The Influence of Global Warming on the Temperature Dependent Sex Determination of Sea Turtle Species

The mechanism and the possible adaptations of the sea turtles

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Picture taken by Manon de Visser
at Playa Tortuga, Costa Rica

Abstract

Sea turtles are threatened by global warming and climate change in several ways. The most concerning consequence is the rise of temperatures on beaches, which cause most eggs to become female or to die, if the temperature is too high. The result is smaller populations with strongly female biased sex ratios, a phenomenon already observed on several locations for different species. Adaptation might occur by means of simple natural selection, whereby individuals that fit the new environment better are selected for. The species do in fact also change migratory patterns, timing of nesting in nesting seasons and areas according to rising temperatures. Turtles appear to make decisions about the microenvironment in which they build their nests, so individual adaptation might also occur, for example if turtles dig their nests deeper or search for a nest-site in the shadow. Interspecific mating is also observed for loggerheads and olive ridleys. Still, a lot of other consequences of global warming like drastic climatic changes, a rising sea level, rising water tables, hurricanes and affected food sources could cause sea turtles to go extinct. Sea turtle numbers have already experienced massive declines, which means more research and more conservational projects are needed to protect these animals. Sea turtles are economic and ecologically important and they are flagship species, so they should be protected. While sea turtle farms are no solution, the building of more hatcheries, in situ protection of eggs and protection of nesting and foraging habitats are on the contrary, promising strategies. It also seems that sea turtles are more vulnerable to global warming because of its combination with non-climate related threats, like fisheries bycatch, poaching of eggs and turtles, pollution and diseases. These are, therefore, also points of interest for research and conservation. Besides sea turtles and other TSD species, global warming even seems to affect sex ratios of GSD species, probably even humans. This means the effects of global warming should be taken seriously for a variety of animal species.

Introduction into Temperature-Dependent Sex Determination

In the animal world with sexual reproduction, different mechanisms exist to determine the sex of an organism's offspring. *Genotypic Sex Determination* (GSD) is the mechanism by which an offspring's sex is determined by its sex genes, which lie on sex chromosomes. Mammals have the so called *male heterogamety*, which means that males have two different sex chromosomes (XY) and females two times X (XX). On the Y chromosome lies the master gene SRY that cause male development. For birds, it is the other way around. Maleness is determined by having two Z chromosomes (ZZ) with the double dosage *DMRT1* gene. Females have one Z and one W (ZW). Therefore, this mechanism is called female heterogamety. In fishes, amphibians and reptiles both male and female heterogamety is observed (Quinn *et al.*, 2011). Sex chromosomes are inherited from the parents and therefore, the sex of the offspring is irreversibly fixed. In 1930, Ronald Fisher described his thoughts about sexual selection. If the parental expenditures are equal on both males and females, an equal sex ratio (male:female = 1:1) will be the result, as is the case for GSD. This is called a 'Fisherian' investment sex ratio. If sex ratios start to deviate from this Fisherian sex ratio, selection pressures will always lead to an equal sex ratio. (Fisher, 1930) Hamilton explained this equal sex ratio as follows: If, for example, more females will be produced than males, this will give males better mating prospects than females. Males will therefore produce relatively more offspring. If this is the case, it is most likely advantageous to have genes with male-producing tendencies. These genes will spread in the population, so more male births will occur. This will bring the sex ratio back to the Fisherian sex ratio, with which the advantage of producing males will disappear. (Hamilton, 1967)

Environmental Sex Determination (ESD), on the other hand, is the mechanism by which an offspring's sex is determined by environmental factors. This means that the sex of the offspring is not irreversibly fixed from the moment of fertilization. A well-known phenomenon is *Temperature-dependent Sex Determination* (TSD). For TSD, the sex determination depends on the environmental temperature the embryo experiences (see BOX 1 for more information). TSD exists of three different patterns. One pattern is the 'female-male-female' (FMF) pattern, whereby females develop under extremely high and low temperatures and males develop under intermediate temperatures. This is

BOX (1): Temperature Dependent Sex Determination (TSD)

In TSD, temperature influences genetic mechanisms that enhance the production of steroids, steroidogenic enzymes or steroid hormone receptors. This has a direct effect on the hormonal environment of the embryo, which influences sexual development. In reptiles, steroid hormone levels decline during embryonic development and the degree of this decline depends on incubation temperatures. For example, adding estrogens to eggs of many reptile species activates female development, even though the environmental temperature is male-promoting. On the contrary, androgens do not seem to activate male development at female-promoting temperatures. Still, male development is blocked without androgens.

Also, genes involved in gonad formation are important thermal switches. For example, *Wt1* and *Sf1*, which both contribute to testis formation by activating the transcription of the *Anti-Müllerian hormone* (AMh) gene, are important in TSD turtles. AMh suppresses the development of the female reproductive tract. In alligators and turtles, *Dax1* (important for the formation of ovaries by repressing *Wt1* and *Sf1*) and *Dmrt1* (involved in testis formation) expression occurs prior to gonadal differentiation in both sexes. Their expression is temperature-sensitive. The mentioned genes are also important in GSD species. More research is needed to fully understand the roles of these genes and with that, the molecular basis of both GSD and TSD.

(Ramsey & Crews, 2009; Warner, 2011)

the case for crocodile species, some turtles and some lizards (Hulin *et al.*, 2009). In another pattern, the ‘female-male’ (FM) pattern, females are produced under low incubation temperatures and males are produced under high temperatures. The FMF and FM pattern are less common. The most common pattern of TSD is the ‘male-female’ (MF) pattern, where it is the male that develops at low incubation temperatures and the female that develops at high temperatures. This pattern is seen in most lizard and turtle species, including all marine turtle species (Girondot & Pieau, 1988; Warner, 2011).

In sea turtles the sex of the offspring is determined by the environmental temperature in the middle third of the incubation period, the *thermo-sensitive period* (TSP). (Merchant-Larios *et al.*, 1997; Mrosovsky & Pieau, 1991) The *pivotal temperature* (PT) is the temperature at which 50% of the eggs will develop as females and 50% will develop as males at population level (see fig. 1). This gives a 1:1 sex ratio. Due to slight genetic variation in PTs within a population, temperature conditions that would lead to differentiation of both sexes should actually be defined by a small interval of temperatures. This would be even more precise than using the estimated PT of the species, but this is often hard to calculate. (Girondot & Pieau, 1988) Anyhow, around the PT a narrow range of a few degrees Celsius exists in which both sexes are still produced. This range is known as the *transitional range of temperatures* (TRT). The PT and the TRT are highly conserved among species (Pike, 2013). The PT is an estimate on population level and therefore, inter-clutch variability is common. For individual nests it is called the *threshold temperature* instead of the PT. (Mrosovsky & Pieau, 1991) For six out of seven species, PT values are close to 29 ^{o}C (Hulin *et al.*, 2009). For one species, the olive ridley (*Lepidochelys olivacea*), the PT is around 30,5 ^{o}C (Valverde *et al.*, 2010). When temperatures during the TSP differ from the PT, but are still within the TRT, this produces a skewed sex ratio. Sea turtle embryos can’t survive in too hot or too cold environments. The upper lethal limit is around 33 – 35 ^{o}C, the lower lethal limit is around 25 – 27 ^{o}C. The range between these limits is called the *thermal tolerance range* (TTR) in which embryonic development is allowed to occur. (Valverde *et al.* , 2010) Once the temperature is outside of the TRT, but still within the TTR, all offspring will develop the same sex (Warner, 2011). For all sea turtle species, nests with temperatures of 27°C ± 0,5 °C are completely male-promoting and nests with temperatures of 32°C ± 0,1°C are female promoting (Merchant-Larios *et al.*, 1997). Since the investment costs to produce males or females are the same for sea turtles, a fisherian sex ratio is expected to be evolutionary stable. After all, the sex is only determined during the incubation period, the period in which embryos develop until the eggs hatch. It is, however, important to realize the fact that the sex of sea turtle offspring is not determined by GSD, but by TSD. The selection pressures favoring this fisherian sex ratio could work on a population level, for example by selecting for a desirable PT value. This is described in chapter 3. It could also work on an individual level by favoring sea turtles that lay their eggs in places with an advantageous temperature, as described in chapter 4.

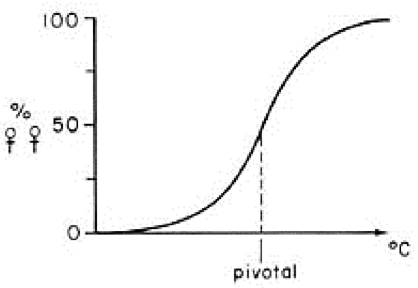


Figure (1): A graph defining the pivotal temperature.
The temperature at which 50% of hatchlings will be male and 50% will be female (the pivotal temperature, PT), is shown. For sea turtles, temperatures higher than the PT will lead to more females and lower temperatures will lead to more males. (Mrosovsky & Pieau, 1991)

Global warming with a rapid increase of temperature could affect TSD species, by production of abnormal skewed sex ratios or even that the temperature reaches the upper temperature limit of 30-35 ^{o}C. For threated species like the sea turtles (see table 1 in Chapter 1) this could lead to extinction, like the extinction of the dinosaurs. Namely, some scientists think that dinosaurs might have had TSD and that dinosaur extinction was most likely caused by severe environmental changes in temperature. 65 million years ago, the cold temperatures would have probably created a skewed sex ratio for TSD species, or it could have halted embryonic development altogether (Miller *et al.*, 2004; Silber, 2011).

