alligator mississippiensis* has its primary geographical contribution located from Northern America down the East Coast and is a common inhabitant of the Everglades in Florida [91, 94]. A suspected increase in temperature of around 2 °C to 2.5 °C could significantly alter the flora and fauna of the wetland of the Everglades. Droughts would leave the American alligator with fewer available nesting sites and reduced food availability, resulting in decreased reproductive function [94].

The other side of the medal includes the danger of rising sea levels. Fukuda et al. (2022) examined the effect of rising water levels in *Crocodylus porosus* along the northern coast of Australia. The crocodile species mainly reside in freshwaters, like rivers and lakes, thus a mix in water quality due to seawater invasion into freshwater habitats could result in a reduction of possible living areas [95]. The authors conclude that sea levels in that particular area are possibly going to rise around 1.90m by 2100, resulting in ca. 60% less available natural territory for *Crocodylus porosus*.

All in all, climate change is a real and menacing risk for the crocodilian species around the world. Still, due to their resilient nature and the new knowledge about TSD which was gathered over the last years of research, there is a good chance of being able to preserve those large reptiles. Crocodilians might not have a possibly genotypic SD in their background, which could take overriding action if TSD fails, but PT and TRT work in the reptiles' favor, by still producing many males at high temperatures and ultimately females at even higher temperatures. Additionally, the long life span and the possibility of having many reproductive cycles in order to reinstate a balanced sex ratio, is a high advantage and should not be underestimated [8, 24, 25].

## 5. Conclusion

SD mechanisms in our ecosystem have proven to be very different and are utilized quite variously among the animals on our globe. By analyzing the results that were gained over the past couple of years, it becomes apparent that crocodylians represent a special clade of reptiles. Their offspring's gender is determined by the temperature during their incubation time. Females are commonly produced at high and low temperatures, while males predominantly hatch when temperatures fall into intermediate levels. Variations between species exist, but generally, the patterns apply to all members of the crocodylians. Researchers have proven that the gender of eggs is not only determined by temperature in artificial incubation experiments but that it is also a common event in nature. Clutches therefore have different sex ratios, all depending on the location of the egg within the nest and the environmental temperature.

Even though TSD has been in the focus of research ever since Charnier (1966) discovered the odd mechanism in a lizard, no clear explanations have yet been given on how exactly temperature poses its effect during embryonic development. Multiple hypotheses have been proposed, with surely all of them being crucial components in the differentiation of the gonad during the TSP. Steroid hormones such as  $E_2$  and T are in direct correlation with gender, as their expressional patterns align with either a female or a male hatchling. Further, AT, an enzyme that knowingly converts T into  $E_2$ , shows higher activity levels in putative ovaries. Adding to that, the application of AI causes the interruption of ovarian differentiation. Genes involved in mammalian SD were also isolated and determined in crocodylians and turtles. Their expression varies slightly between the species, but their role in TSD during the TSP is undeniable. However, what is missing is the pivotal thermosensitive factor that ultimately elicits a response in the bipotential gonad that will either cause the male- or female-determination pathway to be induced. That is, none of the proposed factors, neither steroid hormones nor genes, are solely responsible for the determination of the gonad into the testis or ovary, indicating that they must be upregulated from a higher level.

The significance of TSD is highlighted by its post-hatching effects on the hatchlings. Females with higher body weight, due to yolk absorption at lower temperatures, benefit from reaching their reproductive fitness quicker than males, hence having many reproductive

cycles to balance the population. Males on the other hand draw their benefit from a large body size, by being in an advantage to predators in their territory.

Altogether, there lies significant importance in understanding how TSD operates among species. Being able to determine the TSP and the critical temperature ranges is essential in the possible preservation of endangered species, that are faced with the danger of climate change. Even though such drastic effects seem to be far away, there is a realistic chance for animal species to be critically affected by rising sea levels and continuously increasing global temperatures. Further research in the field of TSD is consequently indispensable, especially since the major thermosensitive factor in the SD mechanisms remains elusive.



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5. **Figure 5:** Figure created by the author of this thesis
6. **Figure 6:** Figure created by the author of this thesis



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## Thesis progress report for veterinary students

Name of student: Josefine Knotz

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Name and title of the supervisor: Dávid Sándor Kíss, PHD

Department: Physiology and Biochemistry

Thesis title: Temperature-dependent sex determination in crocodilians

### Consultation – 1st semester

Timing				Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2023	02	15	Overview of the semester assignments	
2.	2023	02	20	Understanding the use of search engines	
3.	2023	03	8	Preparation of the first draft	
4.	2023	04	9	Understanding the management of citations	
5.	2023	06	30	End-of-year version of the thesis	

Grade achieved at the end of the first semester: .....5.....

### Consultation – 2nd semester

Timing				Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2023	09	19	Thesis version written over summer	
2.	2023	09	29	Edited version from consultation 1	
3.	2023	10	25	Edited version from consultation 2	
4.	2023	10	31	Edited version from consultation 3	



5.	2023	11	13	Final version	<i>lbr</i>
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Grade achieved at the end of the second semester: .....<sup>5</sup>.....

The thesis meets the requirements of the Study and Examination Rules of the University and the Guide to Thesis Writing.

I accept the thesis and found suitable to defence,

*lbr*

signature of the supervisor

Signature of the student: .....*Joelle Herz*.....

Signature of the secretary of the department: .....*ll*.....

Date of handing the thesis in. *2023. 11. 13.*.....

I hereby confirm that I am familiar with the content of the thesis entitled “**Temperature-dependent sex determination in crocodylians**” written by **Josefine Knotz** which I deem suitable for submission and defence.

Date: Budapest, 13<sup>th</sup> of November 2023



Dávid Sándor Kiss

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