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Gonad Development and Hormone Titres in Loggerhead Sea Turtles (*Caretta caretta*) in the NE Atlantic

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Abstract

The study proposed to describe sexual development in pelagic stage loggerhead sea turtles *Caretta caretta* and compare this to hatchlings and adults. It is meant as an ontogenic approach, in order to understand reproductive development and population composition and their dynamics in the pelagic environment. The study focused on the pelagic loggerheads that are found in the waters offshore Madeira Island (Portugal) in the North-eastern Atlantic and use it as a developmental habitat.

The innovating character of this work relied on the lack of any description regarding the gonad ontogenesis and reproductive development for the pelagic stage in any of the 7 existing sea turtle species, all of them in danger of extinction.

Three methods were used to diagnose the sex of each juvenile individual and asses the level of reproductive development: (1) laparoscopy, (2) gonad biopsy and (3) the assessment of two sex steroids circulating levels, namely testosterone and estradiol.

In order to cover all life stages and compare data obtained for the juvenile stage, hatchlings and nesting female adults were sampled at the nearest nesting rookery at Boa Vista Island in the Cape Verde Archipelago. Gonads from dead hatchlings were collected for gonad histology and blood was collected from nesting females for sex steroids assessment.

Laparoscopies revealed to be a valid sexing method for the juvenile stage, since gonads are morphologically differentiated at these size classes. Moreover, laparoscopy was validated using gonad histology.

Gonad histology of juveniles showed that gonads are already completely differentiated into ovaries or testes at the size classes examined, but development seems to be quiescent.

Males present already developed seminiferous tubules with spermatogonia lining the interior of the seminiferous tubule. Female gonads present oocytes at different development stages, but only oocytes up to stage III were observed. The maximum oocyte diameter in each individual correlated with body size, suggesting that reproductive development is an on-going process in juvenile females.

The circulating levels of both testosterone and estradiol in juveniles of both sexes were very low and consistently lower than the ones observed in the nesting females from Boa Vista Island.

No bimodal distribution was found for any of the sex steroids analysed and thus circulating hormone levels were not a reliable tool for sexing juvenile individuals with a non-invasive technique. The ratio testosterone:estradiol did not show a bimodal distribution either.

The levels of testosterone correlated with sea surface temperature. The fact that temperatures observed during this study were below 24ºC might have hindered a differential testosterone pattern between juvenile males and females.

Sex ratios for this population were generated according to laparoscopy results and compared among years and size classes. An overall sex ratio of 2 females for each male was found, but they varied among size classes but not among years. Possible causes for the sex ratios observed are discussed.

This study is a contribution to our knowledge on the pelagic stage of loggerhead turtles, namely on the population structure regarding sex ratio, which is a vital tool for implementing conservation strategies.

Resumo

O estudo propôs-se descrever o desenvolvimento sexual no estadio pelágico de tartaruga comum *Caretta caretta* e compará-lo com o dos neonatos e adultos da mesma espécie.

Pretendeu-se uma abordagem ontogénica de modo a compreender o desenvolvimento reprodutivo e a composição populacional e respectiva dinâmica em ambiente pelágico.

O estudo focou as tartarugas comum que se encontram nas águas da Ilha da Madeira (Portugal) no Atlântico Nordeste e que usam estas águas como habitat de desenvolvimento.

O carácter inovador deste trabalho reside na ausência de qualquer descrição da ontogénese da gónada e do desenvolvimento reprodutivo para o estado pelágico de todas as 7 espécies de tartarugas marinhas existentes, todas elas ameaçadas de extinção.

Usaram-se 3 métodos para diagnosticar o sexo de cada indivíduo juvenil e avaliar o nível de desenvolvimento reprodutivo: (1) laparoscopia, (2) biópsia da gónada e (3) medição dos níveis circulantes de duas hormonas esteróides, nomedamente de testosterona e de estradiol.

Por forma a descrever todos as fases do ciclo de vida e comparar os dados obtidos para o estadio juvenil, amostraram-se neonatos e fêmeas adultas da população nidificante geograficamente mais próxima, designadamente na Ilha da Boa Vista no Arquipélago de Cabo Verde. Foram amostradas góndas de neonatos mortos para histologia da gónada e recolheram-se amostras de sangue das fêmeas nidificantes para medição de esteróides sexuais.

A técnica laparoscópica revelou ser um método de diagnóstico do sexo válido para o estadio juvenil, uma vez que as gónadas se encontram morfologicamente diferenciadas nestas classes etárias. Adicionalmente, a laparoscopia foi validada através da histologia da gónada.

A histologia da gónada em juvenis revelou que as gónadas estão já completamente diferenciadas em ovários e testículos nas classes etárias examinadas, mas o desenvolvimento aparenta estar quiescente.

Os machos apresentam túbulos seminíferos desenvolvidos com espermatogónias no interior do túbulo. As gónadas das fêmeas apresentam oócitos em diferentes estadios de desenvolvimento, mas observam-se oócitos apenas até ao estadio III. O diâmetro máximo do oócito está

correlacionado com o tamanho do indivíduo, o que sugere que o desenvolvimento reprodutivo é um processo contínuo em fêmeas juvenis.

Os níveis circulantes de testosterona e de estradiol em juvenis de ambos os sexos foram muito baixos, e consistentemente mais baixos do que os observados em fêmeas nidificantes da Ilha da Boa Vista.

Não foi encontrada uma distribuição bimodal para nenhuma das hormonas esteróides analisadas e deste modo os níveis hormonais não são fidedignos como ferramenta não invasiva para diagnóstico do sexo em indivíduos juvenis. O racio testosterona:estradiol não mostrou também uma distribuição bimodal.

Os níveis de testosterona apresentaram uma correlação com a temperatura de superfície do oceano. O facto das temperaturas observadas durante este estudo estarem abaixo dos 24ºC poderá ter impedido uma resposta hormonal distinta entre machos e fêmeas juvenis no que respeita à testosterona.

A laparoscopia permitiu a identificação dos *sex ratios* para esta população, os quais foram comparados entre anos e classes etárias. Encontrou-se um *sex ratio* de 2 fêmeas para cada macho, tendo-se observado variações entre classes etárias mas não entre anos. As causas possíveis para os *sex ratios* observados são discutidas.

IV

Este estudo é uma contribuição para o conhecimento do estadio pelágico da tartaruga comum, nomeadamente da estrutura populacional relativa à razão dos sexos, ferramenta esta vital para a implementação de estratégias de conservação.

The water was warm. There was plenty to eat.

The turtles had everything turtles might need.

V

And they were all happy. Quite happy indeed.

...

And the turtles, of course... all the turtles are free

As turtles and, maybe, all creatures should be.

(from: Myrtle the Turtle by Dr. Seuss)

Juvenile Pelagic Stage Loggerhead Sea Turtles (*Caretta caretta*)

Dedicated to my grand-mother Cila (or aunt-great-grandmother)

who knitted me my first pet-puffy turtle, somewhere 'half my life ago',

long before I started my 'turtle season' VI

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Dr. Thomas Dellinger, Paulo Branco, Nélio Freitas and João Monteiro, among others, for the photos while general "turtling".

List of abbreviations

AR: androgen receptor

B: corticosterone

Bkm: banded krait minor

CCL: Curved Carapace Length

CITES: Convention on International Trade in Endangered Species of Wild Fauna and Flora

DDE: Dichlorodiphenyldichloroethylene

DHT: dihydro-testosterone

E_2: estradiol

EDC's: endocrine disrupting components

EEZ: Exclusive Economic Zone

ER: estrogen receptor

ESD: environmental sex determination

GPS: Global Positioning System

GSD: genotypic sex determination

GSI: Gonadossomatic Index

IUCN: The World Conservation Union

mRNA: mitochondrial ribonucleic acid

mtDNA: mitochondrial deoxyribonucleic acid

nDNA: nuclear deoxyribonucleic acid

NE (trade winds): North-East trade winds

PCB's: polychlorinated biphenyl compounds

PIT: Passive Integrated Transponder

RIA: radioimmunoassay

SCL: Straight-line Carapace Length

SST: sea surface temperature

T: testosterone

TSD: temperature-dependent sex determination

TSP: thermo-sensitive period

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CHAPTER I
Sex in sea turtles

General Introduction

The natural history and the behavioural ecology of marine turtles have received growing attention during the past 4 decades in parallel with declining populations. However there are still many gaps in our knowledge of the life history and ecology of these marine reptiles, knowledge which is essential for their management and protection.

Seven species of sea turtles representing two families, Cheloniidae and Dermochelyidae, are the only living members of what has been a large and diverse marine radiation of cryptodiran turtles. These seven species include the loggerhead (*Caretta caretta)*, the green (*Chelonia mydas*), the hawksbill (*Eretmochelys imbricata*), the Kemp's ridley (*Lepidochelys kempi*), the olive ridley (*Lepidochelys olivacea)*, the flatback (*Natator depressus*) and the leatherback (*Dermochelys coriacea*) turtles (Meylan and Meylan, 1999).

All of them are endangered or threatened on a worldwide basis, and are protected under several national and international laws (Abreu et al., 1995; Groombridge, 1990; Márquez M., 1990). However, comparison of present population levels to historical levels are difficult to make because there is little or no information (Ross, 1995). Except for the Kemps' ridley, each species has a large proportion of widely distributed populations, and the number of large populations with which current populations can be compared is small. A further problem is the taxonomic confusion between species, namely between *Caretta* and *Lepidochelys* which, until recently, led to many misidentifications (Ross, 1995).

Macaronesian waters comprise the seas around and between the Madeira and the Azores Archipelagos, the Selvagens Islands (Portugal) and the Canary Islands (Spain) (Brongersma, 1995). The German botanist A. Engler gave this name to the Azores, Madeira (plus Porto Santo) and the Canaries in 1879, because of similarities in the plant life of these islands. The name comes from the greek *makaros* and *nesios*, and means the blissful islands (Wirtz, 1994).

These islands do not harbour any breeding marine turtle populations although breeding in the Canaries is historically referenced (Dellinger, in prep.; Liria and Lopez-Jurado, 2007). *Caretta caretta* is by far the most common species within Macaronesia. *Lepidochelys kempi*, *Chelonia mydas*, *Eretmochelys imbricata* and *Dermochelys coriacea* are known from relatively few records

(Brongersma, 1995). All of these species are also reported to Madeira (Biscoito, 1987; Cabral et al., 2005; Dellinger, in prep.).

Macaronesian waters are used by juvenile loggerhead marine turtles that stay in the area for a part of their life: they get there when they are young, as post-hatchlings and juveniles, and leave oceanic waters when they become adult (Bolten et al., 1998). Thus, Madeiran loggerheads belong to the Macaronesian loggerhead aggregation which is all constituted by juveniles ranging from small, nearly hatched, juveniles to large juveniles. The loggerhead turtles found in Madeira are almost exclusively (99%) from the nesting beaches of the west coast of the United States of America and from Mexico according to mitochondrial DNA data (Bolten et al., 1998). Since loggerhead marine turtles do not reproduce here we cannot define it as a true population, but for further discussions the juveniles occurring within Madeira or Macaronesian waters will be referred as a population.

Some authors include also the Cape Verde Archipelago (Republic of Cape Verde) within the Macaronesian region owing to its bio-geographical similarities with the aforementioned European archipelagos. Recently, a fairly large breeding population of loggerheads has been reported for this archipelago, and specially for Boa Vista Island (Cejudo et al., 2000). Juveniles of green turtles seem to use those waters as a feeding area (Nuria Varo, pers. comm.).

The tagging effort developed at Madeira since 1994 has revealed its first tag returns recently, with a recapture of one sub-adult in North Carolina 8 years after tagging and one recapture during a nesting attempt in Boa Vista Island (Cape Verde) in August 2005, 11 years after tagging (Dellinger and Ferreira, 2005). Thus, it is likely that Madeiran waters are used as a developmental habitat by a mixed-stock population of North Atlantic loggerheads. This might not be surprising, since when Bolten et al. (1998) assigned a source rookery for the juveniles from Madeira and Azores, the breeding population in the Cape Verde was poorly known and had not been sampled, and thus its contribution was not taken into account for the mixed-stock analysis. In fact, that study detected new haplotypes whose sources were not known at the time.

Species Description

Marine turtles were common in the cretaceous, 130 MY ago, and their fossil record extends back at least 200 MY. All present day genera and species originated in the period from the early Eocene to the Pleistocene, between 60 and 10 MY ago. Together with the marine snakes, crocodiles and iguanas, they are the only surviving marine adapted reptiles, and depend entirely on land for reproduction (except for some viviparous snakes) (Márquez M., 1990). These marine reptiles have only secondarily adapted to the marine environment as they evolved from terrestrial turtles over 100 MY (Pritchard, 1997). The minimum chronologic separation of modern sea turtle lines is probably no less than 30 MY, and *Dermochelys* at least 50 MY (Carr, 1995).

The loggerhead was originally described in 1758 as *Testudo caretta* by Linnaeus, but is currently known as *Caretta caretta* (Linnaeus, 1758). Genetic analyses from globally distributed sites have not provided support for dividing C. *caretta* into subspecies (Bowen, 2003; Bowen et al., 1994). Taxonomically it is placed in the

Phylum Chordata

Subphylum Vertebrata

Superclass Tetrapoda

Class Reptilia

Subclass Anapsida

Order Testudines

Suborder Cryptodira

Superfamily Chelonioidae

Family Cheloniidae

Genus *Caretta*

Individuals of the species *Caretta caretta* are diagnosed by the two pairs of prefrontal scales, the conspicuously large heads and the elongated carapace, thickened above the caudal region. The head is very broad and triangular in shape, with powerful jaws. They usually have five pairs of well cornified costal scutes; neural bones are usually 7 or 8. The dorsal scutes do not imbricate, except in very young specimens. The adults' vertebral scutes are smooth and do not overlap. C. *caretta* has five pairs of pleural scutes, the first contacting the pre-central and usually with three or four inframarginal laminae enlarged and poreless, and 12-13 marginal scutes. Two rudimentary claws are present on each flipper but the claw on the first digit has a specialized secondary function in adult males, in which is enlarged and hooklike, and is used for clasping the anterolateral marginal area of the female during copulation. The carapace is reddish-brown and the plastron yellowish-brown. Both the carapace and plastron of the loggerhead are heavily keratinized as a protective barrier against attack and the environment (Dodd, 1988).

Juvenile vertebrals are keeled with a knob-like process on the posterior portion of each keel (most distinct on the anterior vertebrals). In juveniles the knobs generally disappear although the keels are still present, and by the end of the juvenile phase the keels also disappear (Brongersma, 1972). These are supposed to provide what may be protective armor against some oceanic predators. Larger juveniles (> 45 cm SCL) that have begun to feed in shallow coastal waters have lost their thickened shell scutes, and their carapace colouration is often masked by fouling organisms such as algae, hydroids, and barnacles (Witherington et al., 2006).

Distribution

The total range of the loggerhead turtle includes foraging areas, migration corridors, and nesting beaches distributed throughout the subtropical and temperate oceans of the world (Dodd, 1988).

Thus, loggerhead turtles are circumglobal, inhabiting continental shelfs, bays, lagoons, and estuaries in the temperate, subtropical and tropical waters of the Atlantic, Pacific, and Indian Oceans.

The major nesting grounds are generally located in warm temperate and subtropical regions,

with the exception of Masirah Island, in Oman. Nesting does occur in tropical regions, and nearly all nesting occurs between 19 and 36 degrees latitude in each hemisphere (Witherington et al., 2006). Some warm temperate zone nesters are known to migrate to tropical waters in Australia and Africa after the nesting season (Dodd, 1988).

Loggerheads do not nest anywhere on the Atlantic coast of Europe and the anomalous absence of nesting grounds of *Caretta* in the central and western Pacific is unexplained (Ross, 1995).

Life History/cycle

All marine turtles share a common general life cycle that includes a iteroparous reproduction (Hirth, 1980), steroptyped nesting behaviour, laying of relatively large numbers of eggs several times during the reproductive season and relatively strong attachment to particular locations for nesting (i.e., philopatry), but inter- and intra-specific variation exists (Miller, 1995).

After hatching and emerging from the nest loggerhead hatchlings crawl down the beach and enter the ocean. Not much is known from this point onwards, but solitary as well as aggregations of young hatchling loggerhead sea turtles in the sargassum offshore the eastern coasts of the United States are reported (Caldwell, 1968; Carr, 1986b; Schwartz, 1988). Apparently loggerhead hatchlings use drift lines created by upwellings, downwellings, currents, and other types of convergences of different bodies of water (Carr, 1986a; Carr, 1986b; Carr, 1987). These convergence zones produce concentrations of resources that are rich in potential prey items for young turtles and floating material, such as *Sargassum* and debris from land sources, providing shelter for both turtles and prey.

For many years this pelagic phase out in the open ocean was known as the "lost year", and Carr (1986a; 1986b; 1987) speculated that hatchlings and juveniles would probably ride the currents and gyres in the North Atlantic between North America and Europe and go back as a subadult to the developmental habitats in the western Atlantic. Carr (1986) suggested that hatchlings born in the south-eastern USA become entrained in the "Gulf Stream-Azores" current and travel eastward to the Macaronesian area, returning to the western Atlantic in the "North Atlantic gyre".

Nowadays the "lost year" stage of life is known to span 6 to 12 years (Bjorndal et al., 2003a;

Bjorndal et al., 2001a; Bjorndal et al., 2000), during which the juvenile feeds and grows into a subadult.

Historical records of juvenile loggerheads are scarce (summarized by Carr (1986c)), but large numbers are reported from the Azores, Madeira and even between Madeira and mainland Portugal. Juvenile loggerheads are also found stranded on the coasts of northern Europe (Brongersma, 1972; Brongersma, 1982; Dellinger, in prep.).

After this pelagic or oceanic phase, where these juveniles stay in the Macaronesian region inhabiting the oceanic pelagic environment (Bolten 2003), and attain sizes up to 60 cm or more straight carapace length (personal observation) loggerheads migrate to the neritic feeding areas for a benthic phase, close to the same nesting areas where they were born. In the western Atlantic, most juveniles recruit from oceanic pelagic to neritic demersal habitats at a size 50 or more cm CCL (curved carapace length) at an estimated age of about 6 to 12 years (Bjorndal et al., 2003a; Bjorndal et al., 1999; Klinger and Musick, 1995). This ontogenetic shift in habitat utilization characterizes the passage from the juvenile to the subadult life stage.

Mating occurs during a relatively short female receptive period in the vicinity of the nesting beach (Owens, 1980) and the individual's mating season is completed before egg laying begins. The reproductive and ovipositional cycles are triggered and regulated by changes in specific serum gonadotropins and gonadal steroids (Guillette Jr. et al., 1991; Wibbels et al., 1992). Marine turtles show a strong conservationism during the nesting process and the nesting sequence is fairly similar between species, with only minor variations. They nest nocturnally on sandy beaches of mainland shores, islands and barrier islands and dig in material that ranges from fine siliceous particles to spherical pellets of calcareous algae in beaches within tropical areas. The homing ability, also called phylopatry, has been supported by evidence of spatial population structure in Atlantic loggerheads (Bowen et al., 1993; Encalada et al., 1998), as well as for other populations.