This leads to the question whether global warming could have an impact on the remaining sea turtle species. Are they even more threatened because their existence depends on TSD, which can lead to strong skewed sex ratio under raised temperatures? And, more importantly, if it does influence the survival of sea turtles, can they adapt to the environmental changes and reach the desirable Fisherian sex ratio, or should we be concerned about their existence? First, in chapter 1, the economic and ecological importance of sea turtles will be discussed. Chapter 2 describes the possible influences of global warming on the species and after this, chapters 3, 4 and 5 describe whether or not adaptations of the species to global warming and climate changes are expected.

Chapter 1: Economic and Ecological Importance of Sea Turtles

Sea turtles live their life in the ocean, but the females must come ashore to lay eggs. (Madden *et al.*, 2014) Adult female sea turtles have the tendency to return to the area in which they were born to lay their own eggs. The eggs need the sandy beaches for incubation to occur. Without such a suitable incubation environment, reproduction will not be successful. So, the long-term ability of suitable incubation habitats are of crucial importance for the survival of a population or species. (Fuentes *et al.*, 2013; Pike, 2014) Olive ridley sea turtles and in a lesser degree Kemp's ridley (*Lepidochelys kempii*) sea turtles sometimes nest at so called *arribadas*. Arribadas are events during which many females aggregate and come to lay eggs on the same beach at the same time. This happens in a period of a few nights and sometimes there are up to tens of thousands of females nesting synchronously. This happens for example in specific areas of Mexico, Costa Rica, and India and to a lesser extent on other Eastern Pacific beaches. For olive ridleys at least 98% of the annual nesting population lays eggs during these events. The remaining nesting ridleys, plus the females of all other species, lay their eggs by solitary nesting. The areas sea turtles often use for nesting are referred to as 'rookeries' (Eckert *et al.*, 1999; Jensen *et al.*, 2006; Valverde *et al.*, 2010).

Sea turtles have declined tremendously in numbers over the past few centuries. Studies show that only a small fraction is left of the original numbers. (Catry *et al.*, 2009) Graphs showing this actual decline are hard to find, especially graphs about global level declines. To still give an impression, figures 2 - 5 all show a decline in nests laid by sea turtles of different species. Most of all sea turtle species are threatened (See table 1, ("www.iucnredlist.org" 2014)), which is concerning because sea turtles are ecologically and economically important animals. For example, green sea (*Chelonia*

Table (1): An overview of the current IUCN status of each sea turtle species. ("www.iucnredlist.org" 2014)

Species	IUCN status
Leatherback	Vulnerable
Kemp's Ridley	Critically Endangered
Olive Ridley	Vulnerable
Loggerhead	Endangered
Green sea turtle	Endangered
Hawksbill	Critically Endangered
Flatback	Data Deficient

mydas) turtles help in conserving healthy sea grass plants, hawksbill (*Eretmochelys imbricate*) sea turtles maintain coral reefs and loggerhead (*Caretta caretta*) sea turtles create trails that alter the aeration and nutrients of the sediment and has an impact on the ecosystem of the seabed. Olive ridleys play an important role for both sea birds and sea fish, acting as a potential roosting, feasting and hiding place. Besides this, sea turtles lay their eggs in holes on beaches and are therefore a source of high quality nutrients for organisms and insects living in the sand as well as bigger predators eating the eggs. It also improves the development of vegetation and the stabilization of dunes. Also, sea turtles are relevant to different ecosystems because they maintain balanced food cycles. (Barik *et al.*, 2014; Fuentes *et al.*, 2013) The eggs laid are a common prey for predators like flies, beetles, crickets, crabs, birds, rodents, carnivores. Also, especially humans contribute to the decline of sea turtle numbers by poaching eggs. (Eckert *et al.*, 1999; Madden *et al.*, 2014) The eggs or nests are also used by certain organisms for purposes other than predation, they can provide a good quality habitat. (Madden *et al.*, 2014) Sea turtles also play a cultural and educational role in terms of their value to certain human societies and in the tourist industries. (Eckert *et al.*, 1999;

Fuentes *et al.*, 2013) In addition to poaching, since the 20th century an increase in commercialization of sea turtle products has caused a lot of populations to decline (Eckert *et al.*, 1999). Specific areas must be protected to protect sea turtles, which means they are flagship species for both local and international conservation. By protecting sea turtles, coastal areas and seas are protected too. (Eckert *et al.*, 1999) The names of five species have already been mentioned, the remaining two species are the flatback (*Natator depressus*) sea turtle and the leatherback (*Dermochelys coriacea*) sea turtle.

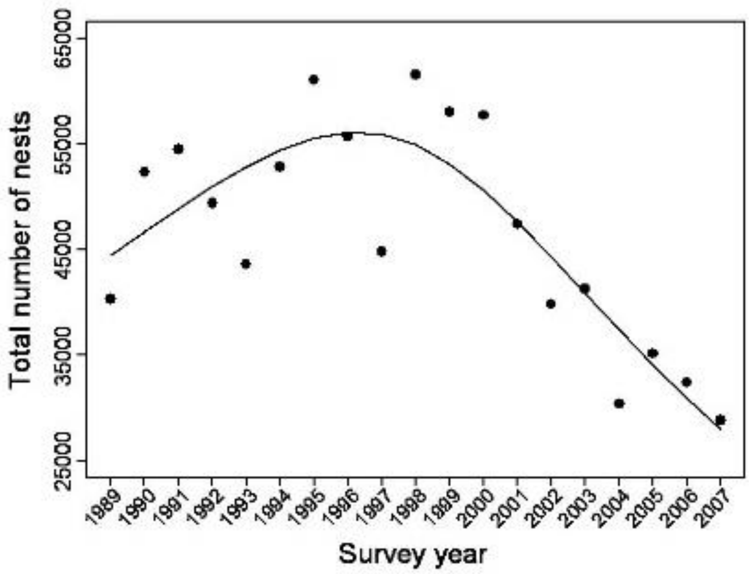


Figure 2: Chart showing the numbers of eggs laid each year by loggerhead (Caretta caretta) sea turtles on Florida beaches from 1989 – 2007. *A dramatic decline is showed. (Godfrey & Appelson, 2007)*

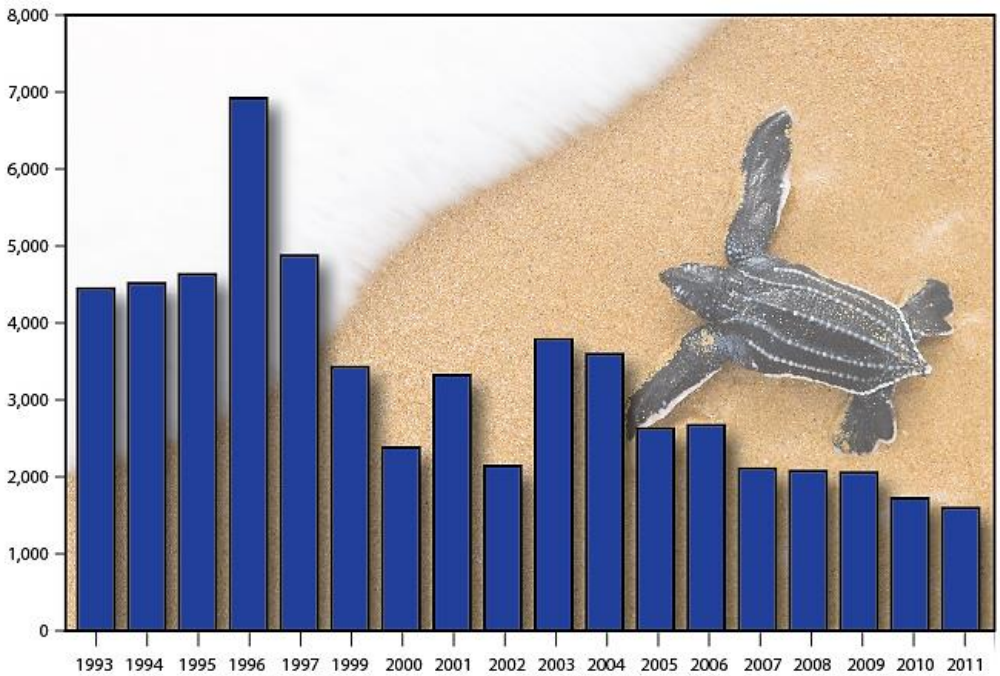
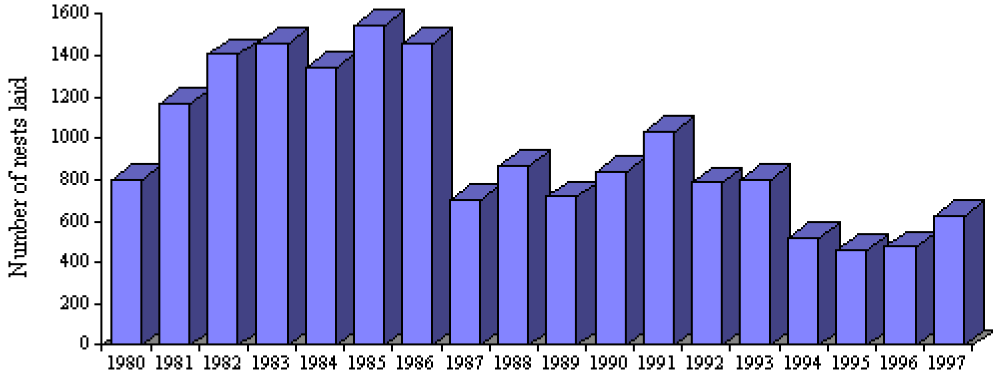
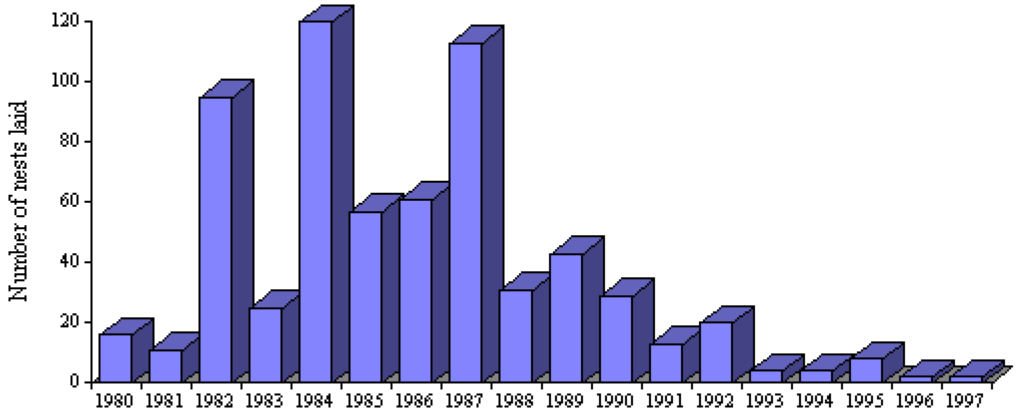