Marine turtles show no sociality (except for the particular case of *arribadas* on *Lepidochelys*) and no parental care but have evolved a successful array of adaptive responses to the demands of successive change in environments throughout the life cycle. They make breeding migrations because the ecologic systems in which successful feeding and successful breeding can occur are

often widely separated (Carr, 1995).

For reference of further discussions, the following size categories are defined as follows, adapted from Dodd (1988):

<u>Hatchling and Post-hatchling</u>: from hatching to the first few weeks of life; characterized by the presence of the umbilical scar.

<u>Juvenile</u>: the pelagic, oceanic life stage, typically referred to as "lost year"; pool of animals of a size and/or age at which little or no sexual development is occurring.

<u>Subadult</u>: from the end of the pelagic oceanic stage to the onset of sexual maturity, also called immature or benthic juveniles; pool of animals that move from oceanic to coastal, benthic foraging grounds.

<u>Adult</u>: attainment of full reproductive maturity, at different sizes, depending on population; the size at sexual maturity for males is assumed to be similar to that of females.

Conservation Status and Threats

The best assessments of marine turtles' abundance worldwide originate from nest counts on beaches. Temporal trends for pelagic loggerheads are much less accurate or even unfeasible due to the difficulties in having reliable, standardized in-water monitoring.

The current status for loggerheads is Endangered [EN A1abd ver. 2.3 (1994)] (IUCN 2007. *2007 IUCN Red List of Threatened Species*. <<u>www.iucnredlist.org</u>>. Downloaded on 17 October 2007), and the species is quoted in the Portuguese Regional Red Data Book (Oliveira et al., 2005). Thus, as well as all other marine turtles, loggerheads are listed under Appendix I (i.e., prohibited from international trade from or to signatory countries) of the Convention on International Trade of Endangered Species of Wild Fauna and Flora (CITES), Appendix II Bern Convention and Appendix I Bonn Convention, as well as various regional or national laws, regulations, decrees and acts. Particularly in Madeira, they are protected since 1985 (Dec. n 18/85/M) which was the first Portuguese law to protect a reptile species.

The main causes for species decline are due to human pressure (Márquez M., 1990). Threats such as severe pressure from local exploitation of eggs consumption and of adult females exploitation

for meat on the nesting beaches and incidental capture by commercial fisheries worldwide have been reducing the nesting populations worldwide. Accidental mortality became an increasing issue in sea turtle conservation with increasing fishing efforts (e.g., trawling, long-lining, gill nets, purse-seines). Thus, current populations are a fraction of historic levels. The considerable loss of nesting habitats to coastal development worldwide presents another threat, as well as climate change. The small size of most populations may be a result of continued pressure (Ross, 1995).

Although historically high numbers of turtles were caught for food and later for tourists as stuffed souvenirs (Brongersma, 1968; Brongersma, 1982; Dellinger, 2007), incidental capture by the commercial black scabbard-fish (*Aphanopus carbo* Lowe, 1839) fishery has been identified as the main source of by-catch within Madeiran waters. This fishing fleet captures over 500 loggerheads turtles every year (Dellinger and Encarnação, 1999; Ferreira, 2001). This accidental capture is the result of turtles attempting to take the bait, or becoming accidentally entangled in the line or hooked in the flippers (Lewinson *et al.* 2004). Measures to mitigate by-catch would allow the conservation of juvenile pelagic stage loggerhead turtles in the Madeira archipelago to be more successful, but would need a governmental backup to be implemented.

Additional sources of at-sea mortality and morbidity include other fishing methods, ingestion of and entanglement in marine debris, oil spills, and other pollution sources.

In fact, this population shows heavy metals contamination such as mercury and cadmium (Dellinger *et al.*, unpublished), but the long-term consequences of these contaminants accumulation at the individual and population levels are not known.

Turtles spend most of their lives submerged within the pelagic realm (Bjorndal, 1999), but nearly all sea turtle conservation efforts have focused on the two, easily accessible, life history stages, i.e., the eggs and the adult females on the nesting beaches. However, a species' endangered status implies the collection of population structure data and use of population modeling to fill information gaps, a prerequisite to its management.

Models developed for loggerhead sea turtles have suggested that it may be more valuable to protect older cohorts at sea, for example through the use of turtle excluder devices (TEDs), than those on the nesting beach (Crouse et al., 1987; Heppell et al., 1996). However, the scarcity of data inhibits these models from providing reliable quantitative analyses of important life history

parameters, like survivorship and age-at-maturity. Thus, rookery protection is of the out most importance for the species preservation but without protection of large juveniles and adults, the conservation of sea turtles will be unsuccessful (Carreras et al., 2004).

Therefore protection and conservation of oceanic stage turtles is extremely important (Crouse et al., 1987). One of the aims of this work is to contribute to a better understanding of the pelagic phase of this endangered species.

Sex Determination and Sex Identification in marine turtles

Sex determination mechanisms

One of the most fundamental traits for any species is reproduction. In sexually reproducing organisms the sex ratio is an important factor for population growth and therefore the existence of male and female organisms. A remarkable diversity of sex determination systems can be found among different animal taxa (Ciofi and Swingland, 1997), and vertebrates have evolved several different sex determining mechanisms. These are usually classified as either genotypic sex determination (GSD) or environmental sex determination (ESD). Even though, cases of mixed sex determination, i.e. combinations of genotypic and environmental sex determination are also observed, although the extent of their relative contribution varies. In these cases, both genetic and environmental cues play a role in an individual's sex phenotype. In fact, genotypic and environmental sex determination systems are now considered the extremes of a continuum (Sarre et al., 2004).

The most common type of GSD (or polygenic sex determination) involves sex chromosomes. In the GSD system sex chromosomes exert an ultimate control on whether the gonad will evolve to a testes or an ovary. The system of genotypic sex determination implies that the sex of an individual depends entirely on its genotype which is determined at the moment of fertilization, i.e., at the zygote stage, and is fixed from that moment on.

The GSD system is the most common sex determining mechanism for most vertebrates (Mittwoch, 1996), and is widespread among warm-blooded vertebrates such as mammals and birds, with

male heterogamety, and the sex chromosomes referred to as X and Y in mammals, and female heterogamety in birds, and sex chromosomes referred to as X and Z.

GSD is also present among reptiles, amphibians, and fish with either male or female heterogamety. In reptiles at least three variations of heterogamety occur for both sexes: XX/XY, XXX/XXY and pseudo XO when males are the heterogamic sex, and ZZ/ZW, ZZZ/ZZW and ZZZ/ZWW when females are the heterogamic sex (Ciofi and Swingland, 1997). Yet many fish and reptile species lack sex chromosomes altogether (Crews, 1994).

Only in the last three decades researchers have come to realize that many vertebrates also exhibit environmental sex determination (ESD). There are two basic types, behaviour-dependent and temperature-dependent, and in both instances gonadal sex is determined after fertilization (Crews, 2000). Environmental sex determination is common among reptiles and is also observed in amphibians and fish. ESD also occurs among invertebrate taxa (see Ciofi and Swingland (1997) for a review).

Environmental sex determination implies that the sex of an individual is determined irreversibly by the environment experienced during early embryonic development, i.e., post-fertilization. When the key environmental factor is temperature, it is referred as 'temperature-dependent sex determination' (TSD). In these cases, incubation temperature serves as the trigger to initiate the cascade of events that leads to the development of ovaries or testes. Other environmental cues are the chemical milieu, such as pH or the social status of the individual.

Temperature-dependent sex determination was first described in the lizard *Agama agama* (Charnier, 1966). In 1971, Pieau found that incubation temperatures influence the sex of hatchling turtles of the species *Testudo graeca* and *Emys orbicularis* (Pieau, 1971). Although the presence of H-Y antigens in some turtles (Pieau et al., 1979) has been taken as supporting the view that there is a genetic sex, temperature is far more important in specifying the phenotype and, whatever the case, the effect of temperature during incubation appears to be permanent (Yntema & Mrosovsky, 1982). Subsequent studies have shown that this phenomenon of temperature-dependent sex determination is widespread in reptiles and it has been demonstrated in more than 70 species from 43 families of reptiles either in laboratory experiments or in field studies (Ciofi and Swingland, 1997), including all crocodilians, some

lizards and most turtles (Bull, 1980; Janzen and Paukstis, 1991) and the tuatara (Cree et al., 1995).

Owens and Hendrickson (1978) were the first to hypothesise that environmental clues might be the mechanism responsible for variable sex ratios observed in marine turtles. TSD was first demonstrated for loggerhead sea turtles *Caretta caretta* (Yntema and Mrosovsky, 1979; Yntema and Mrosovsky, 1980) and subsequently documented for all the remaining marine turtle species (Wibbels, 2003).

TSD mechanisms are not homogeneous, and at least four different patterns have been described (Fig. 1):

a) Females develop at low temperature and males at high temperature, i.e., female-male pattern (FM);

b) Males develop at low temperatures and females at high ones, i.e., male-female pattern (MF);

c) Females develop at low and high temperatures and males at intermediate ones, i.e., female-male-female pattern (FMF);

d) The hatchling sex ratio of some species is not significantly influenced by incubation temperature.

At intermediate incubation temperatures intersexes are rarely formed; rather, the sex ratio varies and the effect of incubation temperature on sex determination is not due to differential mortality. Furthermore, the sex at hatching is believed to be permanent, extending to adulthood (Crews et al., 1995a). In all these cases there is only a very narrow temperature range at which both sexes are produced.
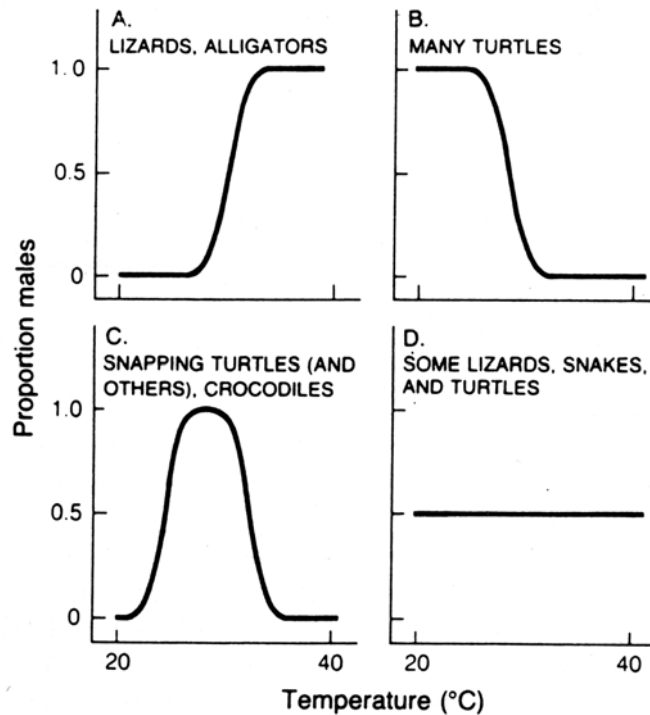
Fig. 1 Response of sex ratio to incubation temperature in reptiles (from Kraak and de Looze (1993)).

All marine turtles exhibit a male-female pattern of sex determination (Wibbels, 2003), i.e., temperatures below the pivotal temperature generate more males and above the pivotal temperature generate more females. Pivotal or threshold temperature is defined as the incubation temperature at which an even sex ratio is generated. For loggerhead hatchlings incubated under laboratory conditions males are produced at temperatures around 27°C, while females form at warmer temperatures around 31°C. A 1:1 sex ratio is produced at a pivotal temperature between the two extremes ($\approx$ 29°C) (Yntema and Mrosovsky, 1982). In the wild, incubation environments of approximately 29°C have also been found to produce an equal number of male and female hatchlings (Limpus et al., 1983; Marcovaldi et al., 1997; Mrosovsky, 1988). However, pivotal temperature can vary among different populations within a species (Limpus, 1985b). For loggerheads, pivotal temperatures have been reported to vary by up to 1.0°C in the wild (Wibbels, 2003).

Reptilian development is temperature dependent and faster at high temperatures. For most species temperatures below 18°C are lethal and temperatures below 24°C reduce hatching success. Thus, nest site selection is critical to ensure high temperatures, as well as adequate

moisture, for the whole process of embryogenesis.

As already stated, cooler temperatures produce males and warmer temperatures produce females, but within fluctuating environments such as sand beach temperature, the sex of the hatchlings is determined by the proportion of development at a given temperature, and not by the duration of exposure to that temperature (Georges et al., 1994). More precisely, sexual differentiation is not determined by temperature throughout incubation but by temperature levels prevailing during a critical period of the embryonic development, the thermosensitive period (TSP) for sexual differentiation. This sensitive period occurs during the middle third of the incubation period (Desvages et al., 1993; Maxwell et al., 1988; Yntema and Mrosovsky, 1982); until this period the gonad is considered bipotential.

TSD and Gonad Development

Sexual differentiation can be seen as a kind of phenotypic plasticity. In vertebrate species that lack sex chromosomes, gonadal sex is fixed during embryonic development, and thus gonadal sex is plastic during a short period of embryonic development (Crews, 2000).

Many studies addressed the underlying molecular mechanisms of TSD, but the physiological, biochemical, and molecular mechanisms by which incubation temperature influence the gonad's fate are still poorly understood, i.e., how the external signal of temperature is transduced into a signal that determines gonadal sex and channels sexual development.

The developing gonad itself does not appear to give the signal for its own differentiation into a testis or ovary, suggesting that an externally driven factor (or factors) moves into the developing gonadal tissue during a sensitive period to induce the normal differentiation, and possibly trigger the initial switch to one sex or the other (Desvages et al., 1993; Merchant-Larios and Villalpando, 1990).

Temperature possibly triggers the testis- and ovary-determining cascades for normal sex determination by acting on genes encoding for steroidogenic enzymes and steroid hormone receptors and modifying the endocrine microenvironment in the embryo (Fig. 2). The temperature experienced during embryonic development also has long-term functional outcomes in addition to sex determination (Crews et al., 1994).

Therefore, steroids play a pivotal role in sex determination in embryos and are a key element of sex determination due to the organizing effects of sex steroid hormones on the tissues that mediate reproduction (Crews, 1994). Only after the gonad is formed do hormones begin to exert an influence that modifies specific structures that eventually will differ between the sexes.

According to Deeming and Ferguson (1988; 1991) the dose of a particular molecule determines sex, whatever the sex determining system is: in GSD the dose is genetically specified and in ESD the efficiency of gene transcription, or translation, or the stability of the mRNA or gene product, or the activity of the gene product, is determined by environmental conditions.

Research on TSD indicates that gonadal sex depends ultimately on which genes encoding for steroidogenic enzymes and hormone receptors are activated during the middle third of embryonic development by temperature – i.e., the temperature sensitive period (TSP). Therefore, incubation temperature modifies the activity as well as the temporal and spatial sequence of enzymes and hormone receptors such that sex-specific hormone milieus, created in the urogenital system of the developing embryo, determine gonad sex. Thus, estrogens are the physiologic equivalent of incubation temperature and the proximate cue that initiates female sex determination (Crews et al., 1995a). In any case, testosterone (T) serves as a precursor molecule destined for conversion to dihydro-testosterone (DHT) (via 5α-reductase) or estradiol (E_2) (via aromatase), and incubation temperature is hypothesized to activate steroid hormone receptor genes [e.g., male-producing incubation temperature upregulates androgen receptor (AR) and female-producing incubation temperature upregulates the estrogen receptor (ER)] (Crews et al., 1995a).
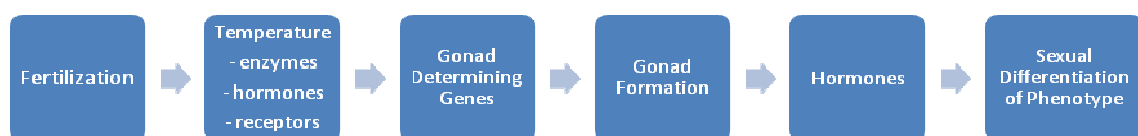
Fig. 2 Model for Temperature-Dependent Sex Determination (from Crews (1993)).

Studies using the administration of exogenous estrogens, antiestrogens and aromatase inhibitors, demonstrated the role of estrogens in sexual differentiation of the gonads in TSD species, and the activity level of the enzyme aromatase was well correlated with gonadal structure, since it increased exponentially in differentiating ovaries, whereas it remained low in differentiating testes (Pieau, 1996). Moreover, estrogens can even override the temperature effect and induce ovarian differentiation at masculinizing temperatures, as when injecting eggs with inhibitors of estrogen synthesis (aromatase) produces male offspring, even if the eggs are incubated at temperatures that usually produce females (Dorizzi et al., 1994; Rhen and Lang, 1994). In contrast, the administration of exogenous estrogen to an egg incubating at a male-producing temperature can reverse the effect of temperature and result in a female hatchling (Bull et al., 1988; Crews et al., 1991; Crews et al., 1989; Gutzke and Bull, 1986; Raynaud and Pieau, 1985; Tousignant and Crews, 1994; Wibbels et al., 1991a; Wibbels et al., 1991b). Similarly, when eggs are incubated at increasing temperatures that progressively produce a larger proportion of females, the dose of E_2 required to reverse the sex of 50% of the animals decreases significantly (Wibbels et al., 1991b). Moreover, the sensitive period for the effects of estrogens and their inhibitors coincides with the time when sex determination usually occurs (Bull et al., 1988; Gutzke and Chymiy, 1988), and there is a high correlation between the thermosensitive periods and increase in aromatase activity (Pieau, 1996).

This hormonally induced gonadal differentiation does start in the gonad itself since assays carried out on the gonads alone, i.e. separated from the adrenal/mesonephros, provided evidence that the gonads themselves respond to temperature shifts by modifying their sexual differentiation and are the site of aromatase activity and oestrogen synthesis during the thermosensitive period. Therefore, estrogens act locally on both the cortical and the medullary part of the gonad to direct ovarian differentiation (Pieau and Dorizzi, 2004).

Whether the effect of temperature in TSD species stands in the aromatase gene or protein itself or in other regulatory proteins is not known. Evidence that aromatase activity may be regulated by the sex-determining gene Sox9 comes from studies in two turtle species: when hatchlings were raised at female-promoting temperatures, the Sox9 expression was down-regulated during TSP, but at male-promoting temperatures, Sox9 expression was restricted to the medullary sex cords

destined to become Sertoli cells (Moreno-Mendoza et al., 1999; Spotila et al., 1998).

The morphological changes that occur during embryonic development of marine turtles – loggerheads included – was already described for both Cheloniidae and Dermochelyidae and is similar in morphological detail and sequence up to the stage 22 (see Miller (1985) for a detailed description of embryonic developmental stages). In general terms, development involves three main phases: 1) structural differentiation of body and organs (organogenesis), 2) functional development of organs and systems and 3) embryonic growth. Embryonic development begins immediately following fertilization, and although cleavage begins within hours after fertilization, development does not advance beyond the 6th stage while still within the female's oviduct. After oviposition, development resumes in a few hours (4 to 8, depending on temperature) and progresses. During stages 22-27 (which comprises the middle third of development and TSP) generic and species-specific characteristics become increasingly evident, such as the shape of the scales and the pigmentation of the carapace (Miller and Limpus, 2003).

Although the genital systems differ greatly in the two sexes, one sex frequently possesses rudiments of the structures characteristic of the other. This is due to the fact that for some time during embryonic life there is an indifferent or bipotential stage. The gonads may attain considerable size without showing specific features of either ovary or testis, and both male and female duct systems may differentiate to a considerable degree in potential members of both sexes. Eventually, however, there appears a definite sexual stage; the gonads become specifically testes or ovaries, and only the ducts and other accessory structures appropriate to one sex or the other continue their development. The nonpertinent structures characteristic of the opposite sex generally cease to develop and may be resorbed, but they are sometimes merely arrested in their growth, to persist as rudiments in the adult (Fig. 3).

The gonads first appear at a stage in embryonic history when most of the main features of other organ systems have been blocked and when the coelomic cavities are well developed. At this time, paired longitudinal swellings, the genital ridges, are formed along the roof of the coelom, lying lateral to the root of the mesentery and medial to the embryonic kidney. Primordial germ cells (PGC's) from wich eggs or sperm develop – large cells with a characteristic clear cytoplasm – may be identified at a relatively early stage in the germinal epithelium of the gonad. Usually,

the definitive gonad is formed toward the anterior end of the abdominal cavity; fat bodies or other structures may arise from abandoned portions of the genital ridge. The germinal epithelium of the ridge, continuous with the mesodermal lining of the rest of the coelom, forms the more important structural elements of the gonad; mesenchyme lining the epithelium forms connective tissues (see Ackerman (1997), Miller (1985) and Miller et al. (2003) for a review).

Merchant-Larios (1989) found that the ultrastructure of *Lepidochelys olivacea* primordial germ cells (PGC's) is very similar to that described for *Caretta caretta* during the migratory stage and the early colonization of the genital crest (Fujimoto et al., 1979). Two morphogenetic events occur in the undifferentiated gonads of *Lepidochelys olivacea* female embryos: surface epithelium thickening and medullary cord fragmentation. The male gonads, on the other hand, keep the same histological structure as the undifferentiated gonad. Thus, they are recognisable as "testes" only because they are not differentiated ovaries (Merchant-Larios, 1989).

Working under laboratory conditions, Yntema & Mrosovsky (1980) demonstrated that gonads of *Caretta caretta* reared for 1-7 weeks after hatching were similar to those of newly hatched specimens and recently Wyneken (2007) showed that captive reared hatchlings up to 120 g had perfectly distinguishable gonads.

It is logistically impossible to follow gonad development in wild animals. At hatching the gonads of C. *caretta* are differentiated as ovaries showing a conspicuous germinal epithelium, or as testes with well-developed primary sex cords; however, the oviducts in males are not resorbed by the time of hatching (Yntema, 1980). In turtle species such as *Sternotherus odoratus*, *Emys orbicularis* and *Testudo graeca*, gonads have also been shown to be histologically differentiated into testes and ovaries by the time of hatching (Pieau, 1971.). It is, then, implicitly believed that gonad sexual determination ends after hatching and sex cannot reverse after TSP.
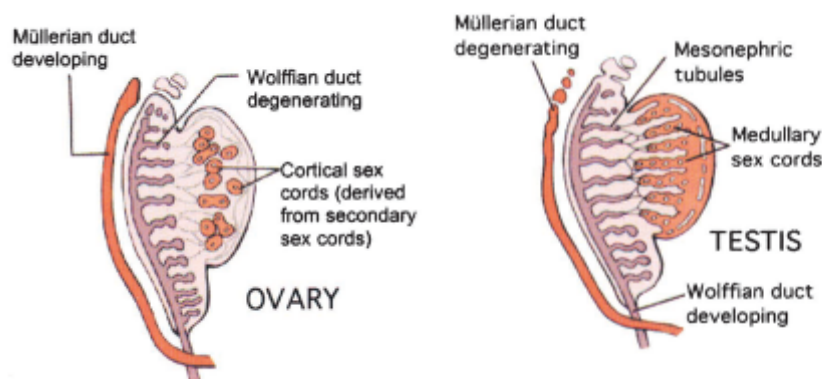
Fig. 3 Diagram of female and male gonadal differentiation in amniote vertebrates (from Crews (2003)).

Sexual dimorphism during ontogeny and sex identification in sea turtles

Ecological modellers of sea turtle populations require accurate quantitative data of population structure for developing predictive models needed for management decisions. Critical demographic parameters such as growth rates, survivorship, recruitment, age at first reproduction, percent of animals reproductively active each year, age and duration of the reproductive life history and sex ratio are essential for the development of population models (Owens, 1997) and are especially important for marine turtles since all species are threatened (Casale et al., 2006). However, demographic models and management plans are limited by the lack of data on the immature pelagic phase for sea turtle populations.

In most immature reptiles, however, identification of sex by external morphology is not possible (Owens et al., 1978), although for population modelling it is of interest to know the sex ratios for the very young cohorts, since they may affect the future reproductive rate of the population.

Loggerheads are bisexual and sexual dimorphism is only obvious in the sexually mature adults: males have longer tails than females (males:females, 3:1) and large curved claws used to hold on to the female during copulation (Fig. 4 A, B). Males have a shorter plastron, probably to accommodate the large muscular tail (Geldiay et al., 1982; Hughes, 1974a). Females, in contrast, do not develop any secondary sexual characteristics (Bolten et al., 1994).
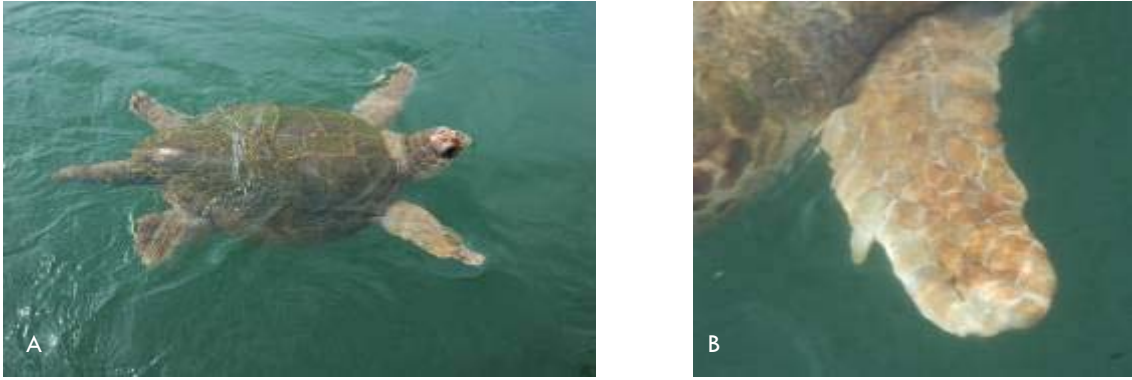
Fig. 4 (A, B) - Adult male loggerhead showing the developed tail (A) and fore-flipper claw (B).

Marine turtles require decades to reach maturity (Bjorndal et al., 2003a; Chaloupka and Limpus, 1997; Limpus and Chaloupka, 1997), and not all individuals mature at the same size even within the same population (Limpus et al., 1994a; Limpus et al., 1994b), since sexual dimorphism in marine turtles becomes apparent very late in the life cycle.

For example, Caldwell (1962) stated that in the Pacific black turtle *Chelonia mydas* secondary sexual characteristics become obvious at a carapace length of about 75 cm, but at the Grand Cayman turtle farm it was possible to misidentify large immature male green sea turtles (120 kg, 80 cm carapace length) as females based on external characteristics only (Owens et al., 1978). Hughes (1974a) reported that sexual differentiation was apparent in South-African loggerhead turtles 60 cm to 67 cm SCL, but the few animals within this size ranges observed (and sexed through laparoscopy) in Madeira Island did not show any secondary sexual characteristics.

Estimates from young captive loggerheads pin-pointed the age of sexual maturity at 6 to 7 years (Uchida, 1967), but these estimates from captive-reared animals are believed to be misleading (Bjorndal and Zug, 1995). Age at sexual maturity for loggerheads is now estimated to span 12-37 years (Bjorndal et al., 2000; Bjorndal et al., 2001b; Frazer, 1983; Frazer and Ehrhart, 1985; Heppell et al., 1996; Parham and Zug, 1997).

Because juvenile marine turtles lack sexual dimorphism, various methods to develop non-harmful sex diagnosing tools have been attempted in order to estimate sex ratios in species with non-dimorphic sexes.

Long before TSD was demonstrated in marine turtles, karyotyping was suggested as a method to distinguish the sexes based on heterogametic chromosomes (Makino, 1952). However, later

studies demonstrated that the karyotpe of *Caretta caretta* consists of 56 nearly identical chromosomes, and there are no obvious morphologically distinctive male and female chromosomes in this species (Bickham, 1979; Bickham et al., 1980). In fact, primary sex determining genes have not been identified in TSD species up to now (Schartl, 2004).

Several assays for H-Y antigen histocompatibility were developed for TSD species (Engel et al., 1981; Engel and Schmid, 1981; Wellins, 1987), since it is a sex-specific antigen in many vertebrates. These assays proved to be an accurate marker for sex but some conflicting results occurred (Zaborski et al., 1982; Zaborski et al., 1988).

Bkm (banded krait minor) DNA fingerprinting was also screened for sex specificity in green and Kemp's ridley sea turtles (Demas et al., 1990). The test proved it could potentially be used as a sexing technique, but was not validated. More tests are needed in order to evaluate whether sex can be reliably identified by molecular markers in these species. In any case, if proven valid, the logistics and costs of these methods would probably hinder their widespread use for examining large numbers of turtles (Wibbels, 1999).

Therefore, given the lack of any genetic or molecular biomarkers suitable and reliable for sex identification in TSD species such as marine turtles, 3 methods are currently accepted:

a) direct visualisation of the gonad in the case of dead animals (hatchlings and juveniles included), preferentially with histological validation (Mrosovsky & Benabib, 1990)

b) direct visualisation of the gonad through laparoscopy of live animals (in the case of juveniles and sub-adults)

c) indirectly by determining hormonal titres, which correlate with sex.

While direct visualization of the gonad, whether in dead animals or under laparoscopy, relies on the macroscopical and/or microscopical differentiation of the gonads either into testes or ovaries, hormonal sexing relies on the fact that males have higher testosterone levels than females. Since sea turtles are endangered species, invasive surgical techniques such laparoscopy should be avoided but, on the other hand, it is good practice, as far as possible, to use laparoscopic validation to independently verify the hormone technique (Owens, 1997). Laparoscopy and testosterone radioimmunoassay (RIA) are also characterized by logistical difficulties and expensive equipment, but they have proven to be practical in successfully sexing

large numbers of immature turtles (Wibbels, 1999).

New sexing technology such as the development of an assay for measuring the anti-Mullerian hormone (Wibbels et al., 2000) or assessing the female-specific vitellogenin (Roldan Valverde, pers. comm.) might prove to be feasible tools in the future for sexing juvenile turtles. Right now, a method for sexing immature sea turtles, both simple and reliable, has not yet been identified.

TSD and sex-ratios: why bother?

A general consequence of TSD is that biased sex ratios are more common in species with TSD than in species with genotypic sex determination (Bull, 1980), and these can vary widely (Mrosovsky, 1994). Biased sex ratios have obvious consequences for mating dynamics and population structure, as well as reproductive rate of natural populations (Orzack, 2002). Therefore, the importance of knowing natural sex ratios cannot be overstated regarding population dynamics for conservation and management purposes, since biased sex ratios can limit or increment a population's reproductive rate. In these endangered marine reptiles even small changes in the incubation temperature can cause dramatic changes in the sex ratio (Bull, 1980). On the other hand, on a more theoretical perspective, knowledge on natural sex ratios provides an important background for explaining the biological importance of TSD systems (Owens, 1997), as well as on life history and evolutionary biology.

For instance, Fisher's principle of even sex ratios applies widely to organisms where equal investment is made in male and female offspring. Since marine turtles have equal egg sizes (within the same species), do not show any parental care and mating occurs in large, effectively panmitic populations (Bowen and Karl, 2007; West et al., 2002), a 1:1 sex ratio would be expected. However, highly skewed sex ratios for natural sea turtle populations have been reported, which provides an interesting floor for debate, namely the issue of adaptively biased sex ratios.

The extent to which ESD operates in the wild is controversial. ESD was demonstrated in the laboratory in the European pond turtle *Emys orbicularis* (Zaborski et al., 1982), but a study on wild populations of this species revealed that in only 17% of the wild individuals the sex was determined by temperature (Girondot et al., 1994). Therefore, laboratory studies regarding

TSD and sex ratios, with controlled environmental conditions, are powerful research tools but do not necessarily correspond to the situations in the field. Knowledge of the situation in the field is thus indispensable in order to assess the potential existence of biased or dynamic sex ratios in the wild.

Although significant advances have been made regarding sex and reproduction in marine turtles, gaps remain for the juvenile phase, since open-water surveys addressing sex ratios and gonad development in wild juvenile pelagic turtles are logistically difficult to obtain, owing to both the at-sea sampling requirement and the absence of an accurate sexing method without deep manipulation of the animal. In fact, most ecological studies and conservation efforts on sea turtles have focused on the two, easily accessible, life history stages found at the beach, i.e., the eggs and adult females, and most of them deal with some aspect of reproduction. As a result, the literature concerning the reproductive biology of sea turtles is immense, albeit uneven (Miller, 1997), and focus only on adult or sub-adult stages, found close to nesting beaches. Additionally, male sea turtles are less well studied than females because they do not come ashore (Owens, 1997).

In order to develop appropriate management and conservation plans, methods to assess relative population abundance and population trends and structure for the oceanic stage are needed (Bjorndal et al., 2000).

Research Objectives

Although several studies of the reproductive biology of the loggerheads have been made on the several nesting populations, few or no studies have been done on the pelagic, oceanic stage population. In fact, although sea turtles spend at most 1% of their lives in or on nesting beaches – in the form of embryos, hatchlings, and adult females that emerge to deposit their eggs – approximately 90% of the literature on sea turtle biology is based on nesting beach studies (Bjorndal, 1999).

The present study investigates gonad ontogenesis as a central goal to understand the process of

gonadal sex differentiation in loggerhead sea turtles and how it correlates with sexual hormone levels. On the other hand, the generation of sex ratios for this population is a keystone objective for demographic models, and 3 different sexing methods were investigated (laparoscopy, gonad histology and sex steroid hormones).

Sex ratios from dead turtles stranded or accidentally caught in fishing gears have been provided for this same population, but sex-ratios provided by the analysis of dead stranded animals might not be a reliable indicator of the operational sex-ratio for a given population, as dead turtles may not provide a representative subsample of the entire population. Also, for dead sea turtles, sex determination can be difficult or unreliable, particularly in severely decomposed carcasses (Heinly, 1990). In a study done by Stabenau (1996) with the Kemp's ridley, gonadal decomposition resulted in sex being determined in only 50% of the stranded sea turtles examined from the upper Texas coast.

No large-scale study has characterized the sex ratio across size classes in wild juvenile loggerheads. Thus, the present study intends to address the above questions, by describing gonadal development through all life stages of loggerhead turtles, but with a special focus on the pelagic stage, which has never been attempted previously.

Gonads of living animals were described through laparoscopic inspection. Moreover, a small gonad area was sampled by localised biopsy using the same laparoscopic equipment with an additional biopsy forceps for histological observation and description.

In order to develop a non-invasive sex diagnosing technique and to find the most reliable hormonal indicator of sex in juvenile turtles as well as to understand how hormonal titres vary with gonadal development, hormone titers were measured concurrently with the laparoscopic and histological sexing techniques in juvenile individuals. Thus, a quantitative study of testosterone and estradiol-17β blood plasma levels in loggerhead sea turtles from the north-eastern Atlantic was conducted to:

    a) establish sex steroids titres as sexing criteria or thresholds levels,

    b) estimate the sex ratio for the wild juvenile population.

Summarizing, the objectives mentioned above were attained in three ways:

    a) Gonad observation through laparoscopy and image collection for gonad morphological

variability assessment;

b) Histological description of gonads and determination of their developmental stage, as well as laparoscopy validation;

c) Determination of sex hormone levels (testosterone and estradiol) and assessment of potential correlations between hormone profiles and individual's sex, as determined by laparoscopy.

Since only the juvenile life stage is observed in Madeira Island waters the closest nesting population in Boa Vista Island (Cape Verde Archipelago) was chosen to assess the hatchling and the adult life stages.

After sampling, all live turtles were tagged with flipper tags and/or Passive Integrated Transponder (PIT) tags.

This research was granted all the necessary permits by the official authorities to conduct the proposed studies, always having the animal's welfare in mind.

Materials and Methods

Considering that all sea turtles are listed under Annex I CITES, Annex II Bern Convention and Annex I Bonn Convention, research on these animals must assure that the individuals are kept alive and are released into the wild again. Thus, due to the logistic constraints inherent to working with protected species and the different life stages under assessment, different sampling approaches were adopted for the Madeira and the Cape Verde populations. Sampling in Madeira Island included blood sampling, laparoscopies and gonad biopsies of juvenile individuals. Sampling in Cape Verde included blood sampling in adult females only, and necropsy of dead hatchlings for gonad withdrawal and posterior gonad histology.

General biometry and tagging were applied for both populations according to the same criteria.

Sexing sea turtles

Currently 3 methods are available for sex identification of juvenile turtles (laparoscopy, gonad histology and blood sex steroids assessment) (Wibbels et al., 2000). These three methods were used in parallel and are described in the next chapters.