Figure 3: Chart showing the number of nests laid by leatherbacks (Dermochelys coriacea) at Jumursba Medi in Indonesia from 1993 - 2011. (www.futuretimeline.net, 2013; Tapilatu et al., 2013)



(A)



(B)

Figure 4: Charts showing the numbers of eggs laid each year by green sea turtles (Chelonia mydas) and olive ridleys (Lepidochelys olivacea) from 1980-1997 on the beaches of Sandspit and Hawkes bay in Karachi, Pakistan. *(A) Chart showing a decline in green sea turtle nests. (B) Chart about olive ridley nests. A dramatic decline was observed.* (Asrar, 1999)

Chapter 2: Possible Influences of Global Warming on the Reproduction of Sea Turtles

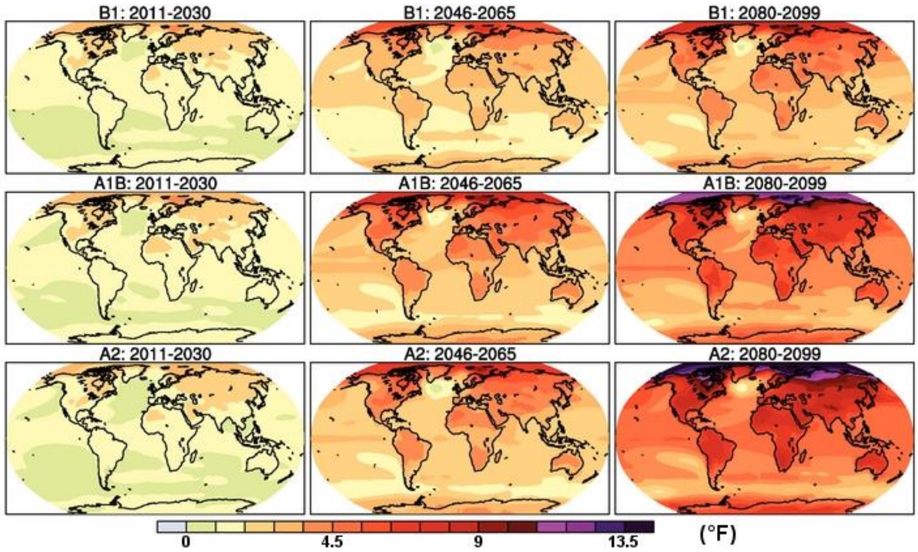


Figure 5: Different charts showing changes in temperatures over the world under three different scenarios of greenhouse emissions (rows) and three different periods of time (columns). *The changes are based on and compared to average global temperatures from 1961 – 1990. See the text for further explanation about the B1, A1B and A2 scenarios. The temperature differences are represented in degrees Fahrenheit. (1 °C = 1,8 °F) (EPA, n.d.)*

Global warming is happening and rapid climate changes are predicted for the upcoming century (Hulin *et al.*, 2009) Also, on a global scale, it is predicted that temperatures increased by 2 °F to 11,5 °F by the end of the 21st century (keep in mind that a difference of 1 °C is equal to 1,8 °F). By 2100, this global average temperature is also expected to increase twice as fast as it did the last century. Air temperatures (at ground level) are predicted to increase even more rapidly over land than oceans and some areas of the world are thought to experience larger temperature increases than the global average. (EPA, n.d.) It is, for example, already said that "Many countries in Latin America face severe challenges in coping with climate-related disasters" (CCAFS, 2013). To protect animals from this possible threat, humans should for example lower this threat by reducing greenhouse emissions. But this would be a big challenge for our world and it would not stop the already apparent and inevitable impacts caused by global warming at this very moment. (Fuentes *et al.*, 2013) Three different scenarios are often used to predict the future. They are defined by the IPCC. The first scenario is B1: low emissions. The second scenario is called A1B: medium-high emissions. The last scenario is A2: high emissions. (EPA, n.d.) See fig. 5, for the different outcomes of global warming for the different scenarios until the end of the 21st century. See also figure 6, which shows the past and future change in global temperature under different scenarios of greenhouse emissions.

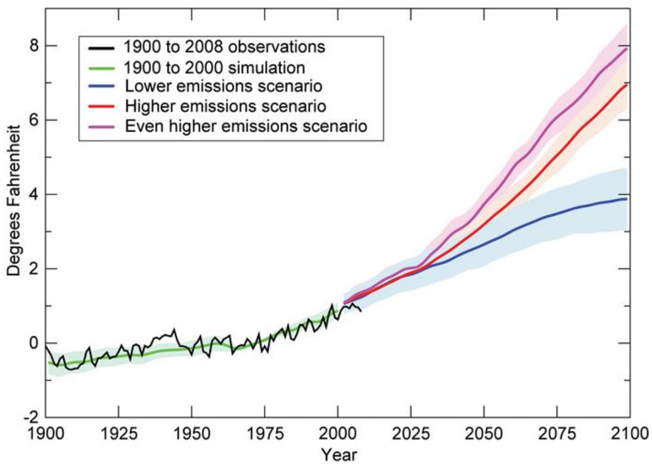


Figure 6: Graph showing past and future changes in global warming under three different scenarios of greenhouse emissions*. The changes are based on global average temperatures from 1960 - 1979. The lines show predicted temperatures in Fahrenheit under the scenario in question. The shaded areas represent their ranges. These predictions are calculated from a set of climate models. The temperatures are represented in degrees Fahrenheit. (1 °C = 1,8 °F)* (EPA, n.d.)

The geographic distribution of sea turtle nesting seems to be limited by the environment and the climate of a region. Especially the temperature and the rainfall are important factors. This is the reason why many scientists are concerned about the possible influences of climate change. The eggs laid by sea turtles are extremely sensitive with regard to environmental conditions. They need good moisture and temperature conditions. Lethal temperatures, which are either too high or too low, will cause the embryos to die, as well as very moist environments, which cause the embryos to be withhold from oxygen. (Fuentes *et al.*, 2013; Pike, 2013)

Sex Ratio Biases caused by Rising Temperatures

Our knowledge of how climate change would influence population dynamics of ectotherms and the way in which they live is limited. It is known that sex ratios of hatchlings can differ from year to year, because of inter-annual variations in local temperature (Girondot *et al.*, 2004; Mazaris *et al.*, 2012; Mrosovsky & Godfrey, 2010). For example, figure 7 shows the sex ratios from 1850 until now and predicted sex ratios until 2100 for loggerhead turtles nesting at Cape Verde. (Laloë *et al.*, 2014) Still, with a slight rise in temperature, sex ratios of the offspring might alter for species with TSD. Female biased sex ratios are already observed for green turtle hatchlings in Northern Cyprus (4 – 14% male), for the entire reproductive green turtle population of the Mediterranean Sea and for hawksbill hatchlings in Brazil (<10% male), representing a sex ratio bias of the whole reproductive population of this species as well. Some of these findings go back for 30 years. The following observations are covering only a small part of the overall nesting populations, but are still worth mentioning; extreme female biased sex ratios are also found for loggerhead hatchlings in the United States, Brazil and the Mediterranean Sea, green turtle hatchlings of Ascension Island and leatherback hatchlings of the Pacific coast of Costa Rica. Higher nest temperatures will lead to a female biased sex ratio, which could create bottlenecks for populations. For example, the secondary sex ratio of a certain generation of turtles of a certain population is, of course, dependent of the primary sex ratio and of the survival of the embryos during development. (Girondot *et al.*, 2004)

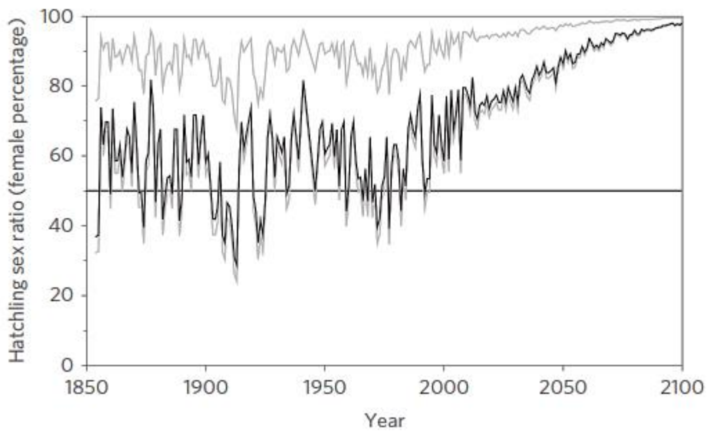


Figure 7: Graph showing sex ratios from 1850 to 2100 for the loggerhead (Caretta caretta) sea turtle nesting population of Cape Verde. *The lower grey line shows the sex ratios for hatchlings that developed on light sand beaches, the upper grey line the ones for dark sand beaches and the black line shows all beaches combined of the Island of Sal.* (Laloë *et al.*, 2014)