Study Areas

Two study sites were chosen in an effort to assess the whole life cycle: Madeira Island (Portugal) and Boa Vista Island (Cape Verde Archipelago). Both archipelagos are located in the eastern Atlantic Ocean (Fig. 5).
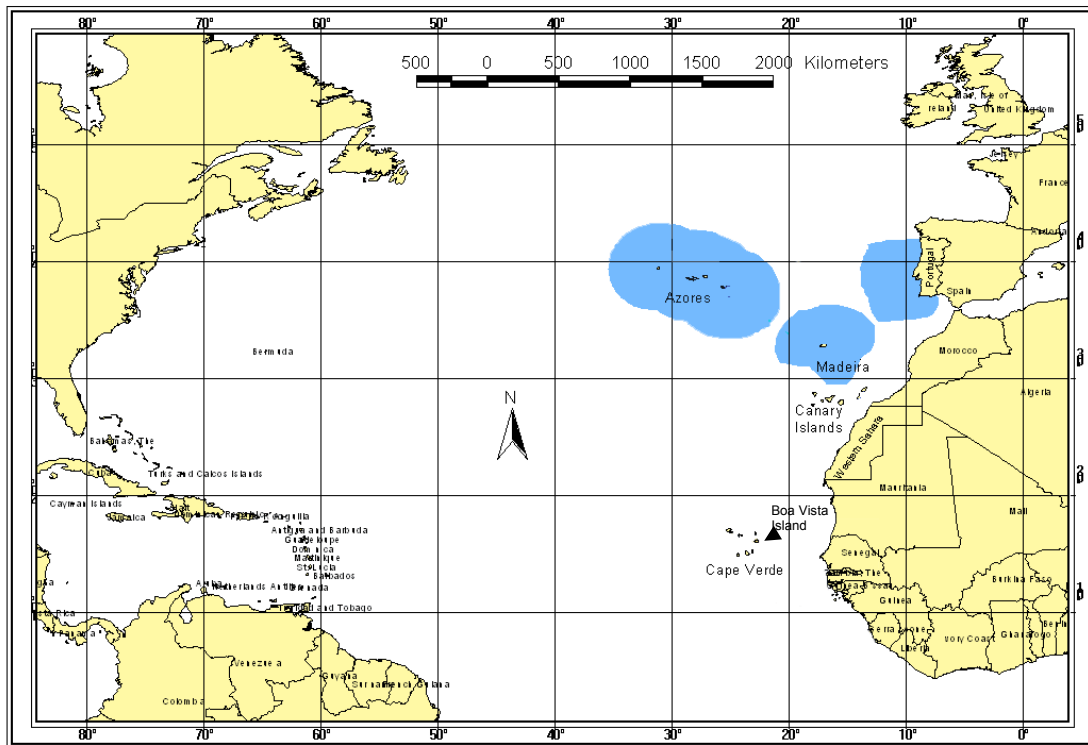
Fig. 5 Madeira and Cape Verde Archipelagos in the north-eastern Atlantic ocean. Blue areas are Portuguese Economic Areas (EEZ's).

Madeira Island (Portugal)

Study area description and population characterization

Madeira Archipelago (Portugal) is located off the north-west coast of Africa (33°N; 17°W), around 1000 km from the European continent and 500 km from the African coastline and is administrated as part of the Portuguese Economic Exclusive Zone (EEZ).

Madeira island is of volcanic origin (6 MY), emerging from the abyssal plain, and thus has no continental shelf. Depths such as 1000-2000 meters can be reached as close as 1-2 nautical miles from the coast, providing a truly pelagic/oceanic nursery environment for loggerheads on these waters.

The archipelago of Madeira is included in the general North Atlantic currents circulation system. The eastern side of the North Atlantic system consists of 4 currents: the Azores current, Portugal Current, the Canary's current and the North Equatorial Current.

The Canary current is the dominant surface current and the NE trades are the dominant wind regimes. The island mass effect phenomena provides a sheltered leeward area on the south coast, making potential spotting of sea turtles while basking easy during special warm and calm weather. The most probable cause of this phenomenon seems to be the obstruction caused by the island's interior mountain range to the dominant NE trade winds (Caldeira et al., 2001). Climate is subtropical with small temperature amplitudes, and the weather is highly influenced by the Azores anticyclone.

The Marine Turtle Project at University of Madeira started a tagging and monitoring program in 1994 focused on the pelagic stage ecology of the juvenile loggerheads offshore Madeira Island.

Sea Turtle Capture

The study was conducted between 2003 and 2006 offshore Madeira Island. Sampling seasons took place randomly, depending on sea and weather conditions. However, data regarding sex ratios obtained through laparoscopy providing from a previous project is also analysed here.

Marine turtle captures were conducted offshore south Madeira Island, and depended entirely on ocean and wind conditions. A 6.5m fibreglass boat with 110CV, 4 stroke outboard motor

belonging to the University of Madeira was used. For safety reasons, the boat crew comprised 2-3 persons, all acting as observers.

Turtles were searched for actively by boat up to 10 nautical miles offshore the Island, and they were captured without regard to their size or location by approaching them at slow speed from behind and picking them with a large dip-net (handle: 2.5m; mouth: 75cm diameter; netting made of thin nylon (gillnet material) (Dellinger et al., 1997). The exact direction of each search depended on sea surface conditions, usually following frontal systems where floating material aggregates.

For every sea turtle captured environmental conditions such as weather and ocean conditions were recorded, as well as behaviour, time, date and GPS position.

Following capture, all turtles were transported to land-based holding facilities at the Marine Biology Station of Funchal where they were maintained in fiberglass tanks filled with sea-water for a minimum of 24 hours to complete sampling procedures. Two large circular tanks (3 m diameter, 1.5m water depth, ~10000l) and 4 smaller squared tanks (~ 1000l) were available to keep the turtles. They were monitored periodically during captivity to assess health and well being. All turtles were returned unharmed to their habitat.

Biometry and Tagging Activities

All turtles were measured, weighted, photographed, and tagged during the holding period. A set of biometrical parameters were taken for posterior correlations with sex (Table 1), as described by Bolten (1999). Straight line measurements were taken using Haglöf forestry callipers (80 and 95cm) for large biometric measurements and Vernier callipers for small biometric measurements, both till the nearest mm. Over the curve measurements were taken using a flexible tailor's tape and weight was determined using an electronic platform balance (Mettler Spider1s60Lst) at 2 g intervals.

The turtles were tagged on the trailing edge of each fore flipper with Monel style 681 tags (National Band and Tag Co., 721 York Street, PO. Box 430', Newport, Ky 41072-0430, USA) obtained from the Archie Carr Center for Sea Turtle Research, University of Florida using tagging pliers.

Loss of monel-style flipper tags has been a problem for most sea turtle species, making it difficult to identify individuals during subsequent observations (Mrosovsky, 1976). Thus, since July 2003 the turtles were tagged with PIT tags (Passive Integrated Transponders, AVID FriendChip™ (AVID Identification Systems, Inc.)), inserted subcutaneously. Insertion places were the shoulder for small size animals (SCL<30 cm) and the region underneath the scales or between the digits of the dorsal surface of the right front flipper for larger animals. These tagging locations have been used previously by other sea turtle research teams (Balazs, 1999).

Table 1 - Biometrical parameters taken for hatchling, juvenile and female adult turtles.

Biometrical parameters	Description
CCLnt Curved-Carapace-Length Notch-to-Tip	Lenght over the Curve of Carapace from anterior point at midline of nuchal scute to the posterior tip of supracaudals [mm]
CCLmin Minimum Curved-Carapace-Length	Lenght over the Curve of Carapace from anterior point at midline of nuchal scute to the posterior notch at midline between the supracaudals [mm]
CCW Maximal Curved-Carapace-Width	Maximum Width over the Curve of Carapace [mm]
SCL Straight-Carapace-Length Notch-to-Tip	Straight-line Carapace Lenght from anterior point at midline of nuchal scute to the posterior tip of supracaudals [mm]
SCL2 Minimum Straight-Carapace-Length	Straight-line Carapace Lenght from anterior point at midline of nuchal scute to the posterior notch at midline between the supracaudals [mm]
SCW Maximal Straight-Carapace-Width	Maximum Straight-line Carapace Width [mm]
HL Head Length	distance between the tip of the beak and the posterior margin of the head [mm]
HW Head Width	distance across the widest part of the head [mm]
TCA	distance tip of Tail to to postcentral scute tip [mm]
TCA2	distance tip of Tail to postcentral scute notch [mm]
CT Post-cloacal tail length	distance from mid-cloacal opening to tip of Tail [mm]
PC Plastron-Cloaca	distance from the midline of the posterior margin of the Plastron to the mid-cloacal opening [mm]

Biometrical parameters	Description
TL TailLength	distance from tip of tail to furthest between tail and capapace [mm]
FFL ForeFlipper-Lenght [mm]	R/L State which fore-flipper was measured: R=right, L=left
FFW ForeFlipper-Width	[mm] measured just before 1st claw
CLW	1st claw length [mm] measured on Right Foreflipper
WT Weight	[g]

Boa Vista Island (Republic of Cape Verde)

Study area description and population characterization

The Cape Verde Islands are an archipelago consisting of 10 volcanic islands and several islets lying in the Atlantic Ocean (14°48'-17°180N, 22°42'-25°18W) about 500 km west of Senegal, West Africa. The climate is dry tropical but ocean conditions are heavily influenced by the cool Canary current that comes from the north, with consistently strong northeast tradewinds.

Five species of marine turtles inhabit the waters of the archipelago, where they feed and/or reproduce, with loggerheads being the most common and with confirmed nesting activity for the islands of Sal, Boa Vista, Maio and São Vicente (López-Jurado et al., 1999b).

Although the existence of marine turtles in these islands was previously known (see López-Jurado et al. (1999a) for a review), in 1977 hawksbill and loggerhead turtles were reported as the species most caught, and an extrapolation from fishermen figures for one island estimated that 1000 adult turtles were caught per year in the whole archipelago (Schleich, 1979). In 1998 three species of marine turtles were recorded for the island of Sal: loggerhead turtle (*Caretta caretta*), hawksbill turtle (*Eretmochelys imbricata*) and leatherback turtle (*Dermochelys coriacea*) although the actual size of their nesting population was unknown (Lazar and Holcer, 1998). Most marine turtles were caught both at sea or during egg-laying and slaughtered both for meat consumption or for the shell's for tourism trade (Cabrera et al., 1999).

The nesting of C. *caretta* has received attention only since the late 1990's, with the first report of an apparently important nesting population in the Boa Vista Island (Cejudo et al., 1999). Nowadays, is considered one of the most significant nesting populations in the Atlantic Ocean (Cabrera et al., 1999; Ehrhart et al., 2003), but is currently under threat from the increasing and

currently poorly regulated tourism boom happening in these islands (McLellan et al., 2004). Several thousands of females are believed to nest in the all archipelago every year (Monzón-Argüello et al., 2007).

"Projecto Cabo Verde Natura 2000" started a tagging and monitoring program in the 1998 breeding season in Boa Vista Island focused on the nesting loggerheads (López-Jurado et al., 1999b) and including three main beaches (Calheta, Ervatão and Ponta Cosme), as beaches on the eastern side of the island are the most important ones (Cejudo et al., 1999), with high nesting density (García-Carcel et al., 2001).

Boa Vista is the most eastern island of the Cape Verde Archipelago, and the nearest one to the African continent. The island is made of sedimentary rocks and is mostly covered with sand dunes or small boulders and it has 55 km of white sandy beaches, most of them used by female loggerheads to nest.

The beaches under monitoring are geo-morphologically different from each other. Calheta is a small beach (500 meters long) and very steep and is apparently a good nesting beach since high tides do not reach most of the turtles' nests. The substrate is almost only sand. Ervatão beach is 1800 meters long and is narrow and flat, which allows many nests to flood during high tides. Ponta Cosme is the largest (3500 meters long) and widest beach.

This population's nesting season starts in mid June and extends till the end of October, with a peak activity in August (Cejudo et al., 1999; Lozano-Fernandez et al., 2001). Hatchlings are seen till late January (Lozano-Fernandez et al., 2001).

The population consists of small-sized females, possibly due to the human predatory pressure for many years, which could have resulted in a decrease of mean body size of females (Ballell et al., 2001; Díaz-Merry et al., 2001). Females appear to be relatively small when compared to the ones nesting in the USA, but still larger than the Mediterranean ones (Cejudo et al., 1999), with mean body sizes averaging 75.8 cm of SCL (Lozano-Fernandez et al., 2001).

General nesting behaviour of loggerheads in Boa Vista is very similar to that already described in preceding works on this species (Díaz-Merry et al., 2001). Females exhibit internesting periods averaging 15.3 days, and clutch sizes of 82.7 eggs average (Lozano-Fernandez et al., 2001) and incubation period for averages 59.0 days (García-Carcel et al., 2001). Hatchlings' size

averages 42.0 mm SCL (Abella et al., 2001).

The beaches of Ponta Cosme and Ervatão have features which make them unsuitable for nesting, due to the short range of useful sand and because they are very frequently flooded by tides and rains (García-Carcel et al., 2001); hatching success on these two beaches is rather low (46.9%) (García-Carcel et al., 2001).

Sea Turtle Capture

In the Cape Verde islands females come ashore to breed, making it fairly easy to sample female adult individuals. Males keep offshore, probably close to the nesting beaches, making any male sampling logistically difficult. Hatchlings can be easily collected from nests. Due to the status conservation of the species, only dead hatchlings were sampled. The field seasons in Boa Vista Island took place between 16th August and 29th September 2003, and 25th August and 2nd October 2004.

Beach surveys were done only in the closest beaches to the campsite (Ervatão and Ponta Cosme) due to logistic constraints with transportation to Calheta. Samples (only dead hatchlings) from Calheta were collected by other samplers who delivered them to me afterwards.

A major part of the work was done overnight. Night patrols were done in two shifts, and beach monitoring consisted of:

> a) tagging with Monel flipper tags and with PIT tags (Passive Integrated Transponders) and measuring (biometry parameters) all female turtles arriving at the beach and nesting;
>
> b) marking nest locality, counting the number of eggs laid and measuring sand and atmospheric temperature and
>
> c) blood sampling.

Nesting female adults

Nesting females were sampled immediately after the nesting process was finished, in order not to disturb the animal's reproductive process, i.e., when they were returning back to the sea.

Blood samples were taken from the dorsal cervical sinus into Vacutainers for posterior sex

steroids assessment. Samples were centrifuged the following day using a 12V portable centrifuge and kept in a refrigerator at the campsite. Transfer into a freezer could take 24-48 hours, and thus some hormone degradation might be expected due to poor preservation of the plasma samples.

The same biometrical parameters taken at Madeira Island were also taken, except for weight, fore-flipper and head parameters due to logistical constraints.

Dead hatchlings

Live hatchlings emerging from the nest were also monitored overnight, which included counting the number of animals emerged and measuring and weighing ten random individuals in each clutch. However, these hatchlings did not accounted for this study as it was not possible to obtain a blood sample large enough for radioimmunoassay without endangering the individual survival, and gonad observation would imply sacrificing individuals.

Instead, and as a large number of hatchling individuals die inside the nest while hatching or emerging, only dead hatchlings were chosen for sampling, i.e., necropsy for gonad withdrawal. The same biometrical parameters taken at Madeira Island were also taken; weight was measured using a 30g dynamometer (1 g intervals).

CHAPTER II

Laparoscopies

Introduction

Like many other reptiles, loggerhead turtles lack heteromorphic sex chromosomes (Bickham, 1979) and exhibit temperature-dependent sex determination (TSD), i.e., the sex of the offspring is influenced by the incubation temperature of the eggs (Yntema and Mrosovsky, 1980). The only way to positively determine the sex of each individual is through the analysis of their internal morphology.

For adult marine turtles sex diagnose becomes evident, since males develop secondary sexual characteristics during puberty. The most obvious secondary sexual characteristic is the large and muscular prehensile tail which extends well beyond the carapace in an adult male. Even though the actual tail lengths vary with species and possibly populations, the tail of female sea turtles is short and, at most, will project only slightly beyond the edge of the marginal scutes (Wibbels, 1999). Males also develop strongly curved flipper claws used in holding the female during copulation (Owens, 1997). Some pubescent or sexually developing turtles might be identified as males by the softening of the medial plastron; however, such identification requires an experienced observer (Wibbels *et al.*, 1991a). In contrast, mature females do not exhibit secondary sexual characteristics, but typically bear scratches and scars on the anterior edge of the carapace caused by the male during copulation (Owens, 1997).

However, this sexual dimorphism is attained only at adult or sub-adult stages (Owens, 1997) and tail length is not an accurate sexing technique for immature sea turtles (Limpus, 1985a; Wibbels, 1988) making the identification of sex of juvenile marine turtles using external morphology very difficult. Loggerheads attain sexual maturity estimated at the age 20 or more years (Bjorndal et al., 2001b). In fact, marine turtles do not begin to breed at a uniform or minimum size and thus body size is not a reliable indicator of sexual maturity and breeding status (Limpus et al., 1994a; Limpus et al., 1994b). Thus, in contrast to adults, the sexing of immature and hatchling turtles represents a significant logistical challenge and an individual's sex can only be identified using more or less complex techniques, in order to assess a population's sex ratio. One of the

fundamental needs in marine turtle sex ratio assessments is a reliable tool for sex identification in individual turtles.

Despite the lack of external sexual dimorphism in juveniles, internal dimorphic morphology is determined at hatching (Wyneken et al., 2007). Therefore, the only reliable method for sexing juvenile turtles is by direct observation of the gonads (Mrosovsky and Benabib, 1990; Van der Heiden et al., 1985), which requires sacrificing the turtle, use of surgery (i.e., laparoscopy) or necropsy of stranded carcasses (Shaver, 1991; Stabenau *et al.*, 1996).

However, and although the information on sex ratios is critical for population modelling and monitoring, the practical use of this methodology faces ethical challenges as it requires destructive sampling and loggerheads are a protected species (Oliveira et al., 2005). In addition the number of individuals required to sacrifice in order to obtain a statiscally significant sample size to enable sex-ratio assessments is rather large.

A variety of nonlethal methods have been proposed and/or developed for determining the sex of immature sea turtles.

Most data on hatchlings sex ratio have been gathered by estimating sex ratios of nests based on mean daily nest temperature using buried thermometers. However, mean daily temperature in natural nests of turtles with temperature dependent sex determination is a poor predictor of hatchling sex ratios when nest temperatures fluctuate, which is commonplace in the wild (Georges et al., 1994).

The most definitive method is the direct observation of the gonads by laparoscopic examination (Wibbels, 1999). Ultrasonography and other diagnostic imaging methods are not indicated, as it is not possible to distinguish the gonad from other organs in such small animals. In contrast to ultrasonography, during endoscopic exploration the inner organs appear in their natural size, shape, position and colour and even very small structures (less than 1 mm size) like juvenile ovaries are visible (Schildger, 2001).

Thus, juveniles' sex can be diagnosed by direct observation of the gonad, such as under laparoscopy examination. Laparoscopy, i.e., the examination of the pleuroperitoneal cavity via an endoscope (coelioscopy), allows *in vivo* observation of the gonad with minimum stressing of the animal and a macroscopic classification of whether the gonad is an ovary or a testis. This

small surgery technique falls within the direct methods to sex an individual, and allows the collection of images for gonad morphological variability assessment among individuals. It was first applied to the green sea turtle *Chelonia mydas* (Wood et al., 1983) in order to predict the sex and stage of gonadal development. It has proved to be a valuable tool for sexing immature sea turtles and in assessing the reproductive state of marine turtles since the gonads can be viewed directly through the laparoscope (Wibbels, 1999). This surgical procedure has been used in a number of studies in order to assess the reproductive status of *Caretta caretta* (Blanvillain et al., 2007; Wibbels et al., 1989; Wibbels et al., 1990b; Wibbels et al., 1987b; Wyneken et al., 2003), *Chelonia mydas* (Hamann et al., 2001; Hamann et al., 2002; Limpus and Reed, 1985; Wood et al., 1983), olive ridleys *Lepidochelys olivacea* (Plotkin et al., 1996) and even the tuatara *Sphenodon punctatus* (Cree et al., 1991) and hatchling and immature desert tortoises *Gopherus agassizii* (Rostal et al., 1994), among others.

Complete familiarization with sea turtle anatomy is essential prior to doing surgery, as the anatomy of reptiles varies among orders, families and even species making the knowledge of the basic features of reptilian anatomy therefore vital for the surgeon (Bennett, 1989). It is a potentially dangerous procedure and should not be attempted until proper veterinary training has been obtained (Wood et al., 1983). This study is a first assessment of the sex ratio in an oceanic population of any turtle species.

Objectives

The objective of this work was to diagnose individual sex through direct *in vivo* laparoscopic observation of the gonads of loggerhead turtles to enable assessment of the Madeira population sex-ratio. Gonad biopsies were also obtained for microscopic validation of the laparoscopy technique.

Materials and Methods

Juvenile loggerhead turtles were captured in the sea offshore Madeira Island (Portugal), north-eastern Atlantic following Dellinger (1997), and were brought in to the laboratory.

Animals were kept overnight in small boxes or tanks filled with water in order to prevent dehydration. Laparoscopies were performed the following day, allowing the animals to fast for a period of 24 hours approximately. Fasting is recommended prior to any surgical procedure and thus no food was provided to the animals; in free ranging animals fasting for a few hours is sufficient (Bennett, 1991; Schildger, 2001).

The laparoscopy equipment consisted of a telescope [HOPKINS® telescope 30°, 2.7mm, length 18 cm (64018 BS)] and a fibre optics projector and light cable [cold light fountain VETERINARY 69495 NL; fiber optic light cable, 3.5mm diam. (69111001)], trocar and trocar sleeve [trocar 3.5mm length and trocar sleeve 5.5 cm (62114 KP)], as well as standard surgical instruments. A biopsy forceps and double cannula was needed for biopsy sampling [examination sheath 14.5 Fr., with built in instrument channel 5 Fr. 67065CC) and obturator (67065 CO) and biopsy forceps, oval jaws 5 Fr. (67161 Z)], everything from Karl-Storz.

As reptiles are susceptible to various microbial infections, aseptic techniques were routinely employed (Bennett, 1989; Wood et al., 1983). Equipment was sterilized by soaking in a solution of 70 % ethanol and povidone iodine (Betadine®) for 10-30 minutes prior and between each use and surgical sterile gloves were used.

Laparoscopies were performed as previously described by Wood (1983) and reviewed by Owens (1999) in order to determine each animal's sex, as well as to get video images of the gonad. Animals were subjected to the surgical procedure only when the turtle's health condition was considered safe to overcome laparoscopy.

The plastron and the caudal area of the animal was first washed and scrubbed carefully with povidone iodine solution and rinsed with water. The right inguinal area was then thoroughly cleaned and disinfected with chlorhexidine and sterile cotton pads.

The animal was anaesthetized locally with 2 or 3 infiltrations in the incision area by intramuscular injection of lidocaine 2%. The total dose injected depended on the size of the turtle:

- 0.5 ml for turtles around 30 cm CCL or less

- 0.7 ml for turtles from 30 cm CCL up to 60 cm CCL

- 1 ml for turtles larger than 60 cm CCL.

A period of 3 to 5 minutes was allowed after infiltration before any incision was made. If needed, in case of a longer surgical procedure or a more reactive individual, a supplementary dose was administered during the procedure. The use of general anaesthetics was avoided for this particular surgery since a local anaesthetic incurs less risk of mortality, is adequate for reducing apparent pain, and allows a much shorter post-operative observation and recovery periods (Owens, 1999; Wibbels et al., 1990a; Wood et al., 1982b).

The turtle was immobilized in an inverted position with the plastron facing forward and tied firmly onto a purpose-built 'turtle-rack' with the right back flipper extended upwards and to the left. A 1 cm long incisure in the right inguinal area was done with a scalpel blade and the trocar was inserted into the pleuroperitoneal or coelomic cavity, by cautiously pushing through the skin and muscle layers and peritoneal wall with slight rotations into the body cavity (Fig. 6).

Fig. 6 (A, B, C, D) - Sequence during laparoscopy procedures: a) turtle tied to the 'turtle-rack'; b) turtle positioned for surgical procedure; c) lidocaine administration; d) positioning the endoscope for gonad visualisation.

The trocar could then be retrieved from the trocar cannula so that the endoscope could be inserted and the gonad could be inspected macroscopically. A 2.7 mm diameter, 30° rigid endoscope was used.

Insufflation improves the optical conditions, especially in small individuals (Owens, 1999). When needed a space to view the gonads was created by partially inflating the cavity with filtered air (Gelman 0.2 Millipore filter) from a pump and delivered via a plastic air hose connected to the trocar cannula valve. Gonads were located, in most instances, by first finding the caudal tip of the lung and then moving the endoscope posteriorly over the kidney and attached gonad. Up to 5 macroscopical characteristics were recorded:

a) estimated gonad size,

b) gonad colour,

c) follicles presence/absence,

d) estimated follicles sizes,

e) oviduct (paramesonephric duct) presence/absence.

After this first phase the whole apparatus was retrieved from the turtle and a second double cannula was inserted into the coelomic cavity through the same incision. This double cannula allowed the insertion of a biopsy forceps in order to withdraw a small gonad biopsy (up to 1 mm diameter). The gonad biopsy was kept in an eppendorf and fixed in Bouin's solution until histological processing.

Video images were recorded whenever possible for posterior reference using a handycam attached to the endoscope ocular with an adaptor ring.

Finally, the incision was sutured using surgical gut (sterile absorbable # 2-0 suture, 26 mm curved cutting needle (triangular point)) and one to two surgical knots and an antiseptic (Betadine®) applied to the wound. If insufflation was needed all the air was removed from the coelomic cavity before suturing by gently pressing the lower region of the plastron.

The procedure took usually 15 to 25 minutes per individual. The animal was then left to rest for up to 24 hours in individual dry receptacles (Wood et al., 1982a) to allow monitoring of its recovery and then released back in the wild after all the sampling procedures were finished (biometry and tagging).

Results

The results shown include the laparoscopies performed largely by the author, first as part of the project PRAXIS/P/BIA/11310 between 2000 and 2002 and then specifically for this thesis between 2003-2006 (Fig. 7). During this period 224 juvenile turtles were examined and subject to laparoscopy in order to identify each individual's sex. Animals ranged from 171-687 mm SCLnt (Mean=359.28 mm; SD=107.389) (Fig. 8) and weighted 824-48450 g (Mean=8980.80 g; SD=7157.234).
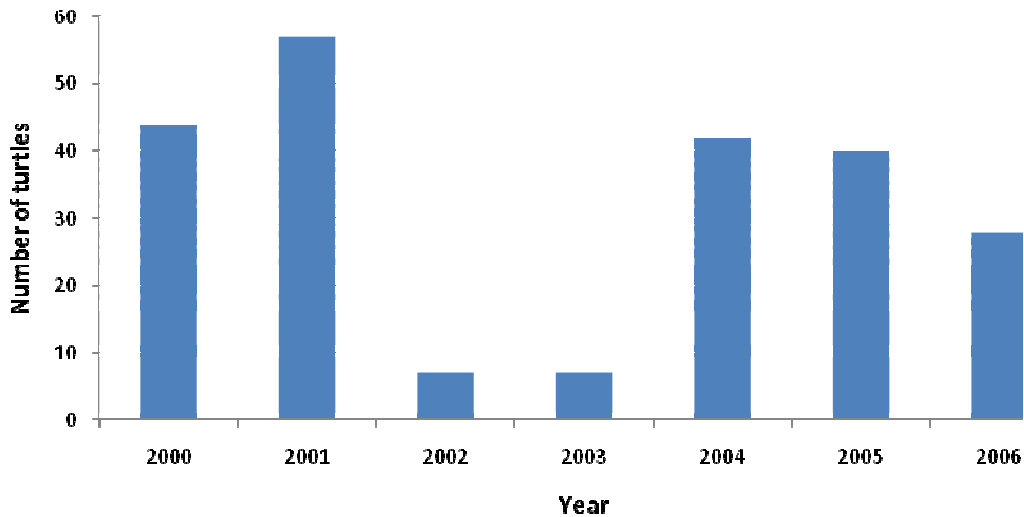
Fig. 7 Frequency of turtles subject to laparoscopy per year.
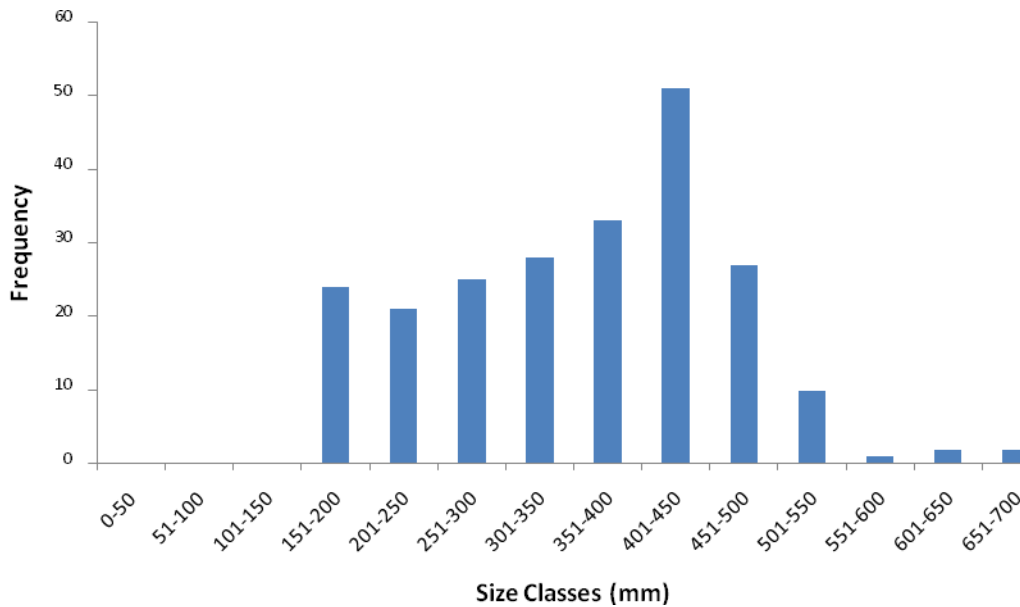
Fig. 8 Size classes (SCL) frequency of turtles subject to laparoscopy (2000-2006).

Marine turtle gonads are paired organs located in the coelomic cavity, caudal to the lungs and attached to the peritoneum overlying the paired kidneys. Under laparoscopic examination, males were identified by the smooth gonad, variable in colour from white pale to bright orange, often irrigated by a more or less developed capillary web (Fig. 9). In females the gonad had a granular aspect, with more or less developed follicles easily visible with the naked eye, from 1 to 2 mm in diameter (Fig. 10). The oviduct/paramesonephric duct was commonly observed in

females, but seldom in males. Females presented ovaries with non-expanded stroma. No variation of macroscopical appearance was observed within sexes, as well as no vitellogenic follicles, corpora lutea, corpora albicantia or atretic follicles were present.
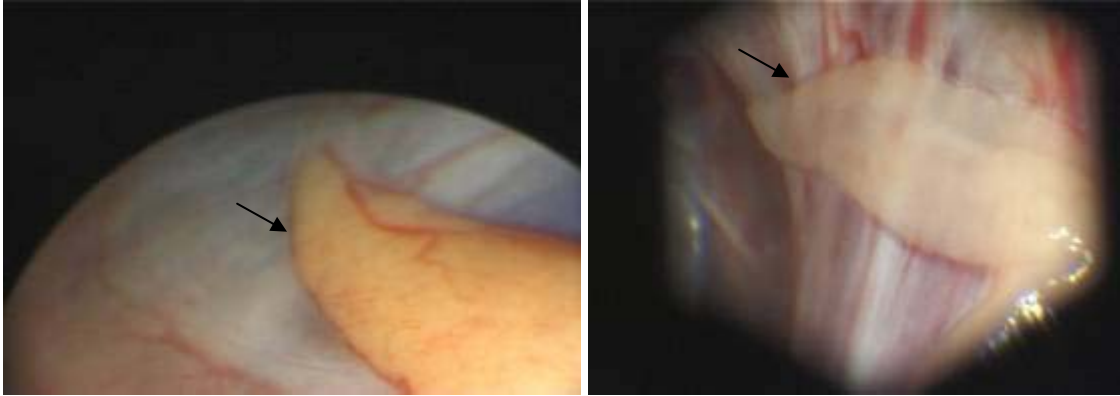
Fig. 9 Photos of male gonads under laparoscopic observation: testis (arrows) can vary from bright yellow to pale white, sometimes showing a capillary web (left); testis are usually fusiform in shape, but note the folded testis on the right picture (right-down side).

Fig. 10 Photos of female gonads under laparoscopic observation: ovary (arrows) is usually transparent, variable in size and shape, and showing many follicles of different sizes; note the overlapping, folded ovary on the left picture.

Of the animals subject to laparoscopy, 138 were identified as a female phenotype (61.3%) and 69 as a male (30.7%) phenotype.

For technical reasons it was not possible to observe the gonads of 17 (7.6%) animals, and thus these were classified as undetermined. These cases correspond to animals where the trocar was not sharp or long enough to go through the several skin and muscle layers into the coelomic cavity in some of the larger turtles making it impossible to observe the gonads.

Thus, if the 17 undetermined turtles are excluded from the analysis, a total of 207 animals is obtained, 66,66% of them females and 33.33% males, representing a sex ratio of 2 females

for each male (2F:1M). This sex ratio is significantly different from a 1:1 sex ratio (χ^2=23.0, df=1, p<0.0001).

Between 2003 and 2006, 82 gonad biopsy samples were collected for posterior histological confirmation (see Chapter III for gonad biopsies analysis).

Only a few animals were not considered physically fit to survive the surgery. Of the 224 laparoscopies performed only 2 turtles died subsequent to the surgery, giving a rate of <1% mortality. *Post mortem* examination of these 2 turtles was supervised by a veterinary but revealed inconclusive, as no striking of vital organs during trocar entry as the potential cause of death was detected.

Discussion

From two hundred and seven positive sex identifications a sex ratio of 2:1 (F:M) was obtained, which was obviously skewed towards females, and significantly different from an expected even or Fisherian 1:1 sex-ratio. This is not surprising, since female-biased sex ratios are common in marine turtles (Wibbels, 2003) and strongly non-equal population-wide primary sex ratios are known, both empirically and theoretically, to result from ESD (Charnov and Bull, 1989b). Moreover, when skewed sex ratios are observed, they are usually skewed towards females (Bull and Charnov, 1989).

The main disadvantage of the laparoscopy technique is that the procedure is invasive and potentially hazardous to the turtle. Moreover, it is logistically difficult and should not be attempted without proper veterinary training (Owens, 1999). Although there are risks associated with the use of laparoscopy it proved highly successful on these juvenile oceanic loggerheads, as sex could be diagnosed in 207 (92.4%) animals out of 224 examined. The animals whose sex could not be assigned was not due to difficulties in sex diagnose but to technical difficulties relating to the trocar size and/or sharpness. Moreover, the mortality rate was <1%, which is below the expected mortality for this kind of surgical procedure in marine turtles, as even experienced laparoscopists can expect a mortality rate in the order of 1-2%, under good conditions (Owens, pers. comm.; (Owens, 1999). The two most common causes of mortality include excessive bleeding due to incorrect trocar placement and death due to non-specific symptoms in

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a turtle that has already been compromised due to other conditions. For example, an overheated turtle may have a gas-distended lung or gut which can easily be perforated even with the best surgical technique. In addition, sea turtles with a heavy parasite load, a severe bacterial infection or acute obesity may succumb very easily during surgery (Owens, 1999).

The fact that females had ovaries with non-expanded stroma and that no vitellogenic follicles, corpora lutea, corpora albicantia or atretic follicles were present is consistent with the expectations for the sexually immature status of these juvenile loggerheads (Limpus and Limpus, 2003). Estimated size for juveniles follicles (up to 2 mm diameter) is also compatible with the estimated size for more advanced developmental stages, such as the sizes referred for vitellogenic follicles (0.3-3.0 cm in diameter) for mature females also under laparoscopic evaluation (Limpus and Limpus, 2003). However, these sizes should be examined cautiously as they depend on the magnifying power specific of each laparoscopic telescope and on the accuracy and experience of the observer (for more accurate and reliable diameters of follicles size see values obtained under microscope evaluation in Chapter III).

The fact that all the animals were sampled during the warmer months might explain the absence of variation of gonad macroscopical appearance, namely regarding colour and granularity within the same sex, since gonads morphological appearance seem to be temperature dependent and vary seasonally, as observed in captive-reared post-hatchlings (Wyneken et al., 2007). Given the lack of function of paramesonephric ducts in males it is not surprising that they were seldom observed in males - they tend to regress in males but persist as the Müllerian duct in females (Miller and Limpus, 2003).

Despite the logistical constraints laparoscopy has been used successfully by a number of researchers and has been used to sex hundreds of sea turtles on ongoing projects worldwide. Further, the use of laparoscopy is currently a necessity for validating other nonlethal sexing techniques for immature sea turtles such as assessment of sex steroids levels (Owens, 1999).

Data obtained through laparoscopy will be validated through histology of the gonad biopsies collected (see Chapter III) and compared with sex steroids blood levels (see Chapter IV). Biological and adaptive significance of skewed sex ratios will be further discussed on Chapter V.

CHAPTER III

Gonad Histology

Introduction

The ontogeny and histological morphology of the gonads of many reptiles has been studied thoroughly, including marine turtles (Ewert, 1979; Ewert, 1985; Fox, 1977; Miller and Limpus, 2003; Raynaud and Pieau, 1985). Miller (1985) described a sequence of 31 embryonic developmental stages for marine turtles, emphasising on the Cheloniidae and Renous provided descriptions of the development of *Dermochelys coriacea* (Renous et al., 1989). The gonadal morphogenesis of the olive ridley *Lepidochelys olivacea* was subject of a detailed histological and ultrastructural description (Merchant-Larios et al., 1989; Merchant-Larios and Villalpando, 1990).

Although the sex of the hatchling is determined at hatching in all marine turtles, including loggerhead turtles (Yntema and Mrosovsky, 1980), with the middle third of the incubation period being of paramount importance for sex determination (Yntema and Mrosovsky, 1982), the possibility of sex reversal is still open for at least several reptile species, including turtles, namely by exposure to exogenous estrogens (Bull et al., 1988; Crews et al., 1991; Crews et al., 1995b; Crews et al., 1996; Richard-Mercier and al, 1995; Sheehan et al., 1999; Tousignant and Crews, 1994). In the European pond turtle *Emys orbicularis* artificially induced sex reversal persists for at least one year after hatching (Belaid et al., 2001).

During embryonic development the gonadal ridge develops from epithelial, mesothelial and mesenchymal cells located between the base of the forming mesonephric tubules and the base of the dorsal mesentery ventral to the subcardinal veins during stage 17. At that time blood vessels invade the genital ridge together with a perforation of mesenchymal cells and collagen fibres and the basal lamina separate the epithelial medullary cords from the loose mesenchymal cells of the gonad stroma (Merchant-Larios et al., 1989; Miller and Limpus, 2003).