However, some evidence suggests that a skewed sex ratio would not be a threat to a population, because male turtles breed with multiple females each year. (Pike, 2014) This is maybe only the case for slight increases in sand temperatures, or for short-term increases in temperatures, which will only cause a small bias in sex ratios. A recent study suggests that global warming will have a positive conservational effect on sea turtle populations (Laloë *et al.*, 2014). They predict increasing nesting numbers as a result of the female biased sex ratio, which will positively influence the natural rates of population growths. Although it is not known how many male turtles are needed to fertilize all fertile females in a population, it is known that having too few males in the population is a limiting factor for reproduction. This conclusion was based on a research of Wood & Wood in 1980, where the operational sex ratio (the number of reproductive males and females at a time in a population), became strongly biased after testing the reproductive success of a population with a female-biased sex ratio under artificial conditions. So, on the long term, or if the rise in temperatures is very big, it could probably still lead to a total population collapse because the sex ratio bias is too great to recover from. After all, if survival of the embryos and hatchlings is high, a slight change in the mean survival of one of the sexes will produce a strong bias in sex ratios. (Girondot & Delmas, 2004; Mrosovsky *et al.*, 2009)

Overcooked Nests caused by Rising Temperatures

In addition, the survivorship of the embryos and hatchlings themselves is threatened. An even more concerning consequence of global warming is that it could kill entire clutches if the sand temperatures on the beaches become too high. For instance, at Ostional Beach in Costa Rica the temperatures in the dry seasons are sometimes already crossing upper lethal limits, which causes nests to 'cook' and embryo development to fail. (Pike, 2014; Valverde *et al.*, 2010) All seven sea turtle species react similarly to high temperatures, with 35 ^{o}C as a vast upper lethal limit (Pike, 2014). The reason why this is happening on this particular beach could be the fact that it is a place where olive ridley arribadas take place. (Honarvar *et al.*, 2008) The already apparent dangers of the high density of the nests (increase in sand temperature, decrease in available oxygen, etc.) could become even more dangerous in combination with global warming (Valverde *et al.*, 2010). It also appears that, at the moment, the effects of climate change are stronger for tropical nesting beaches as compared to temperate nesting beaches around the world. The hatching success decreases more dramatically for tropical beaches than for temperate beaches (see fig. 8). Nonetheless, both types of beaches experience the same magnitude of increase in temperature, as you can see in the figure.

This should be taken seriously, since the beaches with the most nesting sea turtle species are in the

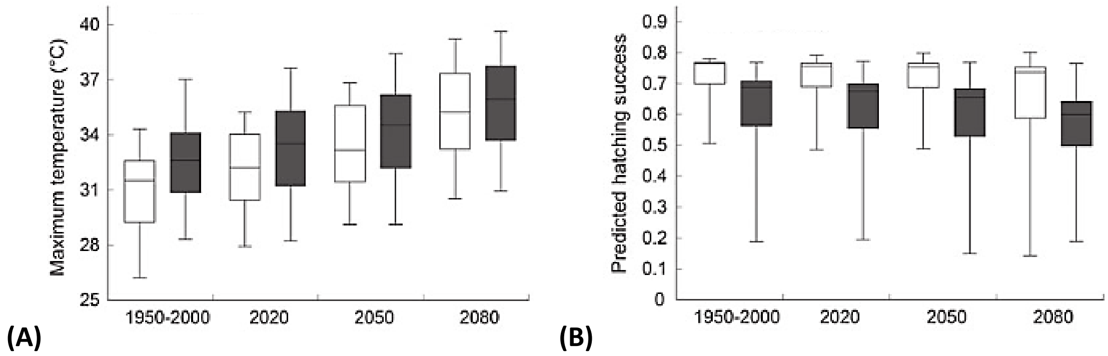


Figure 8: Two graphs about a high-mission future (A2) scenario for temperate (white) and tropical (black) beaches under an A2 (high-emission) global warming scenario. *(A) Future change in temperature during the summer, when most turtle populations nest. (B) Expected hatching success for these beaches.* (Pike, 2014) *Figure slightly modified.*

Indopacific, the North-East of Australia and the Caribbean plus the Gulf of Mexico. It seems that temperate regions receive less nesting species. For instance, the South-Eastern United States, Southern Africa and the Mediterranean Sea only have two nesting species (see fig. 9) Also, the range of suitable climate conditions differs for the different sea turtle species. For example, Kemp's ridleys and flatbacks have a small niche width, probably because of the narrow geographic range they live

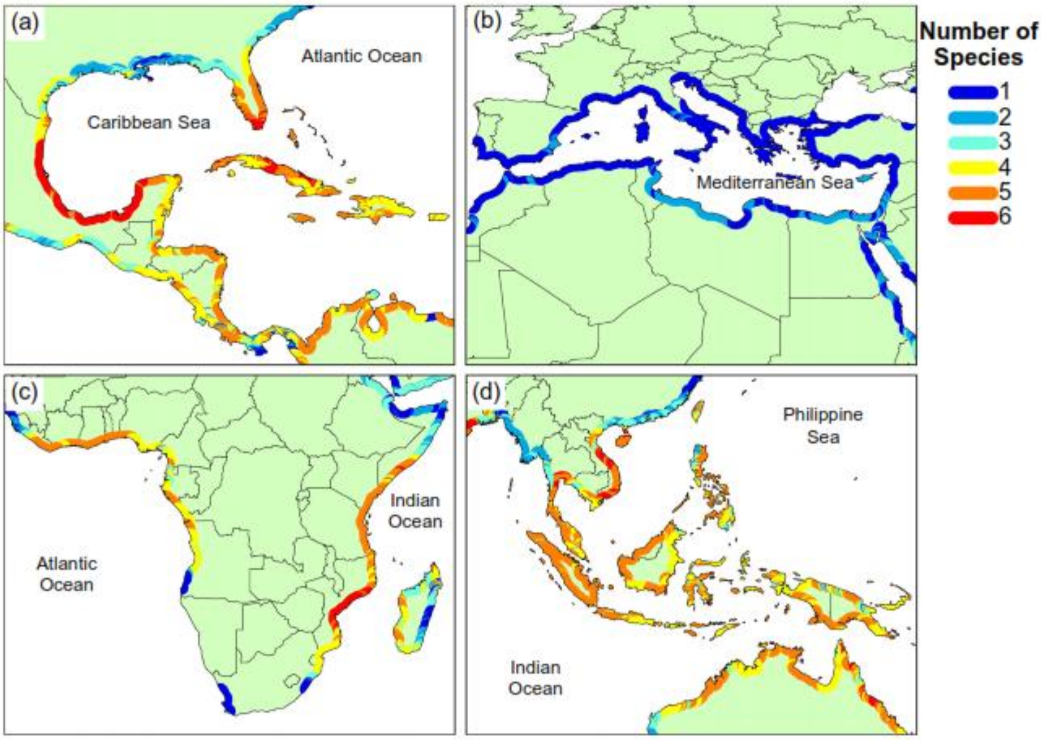


Figure 9: Maps of the world showing global nesting hotspots for sea turtle species, calculated by MaxEnt modelling of all existing species. *(A) Caribbean region, (B) East-India, (C) Pan-tropical Africa and (D) Australasia. The numbers of species nesting at different areas in these regions are shown in different colors. (Pike, 2013)*

in. On the other hand, loggerheads have a large niche width. The olive ridley has an intermediate niche width. (Pike, 2013) This could mean that species with a narrow niche width are even more sensitive to climate change.

What should be noted is that it is not the crossing of the upper lethal limit itself that kills an embryo, it is the amount of time spent at too high temperatures. This means that embryos do have physiological mechanisms that can protect them from lethal damage for a certain amount of time. How these mechanisms work is still unclear. In addition, the occurrence of cooling periods supposedly helps the nests to cool down after crossing the upper lethal temperature. For instance, occasional rain or shadow are the reason many embryos are rescued from overheating. (Valverde *et al.*, 2010) It is known that nests laid earlier in the season are relatively colder and therefore produce more males. But, since more females will come to lay eggs, these nests are often accidentally destroyed by other nesting turtles, especially in high density nesting beaches. (Girondot *et al.*, 2004) This means that the overall sex ratio is most likely female-biased, an outcome that will only become stronger with rising temperatures.

Rising Water Levels and Changing Landscapes

The rising temperature of nesting beaches is not the only consequence of global warming. Also, the existence of sea turtles is threatened because of the rising sea level. This would lead to a reduction in available nesting areas. The quality of the habitat left could be decreasing, for example because of the higher water table in the ground, which increases the moisture of the sand. (Pike, 2014)

Since the development of the eggs consists of an immobile life stage, reproductive success depends on the nest-site choice of the mother. Females do choose their nesting areas based on temperatures, but climate change might modify the landscape of the beaches in such a way that females will not be able to find the proper nest site, if microhabitat characteristics are altered. In the context of TSD, this could lead to biased sex ratios worldwide or even to increased embryonic death. (Pike, 2014) Other predictions are more cyclonic activities and hurricanes, flooding, severe rainfall and negative impacts on food sources as results of climate change. (Fuentes *et al.*, 2013; Mrosovsky & Godfrey, 2010) It could even lead to changes in reproductive periodicity, shifts in latitudinal ranges and disturbed hatchling dispersal and migration. (Fuentes *et al.*, 2013) With the fact in mind that almost all sea turtle species are endangered or vulnerable (see again, table 1), all of these predictions should be taken seriously.

Chapter 3: Adaptation on a Population Level

To protect species from the possible influences of global warming, it is important to understand whether and in which ways a species or ecosystem is vulnerable to climate change.