During this phase the gonad is still bipotential, since sexual differentiation occurs only during the thermosensitive period of embryogenesis (Yntema and Mrosovsky, 1982). This thermosensitive period (TSP) occurs between stages 22-27 (Miller, 1985), when the cells of the gonadal primordial propagate both to the cortex and the medulla. The time for sexual differentiation

seems to be species-specific, since sexual differentiation for *Lepidochelys olivacea* is reported slightly later, during stages 24-29 (Merchant-Larios et al., 1989). The gonadal primordia has two regions: the outer cortex, formed by a single layer of cuboidal epithelial cells with embedded germinal primordial cells, and the inner medulla, derived from mesenchyme cells within the undifferentiated gonad (Merchant-Larios et al., 1989; Miller and Limpus, 2003).

The primordial germ cells form at the caudal end of the embryo and accumulate at the base of the gonadal ridges and can be distinguished from surrounding somatic cells due to their large size and large round nuclei (Merchant-Larios et al., 1989; Miller and Limpus, 2003). These cells migrate by amoeboid movement during the second day of incubation from the endoderm of the yolk sac into the lateral border of the area pellucida, and continue migrating until the primordial germ cells have moved into the gonads (Miller, 1985). Gonadal organogenesis implies that, in future females, the ovary results from the simultaneous proliferation of cells in the cortex and regressive modification in the medulla: the cells become more columnar in shape and the layer becomes thicker; primordial germinal cells are interspersed among the cells of the cortex and the medullary area shows some differentiation of cells forming the primitive medullary sex cords (Miller and Limpus, 2003). In males the testis results from the simultaneous regression of the cortex and the differentiation of primitive sex cords in the medulla. In the cortex cells regress to become a flattened epithelium, whereas cells in the medulla differentiate to form hollow sex cords that will form the seminiferous tubules (Miller and Limpus, 2003).

The degree of sexual differentiation at hatching is controversial: while some authors argue that gonads may be considered morphologically and physiologically immature in all sea turtle species (Merchant-Larios, 1999; Mrosovsky and Benabib, 1990), other authors state that the gonads of hatchling marine turtles are morphologically defined by the time the turtles reach the beach surface. At emergence the gonad appears as a whitish, elongate structure on the ventral surface of the kidney although it cannot be distinguished by eye as an ovary or a testis (Miller and Limpus, 2003; Wyneken et al., 2007). Moreover, the gonads of different species of sea turtle reveal variations in the degree of differentiation at hatching, with the most differentiated gonads being those of C. *caretta* and the least the ones of *D. coriacea*, while an intermediate differentiation may be observed in *L. olivacea* and C. *mydas* (Merchant-Larios, 1999).

Most data on sex ratios have been gathered by estimating sex ratios of nests based on mean daily nest temperature, using buried thermometers. However, mean daily temperature in natural nests of turtles with temperature dependent sex determination is a poor predictor of hatchling sex ratios when nest temperatures fluctuate, which is commonplace in the wild (Georges et al., 1994).

Due to the small hatchlings' size, laparoscopy is not a feasible technique to perform sex identification at these size classes, and direct visualization of a subsample of nests within a nesting beach have been used to asses hatchlings sex ratios.

While some authors state that it is possible to macroscopically diagnose the sex of hatchling marine turtles (Whitmore et al., 1985), others argued that only histological sex assignment is an effective sexing method (Mrosovsky and Benabib, 1990) and thus, in order to establish sea turtle populations sex ratios, and despite its laboriousness, gonad histology and subsequent observation of microscopic morphology is considered the most accurate sex diagnosing method (Mrosovsky and Benabib, 1990; Mrosovsky and Godfrey, 1995; Wibbels, 2003). Very recently, Wyneken (Wyneken et al., 2007) proved that macroscopical sex diagnose through laparoscopy in 120 g post hatchlings is an effective sexing method.

Despite the many studies that contributed to the understanding of marine turtle gonad ontogeny and the fact that the general pattern of ontogeny of the gonads of marine turtles can be described, no study has specifically addressed the development of gonads of post-hatchlings and juveniles in the wild, and a gap still persists on the pelagic phase of marine turtles life cycle. The reproductive system of female pubescent loggerheads has been described (Limpus, 1990), and only very recently Wyneken described the gonads – both macroscopically *in vivo* and histologically – of captive-reared loggerhead hatchlings (Wyneken et al., 2007).

Objectives

Although the diagnose of sex trough direct *in vivo* observation of gonad macroscopic morphology is considered a valid method for obtaining sea turtle sex-ratios, this study aims to validate the identification of gonadal sex by the laparoscopic technique through histology according to microscopic morphological criteria.

It also aims to correlate morphological and histological changes of the gonad to turtle size in order to detect ontogenetic changes of the gonad during the juvenile pelagic phase.

Materials and Methods

Juveniles

Gonad biopsies were collected from turtles subjected to laparoscopy using biopsy forceps (see Chapter II for detailed procedure). One biopsy sample was collected from the right gonad of each specimen and fixed and stored in either Bouin's fixative or 10% buffered formalin until histological processing. Care was taken to collect the biosy sample from a representative area of the gonad as observed during laparoscopy.

Hatchlings

Dead hatchlings were collected during the field seasons on Boa Vista Island which took place from 16th August to 29th September 2003 and from 25th August to 2nd October 2004 on the beaches of Ervatão and Ponta Cosme.

Surveys for dead hatchlings collection were done during early morning shifts (6am onwards), when all turtle crawls not observed during night shifts were registered and all nests on the beach were checked for new emergences, predation or mortality due to tide flooding. Only complete hatchlings were collected, as many of them were partially predated by ghost crabs (*Ocypode cursor*), crows, etc, mainly for the vitello.

During 2003 season hatchlings were necropsied onsite and the complex gonad/kidney (Fig. 11) was removed and preserved in Bouin's solution. However, this approach was not successful since during the histological process many gonads were lost. Therefore, in the 2004 season, the hatchlings were only partially necropsied for vitello removal and the whole hatchling was preserved in 10% buffered formalin; only back in the laboratory the complex gonad/kidney was withdrawn (T. Wibbels, pers. comm.).

Fig. 11 Loggerhead hatchling under necropsy: circles depict the paired gonads lying over the kidneys.

Histological processing and sex diagnosis

The gonad samples (1 mm diameter biopsy in case of juveniles and the complex gonad/kidney in case of hatchlings) were histologically processed using standard procedures (see Appendix 1). Shortly, after dehydration in a series of alcohol washes, the fixed tissues were embedded in paraffin, serially sectioned at 6μm on a microtome, and stained using haematoxylin-eosin staining (H&E) (Bancroft and Stevens, 1990; Yasutake and Wales, 1983). The 2003 batch of hatchlings' samples was stained with the periodic acid-Schiff reaction (PAS) staining procedure, as recommended by (Mrosovsky and Benabib, 1990). However, this technique was changed for the haematoxylin and eosin (H & E) staining technique for the 2004 batch (T. Wibbels, pers. comm.) and was more satisfactory. The poor results obtained with the PAS technique were probably due to the fact that this staining technique was only applied to the hatchlings' gonads, which highly degraded.

Two to three slides were prepared from each individual and observed under a compound light microscope for sex assignment using the diagnostic criteria listed in Table 2. Some hatchlings

were discarded due to poor gonad and/or whole-body condition at the time of necropsy, as well as some slides during microscopical observation due to tissue degradation.

Table 2 - Published criteria for hatchling's sex assignment.

Male	Female	Intersex	Species/Author
Simple squamous epithelium on the surface Delicate tunica albuginea may be seen under the simple squamous epithelium on the surface Convoluted primary cords have formed immature seminiferous tubules with diameters which are 2 to 4 times those of the retrogressing primary cords of the female These cords are surrounded by a stroma rich in PAS positive elements	Germinal epithelium forms the outer surface of the ovary except in the region of the mesovarium Germinal epithelium relatively thick on the ventral surface Epithelium may form extensions into the medulla Germinal epithelium is sharply delineated from the medulla by the tunica albuginea (PAS positive) Small primary cords persist in the medulla and lie in a stroma containing PAS positive elements	Not described	*Caretta caretta* (Yntema and Mrosovsky, 1980)
Tubular medulla with tubes not in contact with epithelium Cortex degenerate with squamous epithelium	Dense medulla without tubules Cortex with columnar epithelium Germ cells usually visible	Dense medulla without tubules and cortex degenerated or tubular medulla with tubes in contact with epithelium and cortex partially developed	*Chelonia mydas* (Miller and Limpus, 1981)
Cortex is differentiated into flattened, squamous cells positioned next to the tunica albuginea Medulla is filled with seminiferous tubules with germinal cells situated near the inner membrane The space contained in the lumen of the tubules appears about the same as that occupied by the surrounding tissue	Cortex differentiated into columnar shaped cells with germinal cells spaced among them near the basal membrane epithelium medulla is dense with occasional small strings of cells among blood vessels no tubules with open lumen are present	Not described	*Chelonia mydas* and *Lepidochelys olivacea* Adapted from (Miller and Limpus, 1981) and (Merchant-Larios et al., 1989)

In order to address the pattern of histological differentiation of gonads in relation to size classes in juvenile females, an hierarchy for oocyte developmental stages was adapted from the one

applied to the American alligator *Alligator mississipensis* (Uribe and Guillette, 2000), a TSD species also, and developmental stages were assigned for each female gonad (Table 3). Moreover, the diameter of the largest follicle within a biopsy was measured using a calibrated ocular micrometer for each female as an indicator of ovarian development. Regression analysis was employed to evaluate the relationship between oocyte diameter and individual's size.

Table 3 - Stages of folliculogenesis. Source: summarized from Uribe, M.A.C. and L.G. Guillette Jr. (2000) Oogenesis and Ovarian Histology of the American Alligator *Alligator mississipensis*. Journal of Morphology 245:225-240.

Stage	Characteristics
Stage I: Previtellogenesis	Nucleus contains dense chromatin beginning prophase I of meiosis. Thick chromosomes visible. One nucleolus. Squamous cells begin to surround the oocyte. Oogonia and oocytes have an ooplasm lightly stained. No stroma surrounding stage I oocytes.
Stage II: Previtellogenesis	Nucleus contains lampbrush chromosomes and one nucleolus. Stage II exhibits a yolk nucleus adjacent to the oocyte nucleus Follicular cell nuclei surround the oocytes Stroma surrounds stage II oocytes
Stage III: Previtellogenesis	Nucleus contains lampbrush chromosomes and multiple nucleoli. Squamous cells completely surround oocyte; monolayer is referred to as granulosa (squamous follicular epithelium) Yolk nucleus is diffuse Lacunae adjacent to the theca Theca is thin but evident, containing blood vessels and collagen fibers
Stage IV: Previtellogenesis	Zona pellucida at periphery of oocyte. Granulosa cells are cuboidal containing a nucleus. Theca is developed, comprised of fibroblasts and with small lacunae. Nucleus similar to stage III Ooplasm with finely granular and dispersed appearance Large lacunae surround the oocyte
Stage V: Previtellogenesis	Well defined zona pellucida; is considerably thicker, consisting of two layers; an inner striated layer and an outer hyaline band. Ooplasm is homogeneous Theca and lacunae surround the oocyte

Stage	Characteristics
Stage VI: **Vitellogenesis**	Peripheral granules and centralized vacuoles in ooplasm. Theca has sinuses. Numerous lacunae and bundles of smooth muscle surrounds the oocytes Central ooplasm is lightly granular Early deposition of yolk platelets
Stage VII: **Vitellogenesis**	Granules and vacuoles have increased greatly in numbers. Vacuoles are much larger (up to 25 μm), some containing yolk platelets. Peripheral ooplasm is yolk free
Stage VIII: **Vitellogenesis**	Regional animal and vegetal poles clearly visible. Zona pellucida 18-20 μm thick and have well defined radiata and hyaline layers. Theca contains blood vessels, collagen fibers, and flattened lacunae. Most of the ooplasm contains abundant vacuoles and dense yolk platelets
Stage IX: **Vitellogenesis**	Ooplasm is filled with large (90 μm) yolk platelets of different sizes and shapes Also with polyhedral shape.
Stage X: **Vitellogenesis**	Yolk platelets larger than stage IX (160 μm). Theca is quite thick (180-200 μm), containing muscle cells as well. Surrounding the oocyte there are parallel lacunae and bundles of smooth muscle

Results

Juveniles

Between 2003 and 2006, 82 gonad biopsies were obtained through laparoscopy, representing 36.4% of the total laparoscopies performed during the period 2000-2006. Individuals analysed ranged from 173-551 mm SCLnt (Mean=351.71 mm; SD=87.56; n=82).

Sex diagnosis was based on development of cortical and medullary regions and presence or absence of seminiferous tubules. Males were identified by the presence of immature seminiferous tubules in the medulla and flat, monostratified surface epithelium, whereas females exhibited more or less developed, spherical follicles, enclosed by a membranous structure (Fig. 12)

(Merchant-Larios, 1999; Yntema and Mrosovsky, 1980). According to these morphological criteria, 52 specimens were identified as a female phenotype, 26 as male phenotype, and 3 did not match the sex identified through the laparoscopic technique. The unmatching samples were female-male and vice-versa. One individual was assigned as intersex. The term intersex refers to any specimen which exhibits both male and female characteristics (Limpus et al., 1982). A conservative approach was adopted and the last 4 samples were excluded from further analysis. Thus, a pool of 78 biopsies were obtained, 66,66% of them females and 33.33% males, i.e, an overall sex ratio of 2 females for each male (2:1). This sex ratio is significantly different from a 1:1 sex ratio (χ^2=8.667, df=1, p=0.003).

Regarding the developmental stages, all males presented spermatogonia only (Wibbels et al., 1990b) lining the interior of the seminiferous tubules.

Females presented ovaries with variable developmental stages within a non-expanded stroma. Profiles of oocyte developmental stage and growth were obtained for females. All juvenile females presented oocyte developmental stages up to stage III of previtellogenesis (Uribe and Guillette, 2000). Diameters observed ranged up to 412.5 µm (lower measure limit was 3 µm) (see Table 4 for a summary of histological characterization of juvenile loggerheads gonads).

The diameter of the largest oocyte for each female was compared with individual's size (SCLnt) in order to address the pattern of ovarian differentiation in regard to size classes in juvenile females (Fig. 13). Individuals analyzed ranged from 200-551 mm SCLnt (Mean=375.13 mm; SD=80.41; n=40). A linear correlation between the diameter of the largest oocyte and the size of the female was found, as oocyte diameter was significantly correlated with female turtle body size (OD=0.707*SCLnt-30.42, n=40, R^2=.409, F=27.023, p<0.001).
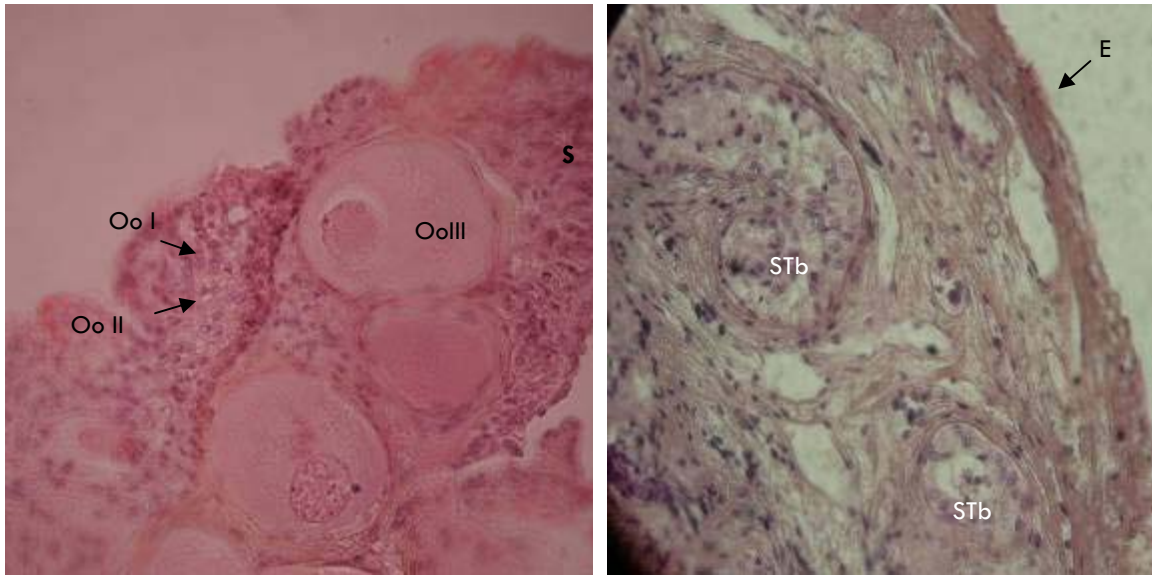
Fig. 12 Sections of a female (left) gonad showing oogonia stage I (Oo I), oogonia stage II (Oo II), oocyte stage III (Oo III) and stroma (S) and of a male (right) gonad showing seminiferous tubules (STb) and flattened epithelium (E) (6 μm section, HE stain, 40X).

Table 4 - Summary of histological characterization of juvenile loggerheads gonads.

Structure	Histological description
Ovary	previtellogenic follicles, variable in size (<412.5 μm); oocytes up to stage III developmental stage; compact stroma
Testis	compact structure with seminiferous tubules with small lumen
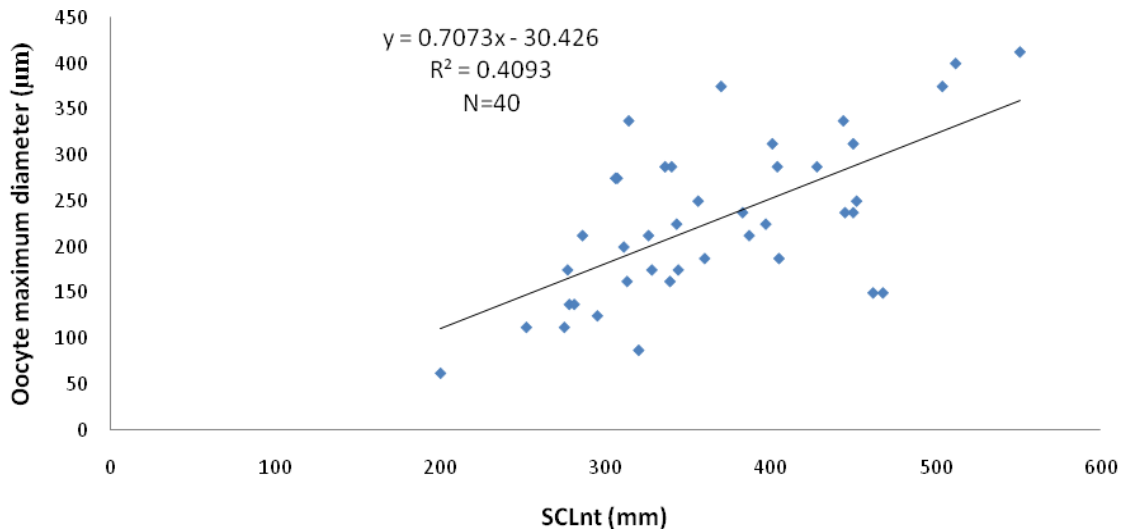
Fig. 13 Maximum observed oocyte diameter (stage III) within a biopsy sample across size classes for juvenile loggerheads.
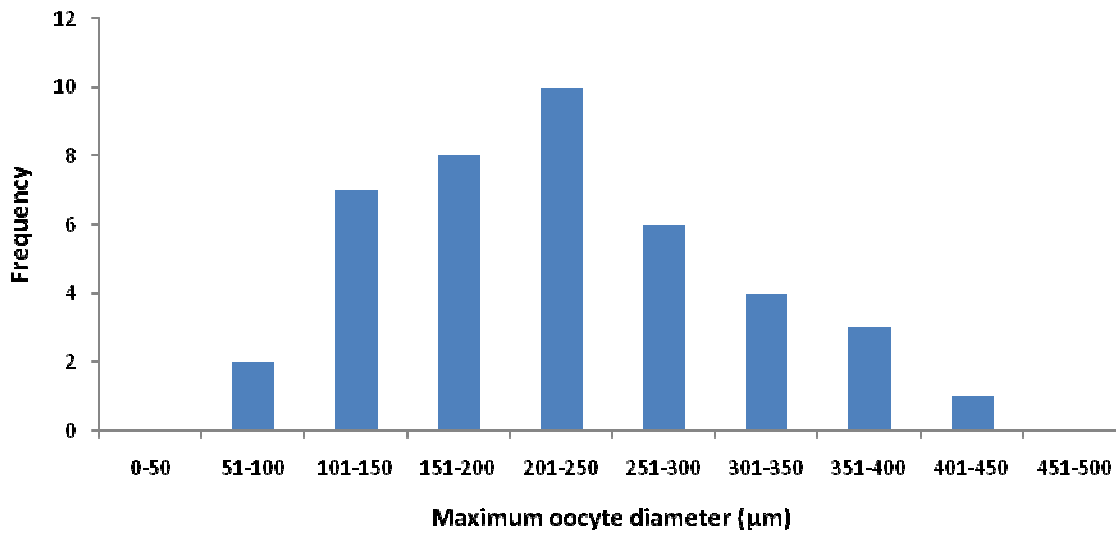
Fig. 14 Maximum oocyte size frequency (stage III) for juvenile loggerheads (n=40).