Resilience of Sea Turtles

The ability of an ecosystem or of a species to either resist or adapt to changes is called *resilience*. It is about the capability to maintain key functions and processes under stress or pressures, which are environmental in the case of global warming. The chances of successfully adapting to or recovering from disturbances is especially determined by three big characteristics of a population or species (See BOX2). The fact that sea turtles have declined sharply in numbers in many regions over the last decades, interferes with all three conditions discussed in BOX2. Also, information is given about an existing *Resilience Index* (RI) and its components. (Fuentes *et al.*, 2013) To make research on sea turtles worldwide easier and more organized, a framework of 58 so called *Regional Management Units* (RMUs) covering all seven sea turtle species was designed by Wallace *et al*. (Wallace *et al.*, 2010). The smaller the RI, the higher the resilience for the corresponding RMU (Fuentes *et al.*, 2013).

Climate changes have also happened in the past and sea turtles overcame these changes. The danger lies in the current combination of threats. The variables most important to the resilience of the species examined seemed to be rookery vulnerability (RV) and threats that are not related to global warming (NT), like fisheries bycatch, poaching of turtles and eggs, pollution and diseases and different coastal developments. (Fuentes *et al.*, 2013) If these variables are higher, the RI will be higher which means the species will become less resilient to climate change. Six out of 13 least resilient RMUs also appeared to be within the world's 11 most endangered RMUs. These six RMUs include olive ridley and loggerhead sea turtles from the Northeast Indian Ocean, olive ridleys in the

BOX (2): Resilience

The best chance for a species to adapt to climatic changes lies in at least three aspects

1) Maintaining genetic diversity. This facilitates the ability to adapt to variable conditions
2) Being geographically distributed over a wide area. This could minimize the impacts of local-specific threats
3) Having a large breeding population. This helps such a population to overcome disturbances, because of the higher numbers of individuals leading to an increased ability to recover.

For sea turtles, a special Resilience Index (RI) is developed, which considers a set of characteristics that are known about 58 different Regional Management Units (RMUs) from sea turtle populations worldwide. The characteristics were: relative population size, genetic diversity of the populations and rookery vulnerability. This last aspect is important, because the disappearance of existing rookeries could have bigger consequences than other areas which are only used occasionally for nesting. Also, threats that are not related to global warming are taken into account in this RI:

$$\textit{Resilience Index}(RI) = \underbrace{(PS*w)+(RV*w)+(D*w)}_{\text{Risk}(R)} + \underbrace{[(F+T+CD+P)/4*w]}_{\text{NonClimatethreat}(NT)}$$

Formula (1): Resilience Index (RI), with PS = population size, ω = weight, RV = rookery vulnerability, D = genetic diversity, R = risk, F = fisheries, T = take/poaching, CD = coastal development, P = pollution and pathogens, NT = non climate-related threat.
(Fuentes *et al.*, 2013; Plot *et al.*, 2012)

West Indian Ocean, loggerheads from the North Pacific Ocean and hawksbill sea turtles from the East Atlantic Ocean and the East Pacific Ocean. The biggest threat for their resilience to climate change seems to be the fisheries bycatch. (Fuentes *et al.*, 2013) The turtles often drown in the fishing nets or are killed to be eaten when they are caught alive. (Catry *et al.*, 2009) Figure 10 gives a simple overview of the abundance of sea turtles from pre-human time period until now and also future scenarios.

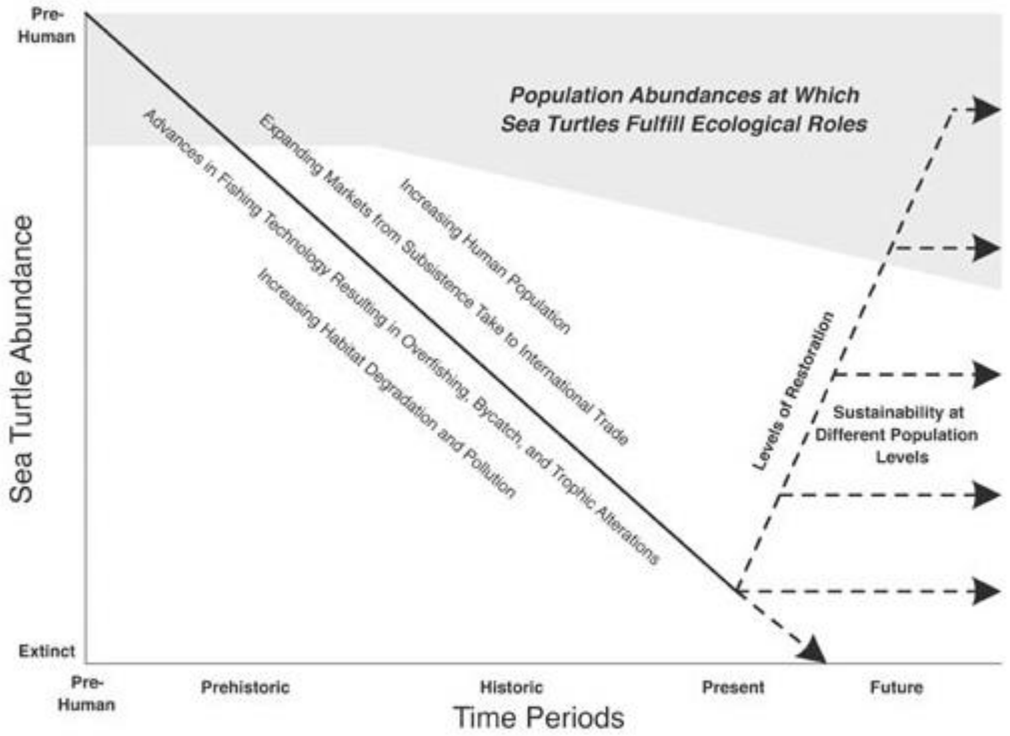


Figure 10: A schematical overview of the global abundance of sea turtles in history, present and possibly in the future. *Along the past decrease, human causes are written. The grey area represents the population abundances of sea turtles at which they completely fulfill their ecological roles in nature. In the future, possible scenarios of how the line continues are shown, which depend on the level of restauration. One scenario is that the populations will go extinct. Another scenario is that the populations are sustained at their present state (horizontal line with low sea turtle abundance) and the last scenario is that the populations will be restored towards abundances at which they can fulfill ecological roles, so ecosystem recovery is also promoted. (Bjorndal & Bolten, 2003)*

Natural Selection

It is thought that the PT (pivotal temperature) is genetically determined and heritable. (Warner, 2011) If this is the case, the two requirements mentioned above for TSD to evolve are met (since inter-clutch variability for the PT appeared common, see introduction). Therefore, the PT may change in response to selective pressures. This could also be the explanation for the existence of different types of TSD (Warner, 2011). Also, genotypes exist for which there is no 100% male temperature, so the female sex will develop at all temperatures (Schwanz *et al.*, 2013). This means that there will probably always be a minor bias towards the female sex. In the context of global warming, this can be concerning because these sex ratios could become more biased. Also, if temperatures are rising to fast, it could be difficult for TSD to evolve, because genetic variation is expressed only at intermediate temperatures. What is meant here is that for mutations that might be advantageous with regard to the new, warmer environment must be passed on to next generations. This can only happen if both males and females are still produced on the long term, which is only possible if the temperatures do not rise too rapidly. Then, not only the PT, but TRT (transitional range of temperature) values could both evolve. The wider the TRT, the higher the proportion of nests that produce both sexes. This is proven to be the case in sea turtles. So, both evolving P and TRT values influence natural sex ratios and are important for TSD species to adapt to climate change, because more mixed nests give the population a higher evolutionary potential. (Hulin *et al.*, 2009) However, studies exist that suggest global warming and climate change are too fast for an evolution of the PT to happen. (Hulin *et al.*, 2009)

Sea turtle species might adapt to global warming by means of natural selection for treats that are heritable and variable. Natural selection could perhaps favor sea turtles that fit the new environment better. For example, in response to selection, TSD itself could evolve. There are two requirements that have to be met before this can happen; 1) In natural populations, there must be variability in the sex determination, and 2) this variability is heritable. What is meant here is variability in how embryos react to incubation temperatures. If these requirements are met, any genetic factor that favors the production of especially the rarer sex, will have a genetic advantage (Hulin *et al.*, 2009). Schwanz *et al.* have indeed shown that species with TSD that live in fluctuating environments (see fig. 11A), will experience selection pressures favoring the rare sex. She and her collaborators found that especially two types of traits are selected for; the first type leads to more plasticity in responding to the environment (fig. 11B), the second type leads to the disappearance of TSD by evolving GSD (fig. 11C). (Schwanz, n.d.). The fact that TSD can evolve to (and from) both male and female heterogamety suggests that there must be a temperature-sensitive element to GSD that also has the ability to change under selective pressures. (Quinn *et al.*, 2011)

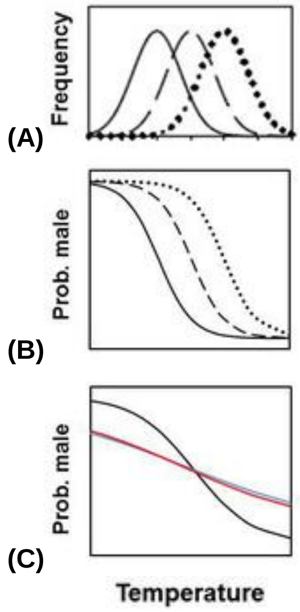
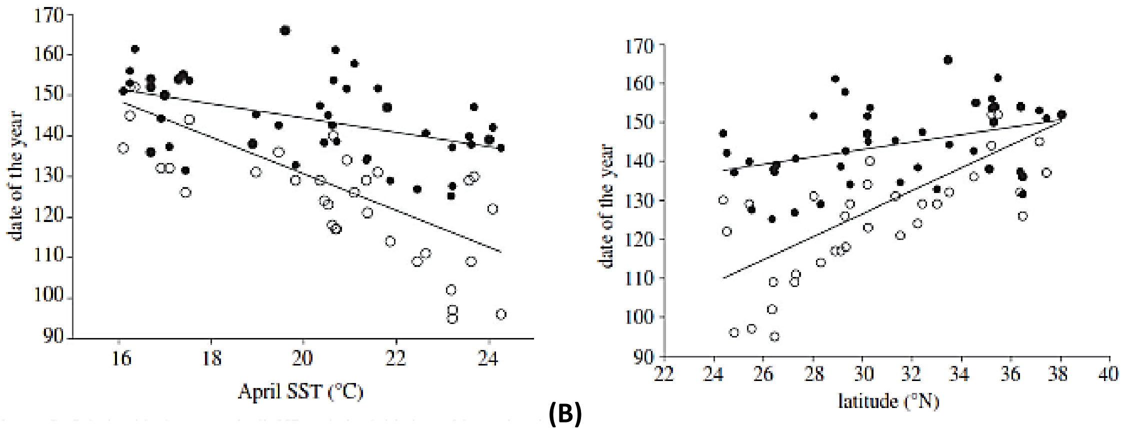


Figure 11: Three graphs from Schwanz et al, n.d.
(A) Schematical representation of sex ratios for environmental fluctuations in temperature (3 different scenarios) (B) Schematical presentation of shifting sex ratios due to plasticity in responding to the environment. (C) less skewed sex ratio for a system closer to GSD (red) than TSD (black) (Schwanz, n.d.)

It is thought that 3 million years ago loggerhead populations at the Indian-Pacific and the Atlantic-Mediterranean areas separated. In times of global warming, the loggerheads would have expanded their geographic distributions towards higher latitudes, where it is colder. During cooling events, however, they would reduce their range towards lower latitudes. (Mazaris *et al.*, 2012). This could be promising with regard to a similar adaptation from sea turtles for current environmental changes. The differences in behavior are also observed in the present, see fig. 12.

Natural selection could also favor turtles with certain maternal nesting behaviors beneficial under the new environmental conditions. (Hulin *et al.*, 2009) For example, turtles that build their nests deeper in the sand, or that choose to lay their eggs on a spot in the shadow, may get a selective advantage since their fitness has improved under the new conditions. In this way both sexes are still produced, despite the rise in temperature caused by global warming. Also, there might be a selection for turtles laying eggs in dryer sand, to compensate for the increased moisture of the sand caused by elevated sea levels and ground water table. There could even be a selective advantage for sea turtles nesting at beaches with light colored sand, since beaches with dark sand are relatively warmer, see Chapter 2, fig 7 (Laloë *et al.*, 2014). Normally, turtles that make such distinctive choices would not be selected for necessarily. Hard evidence for such a selection is very hard to find and might not even exist, more research is needed to find out whether or not sea turtles are already adapting. For example, mutations occur in each generation that are disadvantageous because they lead to these distinctive choices. But when these choices actually become advantageous, the genes holding these mutations would become more frequent in the population because they are now passed on to the next generation. For instance, in the 'normal' situation, such a sea turtle would have laid her eggs in too dry sand, for instance, but now the water levels are rising, this nest-site choice could be more desirable. The problem is that mutations are thought to occur more often in males than in females, because many more cell divisions occur during spermatogenesis than in oogenesis. However, a study about olive ridley paternal and maternal genes revealed that the mutation rates were a lot higher in females than in males. (Hoekert *et al.*, 2002) This could be advantageous since the sex ratios of sea turtles are becoming more female biased.



(A) **(B)**

Figure 12: Two graphs showing the onset of nesting for Loggerheads in the Northern hemisphere from data of 1980 - 2009*. (A) The relationship between the date (day) of the year the turtles start nesting and the sea surface temperature (SST) of April. (B) The relationship between the date (day) of the year and the latitude. White dots show the dates (days) of the first nests of the Loggerheads and the black dots are from the same data after regression, so the mean of the first days.* (Mazaris *et al.*, 2012)

Chapter 4: Adaptation at an Individual Level

It is clear now that environmental conditions of a nesting site are of crucial importance to the developing eggs. Sea turtles often lay their eggs in places with the desirable microenvironment. For instance, close to the vegetation or underneath trees, the shade will create a colder environment for the nest than in open areas, where there is no shade. Also, deeper nests are colder. (Pike, 2013; Théa, 2009) So, it seems that sea turtles could overcome the problem of rising temperature of beaches, just because their behavior makes them choose suitable nesting places.

Whether sea turtles are aware of their decisions is unknown, but there are examples that suggest that they at least search for the required environment before they lay eggs. For example, beaches used by sea turtles for nesting in north-eastern Australia are more exposed to wind and waves caused by this wind, but less exposed to cyclones, than beaches that are not visited by these turtles. In the south-eastern United States, sea turtles tend to lay their nests close to the Gulf Stream, which contains water currents beneficial for dispersing hatchlings towards better foraging areas. After choosing the right beach or location, sea turtles also choose from a range of environmental factors at that location. (Pike, 2013) Another study shows changes in nesting-behavior according to predation. At the Ras Al Hadd Turtle Reserve in Oman, green sea turtles come more often to lay eggs and dig them further away from the water in areas without much predation. In areas where there are a lot of humans and foxes, on the other hand, less nesting occurs and the nests are closer to the surf's edge. (Mendonça *et al.*, 2010)

Case study: Olive Ridley Nest-site Choice at Reserva Playa Tortuga, Costa Rica

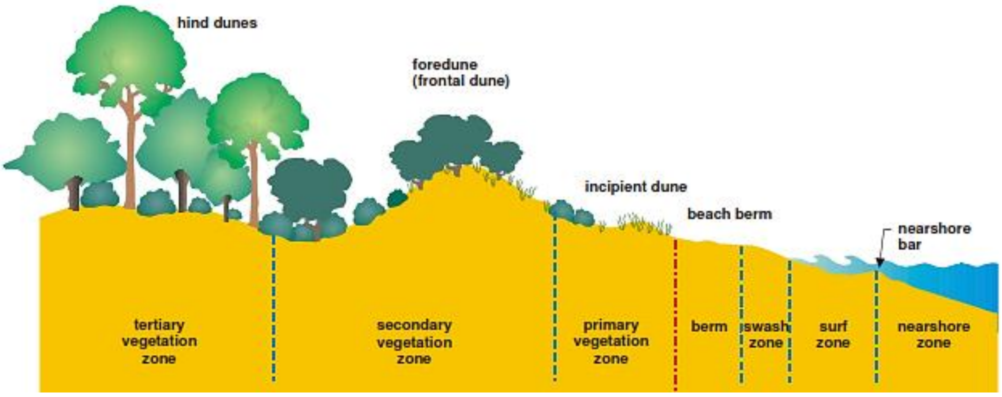


Figure (13): The features of a beach system. From the ocean to the vegetation, the beach is divided in different zones. *(Department of Land and Water Conservation, 2001)* (Figure slightly modified)

A database[1] from Reserva Playa Tortuga (RPT) covering information from 2009 – 2014 was used to examine the nest-site choice of the Olive Ridley sea turtle. Sea turtles seem to choose a suitable nesting place on a beach. A beach consists of different areas, dividing the beach into so called zones from the water all the way up to the heavy vegetation (see fig. 13). The zones used by RPT each year slightly differ from this figure. 'Zone I' has components of [1]both the surf and the swash zone. 'Zone II' is equal to the berm, and the primary vegetation zone is 'Zone III'. At the same time, the beach is divided into sections parallel to the water. After each 100 meter, a milestone is placed. This is why a section is also referred to as 'mojón' in Spanish. A cliff resides at the Northern end of Playa Tortuga, a

[1] *A collaboration agreement was signed, see attachments, appendix 1. The kind of data collected may differ between different seasons, for detailed materials and methods of the project, see the research article of the internship.*

river at the Southern end. Mojón 1 is at the cliff-end and the higher the mojón number, the closer the section lies to the mouth of the river. So, by dividing the beach into zones and mojónes, there are different sectors available as potential nesting sites the turtles could choose from. Mojón data was not available for the 2011 and 2012 seasons.

Data were analyzed using IBM SPSS Statistics, data was organized and graphs were made using Excel 2013.

<u>Results</u>

All available data on zone choice from 2009, 2010, 2011, 2012, 2013 and partially, 2014 were combined. In fig. 14 the proportions of turtles choosing a particular zone is shown. Over these 6 years, 24 turtles laid eggs in Zone I, 282 turtles in Zone II and 102 in Zone III. The observed values differed significantly from the expected values if a zone was chosen by a turtle at random (Chi-Square Goodness of Fit-test with two degrees of freedom = 257,471, p <0,005 with n = 408).

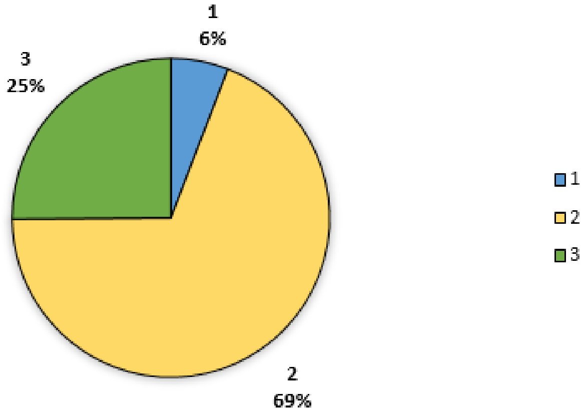


Figure (14): Pie chart showing Olive Ridley zone choice at Playa Tortuga over the 2009, 2010, 2011, 2012, 2013 seasons and a part of the 2014 season. n = 408.