Hatchlings

114 hatchlings were dissected and the gonads were withdrawn for histological analysis; hatchlings' mean size was 41.65 mm (SCLnt) (SD=2.44, n=99) and mean weight 15.26g (SD=2.35, n=114).

Of 96 gonad samples obtained, 13 were assigned as male, 42 as female, 2 were considered as intersex and 39 were considered inconclusive, giving an overall sex ratio of 3.2 females for each male. In terms of years, 2003 had a sex ratio of 2.67F:1M (72.73% females and 27.27% males) and 2004 a ratio of 3. 71F:1M (78.78% females and 21.22% males).

Hatchlings' gonads were sexually differentiated, with conspicuous medullary cords in males and an unstructured medullar area and thick cortical epithelium in females (Fig 15).
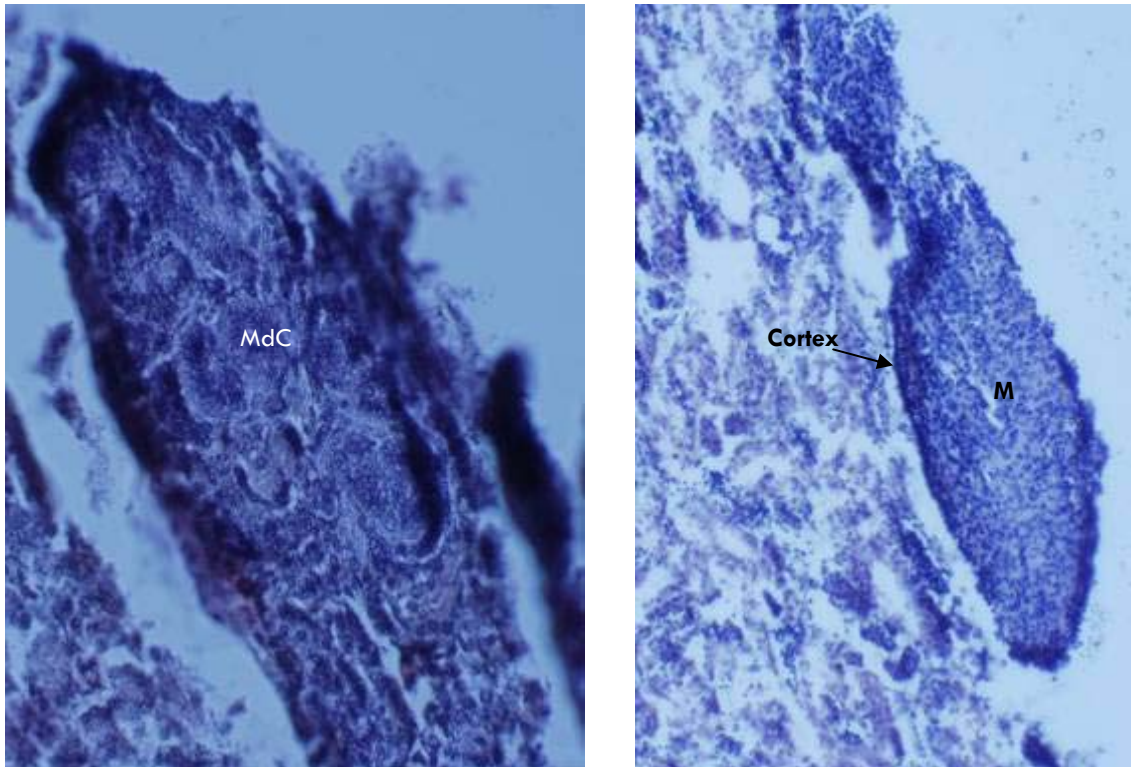
Fig. 15 Photomicrograph of a male gonad (right) and female gonad (left); note the advanced degradation of tissues. Male presents medullary cords (MdC) while female presents a flattened cortical epithelium (Cortex) and unstructured medulla (M) (6 μm section, PAS stain, 40X).

Discussion

Juveniles

In this study, the visual assignment accuracy was verified through histological criteria of the biopsied turtles, which is considered the most accurate sex diagnosing method in order to establish sea turtle populations sex ratios (Mrosovsky and Benabib, 1990). The technique for obtaining a gonad biopsy means that a laparoscopy is done first for gonad localization and subsequent biopsy withdrawal implies that all the laparoscopy apparatus (trocar and endoscope) needs to be withdrawn and inserted again through the same incision, already coupled with the biopsy forceps. This is a very critical phase of the process and needs intense training. There was a clear learning process regarding biopsy withdrawal in this study, since the success rate was low during the first animals attempted and increased over time.

A strong agreement between the histological and laparoscopic technique was found: from the 82 biopsies obtained and histologically examined, 52 were identified as female, 26 as male, and only 3 did not match the sex identified through the laparoscopic technique (plus one individual that was assigned as intersex). The unmatching samples were female-male and vice-versa and were likely due to errors while sample labelling or entering data into the database.

The high percentage of correspondence between sexes diagnosed through laparoscopy and biopsy histology (96.3 %) allowed the validation of the laparoscopic technique, as the overall misidentification rate was negligible, i.e., it does not statistically influences the extrapolation of the overall sex ratio. Hence, the results show that the sex of juvenile loggerheads can be accurately identified macroscopically using laparoscopy, without the added stress to the animal of performing a biopsy. A caution about the histological technique should be born in mind, as that unless complete serial sectioning of the gonad is performed, it might underestimate the proportion of intersexes (Mrosovsky and Benabib, 1990). Since only a small biopsy sample from the whole gonad was observed in these juveniles, the intersex rate might be underestimated in this study. However, the histological verification of sex had to be limited to a small biopsy sample, as reproductive viability of the individual would be impaired or it would imply sacrificing the individuals. Nevertheless the good agreement between the macroscopic and microscopic examination indicates that the overall assignment was accurate and we may be confident that cases of intersex were not overlooked.

Histological examination of the males' testes showed that male gonads were composed of flat monostratified surface epithelium containing seminiferous tubules with numerous quiescent primordial germ cells or spermatogonia lining the lumen's interior. A similar description was also found by Lazar et al. (2003) when assessing sex ratios of loggerheads in the eastern Adriatic Sea using dead specimens. Animals did not yet initiated spermiation as no spermatogenic activity was observed, and they were considered to be immature individuals.

The ovaries exhibited a membranous structure enclosing a stroma (ovarian tissue) with developing spherical follicles at different developmental stages. Juvenile females' follicles presented developmental stages from oogonias type 1 (stage I) and 2 (stage II) up to stage III, and thus all the animals present previtellogenic follicles only.

In contrast with pubescent females, the ovary of juvenile females did not present enlargement of the stroma, and no atretic follicles were observed, which corroborates the non-breeding condition. Therefore, we can state that only juvenile non-breeding individuals are present within Madeiran waters and that there is no reproduction in this region.

The estimated sizes for juveniles follicles according to laparoscopy (1 to 2 mm diameter) were overestimated (see Chapter II for details), as the maximum size observed was 412.5 µm under histological measurement. This overestimation might be due to the wide angle lens of the endoscope, which changes the perceived size according to the distance to the gonad and is difficult to control during surgery. Hence, follicle sizes estimated under laparoscopy should be taken cautiously and only for reference purposes in studies using the same apparatus.

The follicle sizes found for these juvenile females are consistent with the ones found by Hughes, that reported 1-3mm previtellogenic follicles in diameter for mature female loggerheads in South Africa (Hughes, 1974b).

The gonadal state of females is an important indicator of the reproductive status of a population, and the usefulness of the gonadossomatic index (GSI) to infer reproductive stages is commonly accepted. Since gonad weight was not possible to asses in this study, as it would only be feasible for dead animals, alternative or complementary indices of reproductive status usually include histological staging of gonads and maximum oocyte diameter as an indicator of ovarian development. Although it entails complex methodology, histology constitutes the most informative method and, in comparison, measurement of oocyte diameter is rapid, low cost and does not require sacrifice of the individual when using biopsy samples.

The normal distribution observed for the oocyte diameter (Fig. 14) suggests that this measurement is a reliable indicator of ovarian development for this population. However, the rather small tissue sample size available did not allow the determination of oocyte size frequencies, mean oocyte diameter or proportion of oocytes per stage since the number of available oocytes was too small in each slide. As only the diameter of the largest follicle observed for each female was included in the analysis, but we cannot assure that the sample collected the largest follicle present within the gonad, some of the variation observed on the size ranges for the oocytes can be attributed to sampling bias.

Nevertheless, a statistically significant positive relationship between the largest oocyte diameter observed for each female and the size of the animal was found (Fig. 13), which explained 40% of the variation. The positive correlation also suggests that gonad development is an ongoing process throughout the juvenile phase, although within the size range studied development did not progress beyond the stage III previtellogenic phase. It was not possible to determine whether there is an arrested or quiescent development state but judging from the relationship it is unlikely. What triggers further gonad development is not clear for these juvenile loggerheads and needs clarification, but it likely occurs after the ontogenetic shift that characterizes the migration form oceanic to the neritic foraging areas (Bolten, 2003). Absolute or relative levels of sex steroids hormones such as testosterone and estradiol do not seem to explain this, since no correlation exists between oocyte diameter and any of the above mentioned steroid hormones (see Chapter IV for further information on circulating levels of sex steroids in juveniles).

According to Miller and Limpus (2003), during the years before the turtle begins puberty, the changes in gonadal morphology result from growth: as both the ovary and the testis increase in size, their morphological differences become increasingly more visible.

Hatchlings

Marine turtle hatchlings gonads were already described for several species (Merchant-Larios et al., 1989; Mrosovsky and Benabib, 1990; Rimblot et al., 1985; Yntema and Mrosovsky, 1980). Despite species-specificity, namely concerning the differentiation rate, in all cases the ovary consists of an outer germinal epithelium and a medullar area with no tubules with open lumen, whereas the testis consist of an outer simple squamous epithelium and the medulla is filled with convoluted primary cords forming the immature seminiferous tubules.

The identification of a high number of samples (39) was considered inconclusive. This was largely due to an advanced rate of tissue degradation, which was beyond histological recognition and was inherent to the protocol established of collecting hatchlings already dead after emergence from the nest. Emergence occurs 3.68 days on average after hatching for this population (Abella et al., 2001). Supposing these hatchlings died at the moment of hatching or sometime

afterwards, we may conclude that they were collected at least 3-4 days after death, and thus were already decomposing when collected.

The results indicate an overall female bias for the hatchlings examined for both years, and are consistent with the species-specific trend for producing female-biased clutches. The bias observed for this population's sex ratio can be further increased considering the fact that sampling corresponded to the beginning and middle periods of the nesting season for the rookery sampled. In areas where nesting occurs seasonally – as it is the case for the population breeding in Boa Vista Island – mean beach temperatures rise as the season progresses (Mrosovsky et al., 1984), and eggs laid in the early part of the season would produce mostly males and those laid in the middle and late parts, mostly females (Miller & Limpus, 1981). Therefore, we can expect that even more females might be produced for this population for the entire nesting season, as the sampling corresponded to the first third of the reproductive season, and that sex ratios could vary from 10 to 80% female depending on the time of year of nesting (Mrosovsky et al., 1984). In terms of years, 2003 had a sex ratio of 2.67F:1M (72.73% females and 27.27% males) and 2004 a ratio of 3.71F:1M (78.78% females and 21.22% males). This data should be interpreted with caution, however, due to the small sample size; nevertheless, these results constitute the first assessment of hatchlings' sex ratio for this population and have been corroborated in a large extent during the 2005 and 2006 nesting seasons by extrapolating sex ratios from the mean temperature during the middle third of incubation (thermo-sensitive period) on the same beaches, with predicted female biased sex ratios in the order of 71.9% on average (Abella et al., 2007). These results are slightly lower than the ones found for the U.S. nesting beaches, where over 85% of the hatchlings are females (6F:1M) (Hopkins-Murphy et al., 2003).

Madeira Island juveniles and Boa Vista Island hatchlings

Comparisons between the juveniles and the hatchlings' gonads are not straightforward due to methodological constraints in this study. While in juveniles we were able to observe only a small part of the gonad and we were not always able to observe the cortical area and the epithelium, in hatchlings gonads we were usually able to observe the whole gonad and sometimes a transversal cut of the paramesonephric duct in order to diagnose the sex. On the other hand, in

juvenile gonads we were able to observe perfectly preserved tissue, as opposed to decomposing gonads in hatchlings which were many times beyond histological recognition.

In any case we can conclude from the observations of the two stages that while hatchlings present gonads where organization into cortical and medullar region is already apparent, with males presenting conspicuous medullary cords more or less differentiated into seminiferous tubules and females present an unstructured medulla enclosed by a cortical thick area, juveniles present perfectly distinguishable seminiferous tubules with spermatogonia lining the interior of the seminiferous tubule and even the smallest juvenile females present large oocytes and developmental stages up to the stage III.

Despite the highly degraded tissues observed in the hatchlings samples, it is clear that follicles are not yet visible at the time of hatching. This is in agreement with the findings for *Lepidochelys olivacea* gonads, where even 84 days after hatching, oogonia have not yet entered the prophase of the first meiotic division and no follicles are seen (Merchant-Larios et al., 1989). However, in *L. olivacea*, sexual differentiation of the gonad is barely initiated at hatching.

In marine turtle hatchlings in general, as well as in other species, the onset of meiosis is delayed and there is no follicle formation in the ovaries, and in the testes there are no differentiated seminiferous tubules and only medullary cords, with few germ cells, are found. According to Merchant-Larios (1999), the complete differentiation of gonads should occur sometime after hatching. Our findings allow us to conclude that differentiation is completed during the post-hatchling phase and before arriving into Madeiran waters, i.e., prior to 3-6 months of age (Bjorndal et al., 2003a; Bjorndal et al., 2000).

If we compare the results found in this study with the ones found for intermediate size classes in the same species (Wyneken et al., 2007), we can conclude that there is a progressive level of organization for both sexes, since female loggerheads reared up to 120 grams presented a disorganized medulla but a well developed cortex, and with some larger developing oocytes, and males had a poorly developed (usually one cell layer thick) fibrous membrane and the medulla was formed by developing seminiferous tubules.

This study represents a first histological description of the gonad of juvenile pelagic loggerheads turtles. Its great value is the good agreement with the laparoscopy which will enable future

studies of live animals with minimum damage. In the future, a study with whole gonads collected from freshly dead turtles in order to circumvent live animals sampling constraints would enable a more detailed understanding of the reproductive development of these endangered marine reptiles. A wider coverage of sizes and correlation with hormone levels may enable a novel and better understanding of progression of oogenesis in turtles. Comparisons and a more comprehensive discussion on the sex ratios are given in Chapter V.

CHAPTER IV

Identification of sex by measurement of blood plasma Sex Steroids

Introduction

Except for mature adults, sex of marine turtles cannot be determined from external characteristics. Although laparoscopy is a definitive sexing method, it is logistically difficult for large samples, requires surgical training and is a rather invasive procedure. In marine turtles sex ratios may vary depending on the life stage under focus (Wibbels et al., 1991c; Wibbels et al., 1987b). Non-invasive sexing techniques are needed due to the endangered status of loggerhead turtles and, on the other hand, the possible dynamic sex ratios further increase the need to determine populations sex ratios for population modelling.

The ontogenetic morphological changes that occur in the gonad are driven by a complex suite of genetically coded interactive and endocrine changes that occur in the context of endogenous and exogenous clues throughout the turtle's life cycle (Owens, 1997). In recent years several efforts have been made to understand the endocrine regulation systems of endangered marine turtles. The study of reproductive cycles of marine turtles have focused mainly on the behavioural and hormonal aspects happening on nesting beaches or close to the shore, this is to say on female adults and seldom on the adult males as they are difficult to catch. Although the long term and land-based research projects have provided invaluable insight into the reproductive and endocrine physiology of nesting loggerhead turtles, the difficulty to access other individuals rather than the nesting females keeps the whole life cycle puzzle far from being completed. Only a few exceptions focusing on immature loggerheads (Wibbels et al., 1987a; Wibbels et al., 1987b) and reproductive males (Blanvillain et al., 2007) have broadened the scope of endocrine studies on marine turtles. The first studies describing any reptilian hormone cycles were done in captive green turtles *Chelonia mydas* taking advantage of the Grand Cayman Turtle Farm (reviewed in Owens (1997) and Owens and Morris (1985)). Those studies focused on the hormonal changes that play a key role and/or trigger the nesting cycle, and found that the sex of immature captive green sea turtles (4.8 yr old) was accurately predicted using a sensitive radioimmunoassay (RIA) for serum testosterone, since only males had detectable levels of testosterone. Generally, male sea turtles maintain higher levels of circulating testosterone than

females (i.e., the ranges of male and female testosterone levels do not overlap), and this difference is useful in determining sex ratios for these species since sea turtles have temperature-dependent sex determination (TSD) (Wibbels et al., 1987b). Since then many studies have successfully used circulating testosterone levels to determine sex of immature sea turtles of several species (Bolten et al., 1992; Casale et al., 1998; Morris, 1982; Owens, 1997; Owens et al., 1978; Wibbels, 1988; Wibbels et al., 1993; Wibbels et al., 1991c; Wibbels et al., 1987b). The androgen testosterone is responsible for the development of secondary sexual characteristics in male marine turtles (Owens, 1997). In immature marine turtles of both sexes the penis and tail elongate when the animals are injected with testosterone (Owens, 1976; Owens et al., 1978). Estrogen in marine turtles has been reported as an ovarian steroid which causes development of the oviduct in the maturing female (Owens and Morris, 1985). On the other hand, estradiol has been shown to be the primary circulating ovarian steroid controlling vitellogenesis and development of secondary female characteristics in many vertebrates (Bentley, 1980).