Also, a higher number of turtles laid eggs closer to the cliff (See fig. 15), with a negative linear correlation between numbers of nests and mojón number, or distance from the cliff (Spearman's rank test, r.s. = -0,515, p < 0,005 with a total n of 247 nests examined).

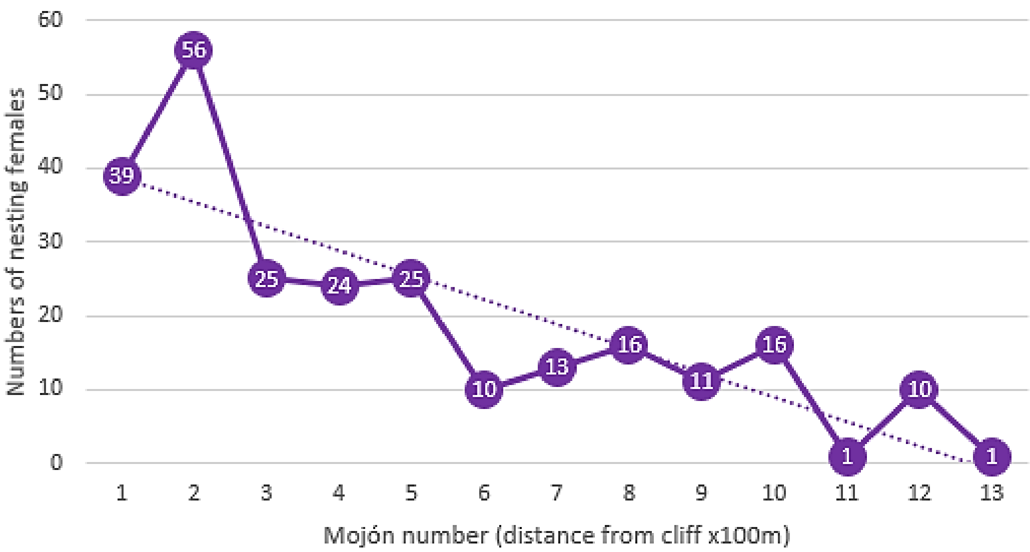


Figure (15): Graph showing Olive Ridley nest-site choice at Playa Tortuga on the beach parallel to the ocean. The further away from the cliff, the less turtles built their nests. In the graph, each data point gives the number of individuals nesting at the concerned mojón. Also, the trend line is shown (dotted line). The data are from turtle seasons of 2009, 2010, and 2013 and partly from 2014. n = 247.

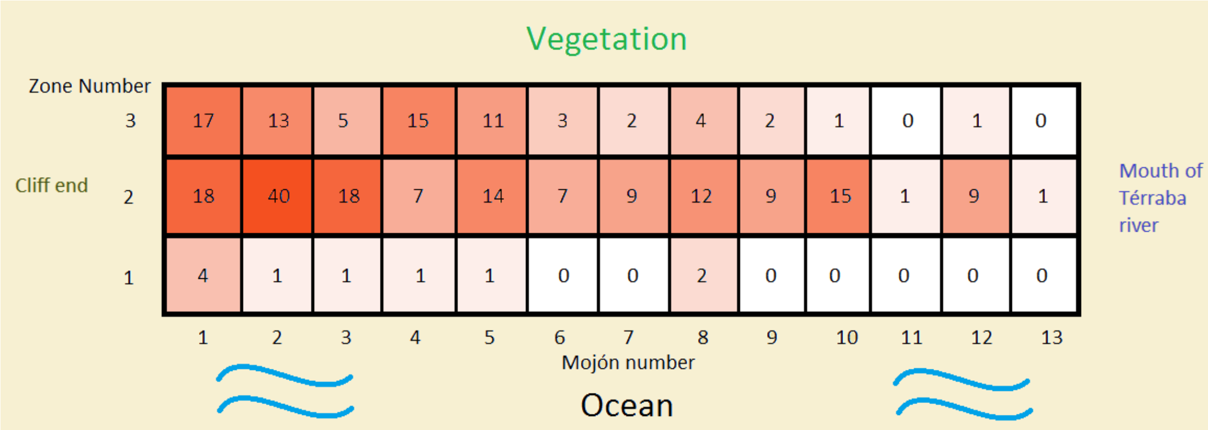


Figure (16): Schematic representation of nest-site choices at Playa Tortuga. What is shown is that the turtles seem to prefer building their nests in zone 2 and on the cliff end of the beach. For this map, information of 243 nesting females was used. The data are from the sea turtle seasons of 2009, 2010, 2013 and partly 2014.

So, this study proves that sea turtles do not make choices at random (see fig. 16). They seem to prefer the cliff-end of the beach and most often lay their eggs in zone 2. The reasons for these preferences have to be investigated further. Maybe the cliff provides necessary shadow, or the sand at the river end might be too moist. Also, when the tide rises, this end of the beach is sometimes flooded. This could also be the reason they prefer not to lay eggs in zone 1, since the sand is more often wet here. It is important to realize while examining figure 16 that the beach did not have the same length each year. Oscar Brenes mentioned the beach was 1,8km long in 1970, 1,5km in 2009, 0,91km in 2013 and 0,63km in 2014. These differences of course cause the numbers of nesting turtles to be lower at the river mouth end as well, but the mojón numbers only go to 13, even in 2009. For example, in 2009, 13 nests were laid from mojón 1-3 and only 4 for mojón 11-13. (The fact that the beach is less long as the years go by might be another concern for Playa Tortuga and its nesting olive ridley population).

Sea turtles have in fact endured dramatic changes in climate in the past. It is thought that they did adapt by redistributing their nesting sites. So, this behavioral flexibility could be of crucial importance for the survival of sea turtle species this time also. However, the main requirement for this to happen is that new nesting habitats suitable for the adapting sea turtles must exist. (Fuentes *et al.*, 2013) Also, it is shown for loggerheads that there is a strong significant relationship between the temperature of the sea surface and the onset of their nesting period. In warmer years, the turtles tended to lay their nests earlier in the season (Mazaris *et al.*, 2012). This is obviously happening on a population level, but it is also an indication that sea turtles, which are ectothermic, somehow get information from their environment and adjust their reproductive cycles and behaviors according to that information. It is therefore not an unlikely thought already mentioned by Girondot, that species might simply adapt to global warming by laying their eggs earlier or later in the season or change nesting location to colder areas on a beach. (Girondot *et al.*, 2004)

Chapter 5: When there is no Adaptation

If a proper adaptation by the species does not exist, this could have serious economic consequences. Besides this, it could disturb a variety of ecosystems. For several years, females would come to lay eggs. These nests would at some point probably all either overcook, drown because of the high water levels or produce almost only females or nothing, since they could be unfertilized. After a while, the sex ratios of existing population could become biased towards the female sex so heavily, that there are almost no males left for these females to mate with. This will lead to a small (nesting) population. In that case, the existence of the remaining sea turtle population is threatened because of the loss of genetic variation. These small populations will deal with inbreeding depression and genetic drift, which will only cause the populations to decline even further and go extinct. (Hoekert *et al.*, 2002)

Example of Interspecific Mating

There is also a study that revealed interspecific mating as a consequence of global warming. In this study, olive ridleys and loggerheads appear to mate with each other as a result of their disturbed sex ratios, and form hybrids. This could just be another way of nature changing as a cause of the environment changing. (Reis *et al.*, 2009) Perhaps, the development of a new species out of two different species is a solution if these species cannot adapt separately. The requirement would be that this new hybrid species is in fact adapted to the new environment. This population of hybrids might be a 'buffer' for the original species in times of environmental change.

Sea turtles are thought to be the most ancient vertebrates that can hybridize under natural conditions. This is probably due to their slow anatomic and chromosomal evolution. The chromosomal numbers and structures are maintained, which may allow species to produce fertile hybrids. However, the interspecific mating is probably only happening because the disturbed sex ratios facilitate it. For example, when there are a lot of females of each species and few males, the females could fail in finding the right mate of the same species. There could be behavioral barriers to hybridization, which would be disadvantageous. Also, it was shown that hybrids were only produced by the reproduction of olive ridley females with loggerhead males. (Reis *et al.*, 2009) According to the IUCN Red list, the olive ridley is vulnerable to extinction and the loggerhead is endangered ("www.iucnredlist.org," 2014). And of course, if a high number of hybrids arises, this will be a threat to the existence of the original species. (Reis *et al.*, 2009) So, it can still be seen as some kind of adaptation, if the hybrids serve as some kind if 'buffer' to the original species, but it is far from ideal since the original species would probably still go extinct.

Conclusions and Discussion

In conclusion, a lot of evidence showed that global warming is a real threat to sea turtles, by skewing sex ratios and also by cooking nests. This last factor could even be a more serious consequence. Sea turtles might adapt at a population level by natural selection, favoring the sea turtles that fit the warmer environment better. This has already happened during climatic changes in the past. Sea turtles might also adapt at an individual level by altered nesting-behavior. It is not known if sea turtles are aware of the choices they make, but they seem to have a preference for certain nest-sites over others. Several studies, including the case study at RPT, suggest that they receive information from their environment and based on this, they choose a nest-site with a microenvironment that is suitable for their eggs and eventually, their hatchlings. Sea turtles could, for instance, change the onset of the nesting season, or move towards higher latitudes to find colder areas. Especially since hatching success is affected more severely for nests on tropical beaches than for nests on beaches in regions with a more temperate climate, migratory patterns can be expected as adaptation. Also, it was observed that light colored sand will become more favorable, so turtles nesting on light-colored beaches gain more advantage over the ones nesting on beaches with dark sand. It is also proven that the resilience of sea turtles could rise by reducing other threats, like fisheries bycatch or poaching. Therefore, conservational projects are also of great importance to help sea turtles.

Conservational Projects

If there is no adaptation at all, maybe the only way to rescue the sea turtles is by means of conservational projects. Therefore, if we want to protect animal species, we should try to learn as much as we can about the influences of climate change and by that, estimate how these species would be doing under future scenarios (Fuentes *et al.*, 2013; Pike, 2014) It is a good thing that all sea turtle species are already protected by law and that a lot of nesting beaches are included in such conservational projects. Also, precautions regarding foreign fisherman settling in areas where bycatch of turtles is common are taken. (Catry *et al.*, 2009) A lot of conservational projects for sea turtles already exist, with a common management decisions system (see figure 17), but more are needed. There are, however, a lot of challenges in trying to maintain species in such a way (these are highlighted in table 2).The possibility of breeding sea turtles in captivity could help conservation. However, these 'sea turtle farms' would be really expensive and it is not shown yet that these farms would actually be beneficial for wild populations. The thought behind it was that many sea turtle hatchlings die in the ocean, so releasing adult sea turtles raised in sea turtle farms can make numbers

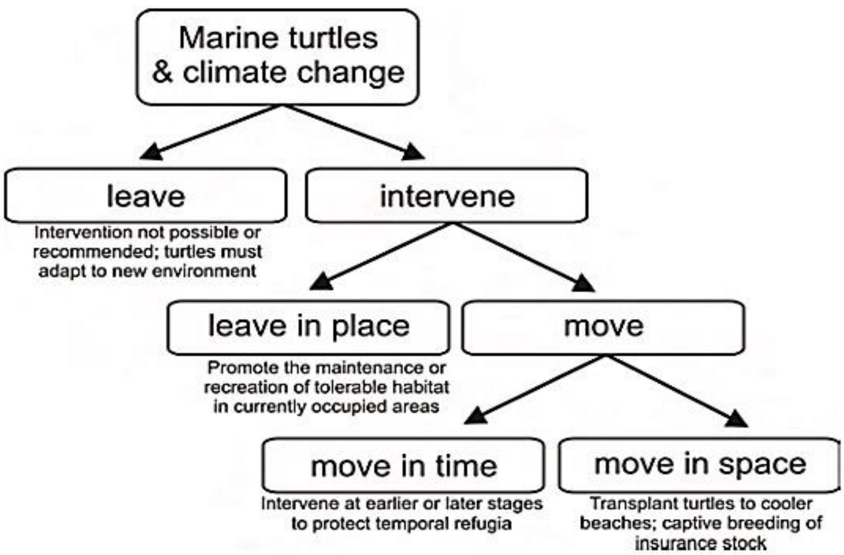


Figure (17): Tree-diagram showing conservational strategies for sea turtles experiencing climate change. *(Mrosovsky & Godfrey, 2010)* in the wild grow faster than they would under natural conditions. However, these released sea turtles seem to hardly join breeding populations because the migratory movements of sea turtles in their juvenile years determine their behavior as adults. They never learned this. Also, diseases and parasites could be introduced from sea turtle farms into wild populations and the possibility of releasing any turtles with a different genetic background into wild populations is concerning. (Eckert *et al.*, 1999) Eckert *et al*. describe a variety of conservational projects in detail, from how to protect eggs in hatcheries as well as *in situ*, to lowering fisheries bycatch and to the protection of foraging and nesting habitats. (Eckert *et al.*, 1999) It has in fact been proven, for example, that conservation efforts in Southern Brazil resulted in a 10-fold increase in nesting sea turtles over the last 11 years. (Plot *et al.*, 2012)

Table (2): Challenges for conservation of sea turtles. On the left the six main challenges are listed, with on the right a more detailed explanation. (Mrosovsky & Godfrey, 2010)

Challenge	Explanation
Regional	Turtles often nest on beaches that are not monitored
Sensitivity to temperatures	A lot of knowledge is needed in such a project to ensure precisely the right temperature for nests and so, maintain desirable sex ratios
Determination of the sex	To be 100% sure if a hatchling is male or female, the gonads must be examined histologically, which means the animal must be sacrificed. So, methods and knowledge are needed about how to estimate sex ratios of clutches, which also brings more subjectivity than histology does.
Permits	Gaining permissions and associated paperwork can take a lot of time and effort. Also, most species are threatened according to the IUCN Red List, which is sometimes an obstacle for research.
TSD	More understanding is needed regarding the advantages of TSD and the way sexual differentiation actually depends on temperatures
Other threats	As mentioned in chapter two, not only rising temperatures but also other consequences of global warming and climate change threaten sea turtles, like sea level rise, flooding, hurricanes, effects on sources of food, etc. These are all different obstacles for conservational projects

TSD and GSD as a Continuum

The proportion of nests that produces both sexes increases when the TRT values increase. The interesting thing is that when this happens, this proportion tends toward the proportion of mixed nests produced in the case of GSD. (Hulin *et al.*, 2009) More evidence is found stating that the mechanisms underlying the development of the sexual phenotype of offspring are similar for GSD and TSD systems. (Warner, 2011) It is estimated that in lizards, TSD evolved independently from GSD for at least five times. In turtles, at least six possible occasions of GSD evolving from TSD exist. (Schwanz *et al.*, 2013) Schwanz *et al*. found a new evolutionary endpoint of sex determination, in which both genotypic and temperature dependent elements are embedded. Some scientists agree that the sex determination is a continuum between GSD and TSD on the developmental level. (Warner, 2011) According to this article, several species are mentioned that in fact possess a mixture of GSD and TSD, in which sex chromosomes are present, but sex is also determined by environmental factors. So, GSD and TSD do not appear as black and white as was previously thought, but differences of opinion remain. It seems more research is necessary about both ultimate and proximate aspects of the evolution of GSD and TSD.

So, of course, the dangers of altered sex ratios do not only go for sea turtles, but for all turtles, other reptiles and all other species with TSD (Pike, 2014). Most of the information combined in this thesis could therefore be extended to all TSD species that face extinction. Their evolution should be rapid, because the extinction for small populations is greater for TSD species than for GSD species. (Girondot *et al.*, 2004). For example, the opposite of what is happening to sea turtles happens to crocodile species. They will experience decreases in number because of a male biased sex ratio as a result of rising temperatures. (Charruau, 2012) The same goes for caimans (Simoncini *et al.*, 2014) and, for example, the tuatara (Grayson *et al.*, 2014). However, global warming does not alter sex ratios of TSD species only. This is to be expected, since it was mentioned that GSD and TSD are not necessarily two separate systems as previously thought. For instance, an article by Barros *et al*. suggests maladaptive long-term effects of skewed sex ratios of the European shag, caused by global warming. (Barros *et al.*, 2013). Another study found male-biased sex ratios in Neotropical primates, even though they are mammals with GSD, because birth seasons and climatic conditioned are affected by climate changes. (Wiederholt & Post, 2011) Another research shows that during warmer years, more male elephant seals tend to be born in the North Pacific Ocean. They also fear an overabundance of males caused by global warming. (Lee & Sydeman, 2009) A very recent study conducted in Japan claims that even human sex ratios are altered and that this is correlated to global warming. Fukuda and colleagues found an increase in the number of male fetal deaths over the years from 1968 until 2012 and they think this is associated with climate changes. According to them, male fetuses are more vulnerable to external stress factors than are female fetuses. (Fukuda *et al.*, 2014) If this is not a reason for us humans to be seriously concerned about the effects global warming can have on the sex ratios of a lot of animals, including ourselves, I do not know what is.

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Attachments

APPENDIX (1)

RESERVA PLAYA TORTUGA • Centro Científico de Investigación y Conservación
Ojochal de Osa • Puerto Cortés • Puntarenas • Costa Rica
Céd Juríd. 3-006-629328 • Teléfono: (00506) 2786 5200
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Collaboration agreement

Between

RESERVA PLAYA TORTUGA & Manon C. de Visser

I. Agreement description

Collaboration agreement between Reserva Playa Tortuga (RPT), NGO located in Ojochal de OSA, Puntarenas, Costa Rica and Manon C. de Visser (The Researcher) Passport number: *NMLD7JPD3*

The agreement allows the researcher to use the data base as part of the information set required to execute the investigation about sea turtles entitled:
Sea turtles, temperature-dependent sex determination and global warming. Both for thesis and research article

needed to obtain the Biology grade of the Utrecht University.

II. Term of agreement

The term of duration of the collaboration agreement covers the period from July 1st 2014 until December 1st 2014.

III. Reserva Playa Tortuga responsibilities

1) The RPT Sea Turtle data base will be available for use of the researcher, during the term of the agreement.

2) Cooperate and assist the researcher to clarify, explain possible details or confusions in the data base.

IV. Researcher responsibilities

1) The researcher has to recognize RPT in the study as a source of information and always respect the author rights.

2) Keep a unique and exclusive access to the data base.

3) Give to RPT a digital copy of the report of the research done using the data base.

4) The Researcher knows that there is not any kind of employment relationship with Reserva Playa Tortuga.

VI. Modification & Cancelation

1)This agreement can be canceled without a previous cause and unilaterally by either party to sign here, having as unique requirement a written notification 15 days before. This notification should include the agreement cancelation date and if is necessary adding final instructions convenient for both parts.

2) All the modifications of the present agreement must be submitted in written form and have to be accepted before execute them.

VII. Execution & Signature

The agreement will be effective with the signature of the RPT director , Oscar Brenes, Manon C. de Visser and the University Supervisor and it execution begins July 14th 2014 until December 1st , 2014.

Oscar Brenes, Director de RPT in Costa Rica & Manon C. de Visser from the Utrecht University manifest compliance with all the clauses in this collaboration agreement, and proceed to endorse it with their signatures to make it effective

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Oscar Brenes-RPT Director

Manon C. de Visser

Utrecht University Supervisor