In spite of the progress in regard to reproductive cycles of marine turtles, little is still known of the endocrine regulation in pelagic marine turtles. The majority of studies related to reproductive endocrinology have focused on the steroid hormones required for the onset of the reproductive cycle and directly related to important physiological and behavioural processes, such as vitellogenesis, follicular development, courtship behaviour and receptivity. Little work has been done to understand the role or profile of steroid hormones in the juvenile loggerhead turtle. Loggerheads are a generalist species, making them important and unique as a model for the other marine turtle species (Bjorndal, 2003). Thus, physiological studies on pelagic loggerheads have a high research potential in identifying critical and possibly unique processes of major concern to all marine turtle species.

Several advantages of the RIA overcome other sexing techniques: the RIA is performed in the laboratory, reducing the time a turtle has to be manipulated, since only blood is drawn, which does not harm the study animals (Owens, 1999). Other advantages of RIA is that steroid hormones such as testosterone are generally very stable, thus samples can be stored for prolonged periods at -20°C or below with little or no degradation, and many samples can be screened at a time allowing large scale studies (Owens, 1999; Wibbels et al., 2000). Moreover,

although it is an indirect method, it has the advantage of being less invasive and less laborious than laparoscopy and gonad histology. Nevertheless, some limitations prevent a thorough utilisation of the testosterone RIA sexing technique: for example, results from RIAs may vary within and between laboratories (Owens, 1999; Wibbels et al., 2000) and testosterone levels can vary slightly between sea turtle species, and possibly between populations (Wibbels, 1988). Moreover, the RIA should be set sensitive enough in order to detect very low hormone levels predicted for very young animals (Wibbels, 1999; Wibbels et al., 1993). Finally, it should be validated by an independent sexing technique such as laparoscopy on a subset of animals from the species and/or population under study and across all sizes range (Owens, 1999). Testosterone levels of a small percentage of males and females can still overlap (Owens, 1999) rendering the diagnosing power of this technique some limitations.

An unpublished study by A. Meylan suggested that RIA could be used to sex green turtles with straight carapace lengths as short as approximately 25 cm (Owens, 1999), but the minimum size of turtles to which this technique can be applied is not established.

Little or no studies dealing with sex-specific hormone levels and/or trends during an important part of the life cycle such as the pelagic or oceanic phase exist for turtles. In order to better understand the basic reproductive physiology of juvenile Atlantic loggerheads, we provide baseline data on two sex steroids for juvenile loggerheads captured in the wild offshore Madeira Island and test whether sex steroid levels can provide a sexing technique. For this we measured testosterone and estradiol levels in individuals whose sex was verified by laparoscopy.

Objectives

The objectives of this study were to describe the blood sex steroids levels (testosterone and estradiol-17ß) and their relationship to sex and size.

Materials and Methods

Juveniles

Loggerhead turtles were captured south of Madeira Island (Portugal), North-Eastern Atlantic Ocean between 2003 and 2006 (see Chapter I for further details). Although blood samples should be collected as soon as turtles are captured in the wild to prevent any alterations of hormone levels due to stress or temperature-related degradation (Owens, 1997), this was not logistically feasible. Thus, sea turtles were brought to land and kept overnight in small tanks filled with seawater. Blood samples were drawn the morning following capture before any other manipulation, in order to minimize any effect of stress on circulating blood steroids. Because of the inaccessibility of most peripheral veins, blood samples are often difficult to obtain, especially in small sized animals (Bennett, 1989). Blood samples were collected from the dorsal post-occipital sinus (Owens and Ruiz, 1980), after carefully cleaning the neck with 70% ethanol or other antiseptic prior to puncturing (Fig. 16 A). Up to 4 ml of blood were collected using sterile disposable Vacutainer needles in 3 different sizes [0.8x25mm (21G1"), 0.8x38mm (21G1.5") and 0.7x25mm (22G1")] with an associated plastic hub. Blood was collected into Vacutainer™ tubes with gel to separate plasma from plasma cells (BD Vacutainer Systems, Preanalytical Solutions, Belliver Industrial Estate, Plymouth, UK) and immediately centrifuged at 4000 rpm (1500g) for 10 minutes (Megafuge 1.0R, Heraeus, Sepatech) to obtain plasma. Plasma was transferred into cryogenic vials (duplicate samples whenever possible) and frozen immediately at -20∘C until hormone analysis.

Adult Females

The field seasons on Boa Vista Island took place from 16th August to 29th September 2003 and from 25th August to 2nd October 2004. Adult females were sampled during night patrols for beach monitoring. Individual turtle sampling (tagging, biometry and blood sampling) took place only after the nesting process ended, including camouflaging the nest, i.e., when they were returning back to the sea, in order not to disturb the animal's reproductive process. As a general

rule, turtles become more tolerant to external disturbance during egg laying and while filling the egg chamber and covering the nesting site (Miller et al., 2003). Blood samples were obtained the same way as for the juvenile individuals using 0.8x38mm (21G1.5") needles (Fig. 16 B). Samples were kept overnight in a refrigerator at the campsite and centrifuged the next day using a portable 12V centrifuge. Later on the samples were transferred to a freezer located in Sal Rei, Boa Vista Island main town (~50 km distance from the nesting beach).

Fig. 16 (A, B). Blood sampling from the dorsal cervical sinus in a juvenile (A) and adult female (B) loggerhead turtle using a Vacutainer system collection tube. Careful cleaning of the neck region is required prior to sampling.

Hormone extraction from blood plasma

Plasma samples were analyzed by specific radioimmunoassays (RIAs) for testosterone and estradiol-17β. Thawed samples were centrifuged for 5 minutes at 1000 rpm for sample homogenization prior to extraction.

Sep-Pak C_{18} cartridges (Waters Corp, Milford, MA, USA) were used for a solid phase extraction (SPE) of steroids in a 1 ml aliquot of plasma: sample was applied to the cartridge previously primed with 2X2 ml of methanol and 2X2 ml of double distilled water. The sample was applied to the Sep-Pak C_{18}, which was then washed with 1 ml of distilled water. The cartridge was then placed into an extraction tube for sample extraction with 3X1 ml ethanol and centrifuged at 600-700 rpm for 3 minutes.

The organic phase was placed into a ~38°C test-tube heater and evaporated to dryness under gaseous nitrogen. Distilled water (200 µl) and diethylether (3 ml) was added to each sample

and vortexed for 10 minutes and sequentially centrifuged at 600-700 rpm for 5 minutes for complete separation of the aqueous and ether phases. Phases were separated using liquid nitrogen (5-7 seconds) and the organic phase was transferred into a disposable RIA glass tube. This procedure was performed twice for complete extraction. The ether phase was evaporated at $\pm40°C$ under nitrogen and thus obtaining the free steroids fraction, and was re-suspended in 500 µl of assay buffer.

In order to assess the conjugate steroid fractions, the aqueous solution left from the free steroids extraction was further analyzed and the sulphate and glucuronide conjugates were sequentially separated following acid solvolysis (for sulphate) and glucuronidase treatments (for glucuronide), respectively.

For solvolysis the dried fractions were incubated with trifluoroacetic acid/ethyl acetate (1:100; v:v) in a water bath at 40°C overnight, evaporated under nitrogen at 40°C, and acetate buffer added plus 3 ml ether. The samples were vortexed for 5 minutes and centrifuged at 700 rpm for another 5 minutes for complete separation of the aqueous and ether phases. Phases were separated by freezing the aqueous phase in liquid nitrogen (5-7 seconds) and the organic phase was transferred into a disposable RIA glass tube. This procedure was performed twice for complete extraction. The ether phase was evaporated at $\pm40°C$ under nitrogen thus obtaining the sulphate fraction (as free steroid); this fraction was re-suspended in 500 µl of assay buffer.

To obtain the glucuronide fraction the aqueous residue (sodium acetate) remaining from the sulphate extraction was incubated on a shaker overnight at 37°C with 10 µml of β-glucuronidase from bovine liver (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada). The glucuronide fraction was then extracted by adding 3 ml ether and following the same procedure as for the extraction of the sulphated fraction.

As with the other fractions the glucuronide fraction was re-suspended in 500 µl of assay buffer and kept at -20°C until radioimmunoassay.

Prior to using this extraction method, direct measurement from plasma and denaturation at 80°C were also tested in order to evaluate whether there were substances that could interphere with the measurement, and if positive the requirement to extract the steroids from plasma.

Steroid radioimmunoassay

After sample extraction duplicate aliquots (100 µl) were incubated overnight at 4°C with 1500 counts per minute (cpm) of tritium-labelled testosterone [1,2,6,7-^{3}H]androsten-4-ene-3,17β-dione (ref. TRK454, Amersham Biosciences), or tritium-labelled estradiol [2,4,6,16,17-^{3}H]oestradiol (ref. TRK587, Amersham Biosciences) and testosterone or estradiol (Research Diagnostics, Flanders, New Jersey, USA) antibodies, respectively. The unbound antibody-bound steroid was separated from free steroid using a charcoal-dextran solution (0.5%-0.05%).

The supernatant containing the antibody-bound steroid complex was decanted into scintillation vials (Sarstedt ref.73.680 HD-PE) with 4 ml aqueous-based scintilant (Ecolite, ICN Biochemicals) and counted on a scintillation counter (Model LS6000IC, Beckman Instruments Inc., Fullerton, U.S.A) for 10 minutes each sample.

A standard curve for testosterone [(4-androsten-17β-ol-one); ref A6950, batch G646 Steraloids (UK) Limited] and 17β-estradiol (1,3,5[10]-Estratirene-3,17β-diol); ref E-8875 Sigma] (with 500, 250, 125, 100, 50, 25, 10, 5, 2.5, 2, 1 and 0.5 pg per tube) was analysed with each run of unknown plasma samples, in order to enable calculation of hormone levels. Sample dilutions (usually 1:5) were prepared in order to fit the standard curve range values and concentration values were corrected for individual plasma volume.

The cross-reactivity of the estradiol RIA was detailed in Guerreiro et al. (2002) and cross reactions for testosterone were described by Kime and Manning (1982).

Corticosterone levels were measured in a sub-sample of juveniles and nesting females in order to assess stress levels.

Environmental data: Sea Surface Temperature (SST)

Since temperature influences the endocrine system of marine turtles (Owens and Morris, 1985), sea surface temperature (SST) data was obtained in Madeira Island in order to evaluate any potential bias on hormone titres due to environmental temperature (Owens, personal comm.) and compare with sex steroid levels. Additionally, the usage of radioimmunoassay to measure serum testosterone levels is restricted to samples collected in turtles in ambient water temperatures in

excess of 24°C, and below that temperature this hormone assay is not reliable (Braun-Mcneill et al., 2007; Braun-Mcneill et al., 2000).

SST data was provided by the Funchal Harbour Administration (Administração Regional dos Portos). Temperature was assessed by means of a multiparameter station installed at a buoy located 2 nautical miles offshore Funchal (32°37.190N 16°56.447W) and Caniçal (32°43.250 16°43.566W), which provide a representative indicator of SST for the pelagic environment. This device provides *in situ* temperature measurements every 3 hours. The closest measurement to the capture time and place of each turtle was chosen (distances were less than 10 nautical miles from capture site and time lags shorter than 90 minutes).

Sex identification and oocyte size

The sex of sampled juveniles was diagnosed using laparoscopy (see Chapter II for further details). The oocyte diameter was measured in gonad biopsies (see Chapter III for further details).

Statistics

Levels of both steroids were compared between sexes within juveniles and between juvenile females and adult females using the Mann-Whitney *U* test. Furthermore, a ratio between testosterone and estradiol was calculated for the different groups and compared among them using the Mann-Whitney *U* test; normality of data was tested using Kolmogorov-Smirnov test (Field, 2005).

Regression analysis was employed to evaluate the relationship between sex steroids levels and SST, as well as between sex steroids levels and juvenile females' follicle size.

Statistical analysis used SPSS 15.0 software (SPSS Inc., 2006) and all tests are 2-tailed unless stated otherwise.

Results

Juveniles

Blood was sampled from loggerhead turtles with carapace lengths ranging from 171-600 mm SCLnt and laparoscopies were performed in all of them for sex identification.

Plasma testosterone and estradiol levels were measured using RIA. Plasma T levels ranged from 3.9-59.6 pg/ml, and for E_2 ranged 2.9-42.6 pg/ml. Results that were statistically significant outliers from testosterone (one male and 2 females out of a total of 36 and 58 respectively) and from estradiol (3 females out of a total of 49) were excluded from the analysis.

Testosterone levels in males ranged 6.9-59.6 pg/ml (M=25.8 pg/ml; SD=13.95) and in females ranged 3.9-44.8 pg/ml (M=22.7 pg/ml; SD=11.6). Estradiol levels in males ranged 3.1-34.4 pg/ml (M=16.1 pg/ml; SD=9.6) and in females ranged 2.9-42.6 pg/ml (M=17.6 pg/ml; SD=10.9).

Shapiro-Wilk tests for normality of the data were performed. Testosterone distribution was normal for males (Shapiro-Wilk (29)=0.952; p=0.207) but not for females (Shapiro-Wilk (48)=0.933; p=0.009), and estradiol had no normal distribution for both sexes (males: Shapiro-Wilk (29)=0.898; p=0.009; females: Shapiro-Wilk (48)= 0.917; p=0.002). However, log-transformation of the data did not render a normal distribution and thus a Mann-Whitney U test was run to test for differences in testosterone and estradiol and the ratio testosterone:estradiol (T:E) between males and females.

Males did not differ significantly neither in testosterone (U=1011.0, n=1=36, n2=58, p=0.800) and estradiol (U=730.5, n=1=30, n2=49, p=0.966) from females. The ratio T:E was slightly higher in males than in females (U=515.0, n=1=29, n2=48, p=0.029, 1-tailed).

Hence, identification of the sex of the turtles based on testosterone and estradiol titres was not possible, since distribution of both steroids concentrations was not bimodal, i.e., there was no clear division between males and females.

No correlation was found between any of the sex steroids levels and size (SCLnt) (Fig. 17, 18 and 19).

Steroids other than free steroids, such as sulphates and glucuronides were screened for in order to eliminate the possibility of under estimating steroid levels. However these non-free steroids were very low or undetectable and, thus, were not considered for the amount of circulating hormones.

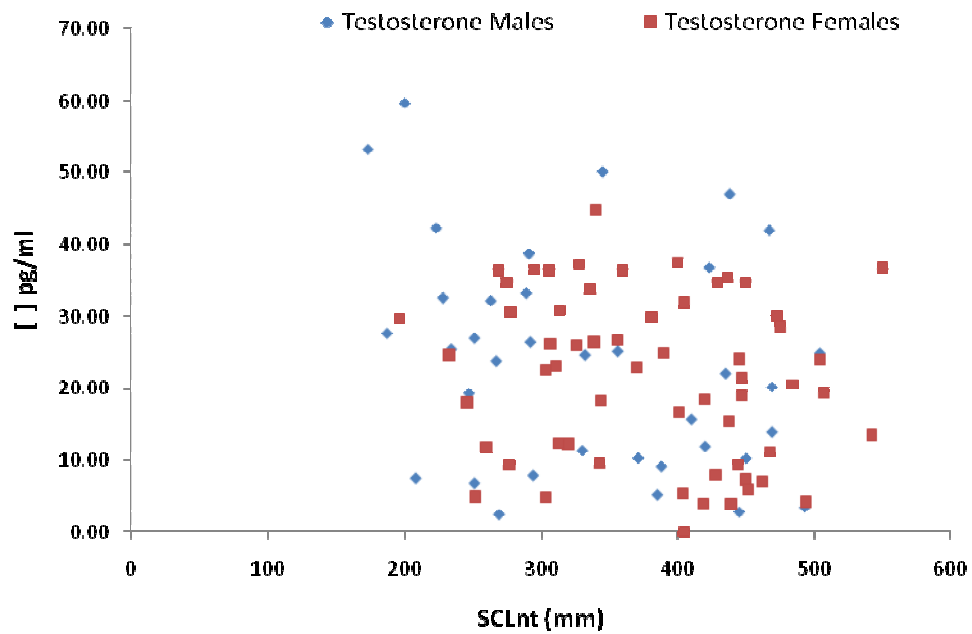Corticosterone levels ranged from 0.74-11.07 ng/ml (M=4.14 ng/ml; SD=3.23; n=24).

Fig. 17 Testosterone vs. SCLnt across size classes in juvenile males and females.
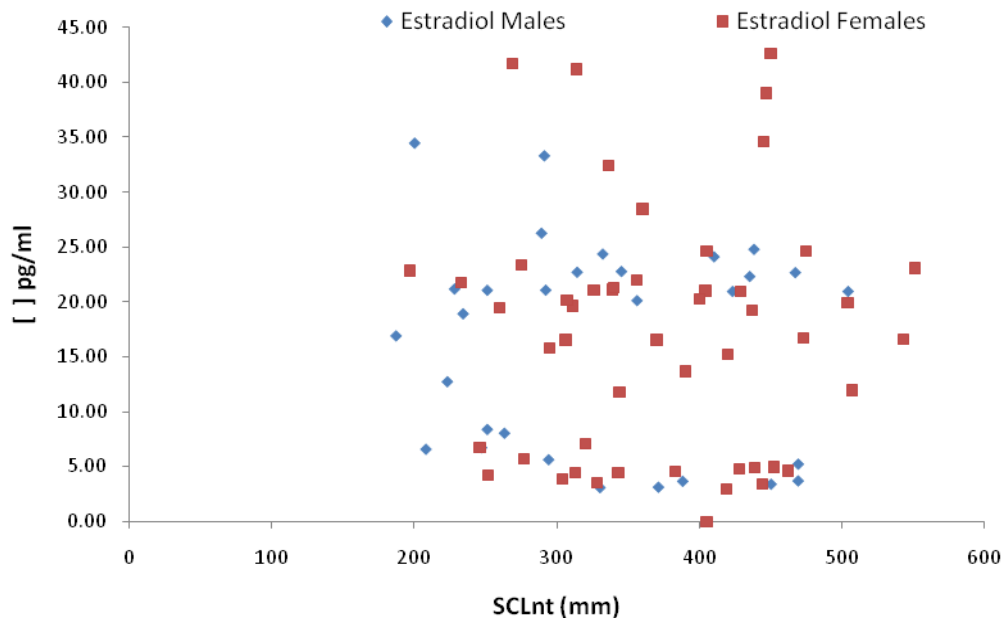
Fig. 18 Estradiol vs. SCLnt across size classes in juvenile males and females.
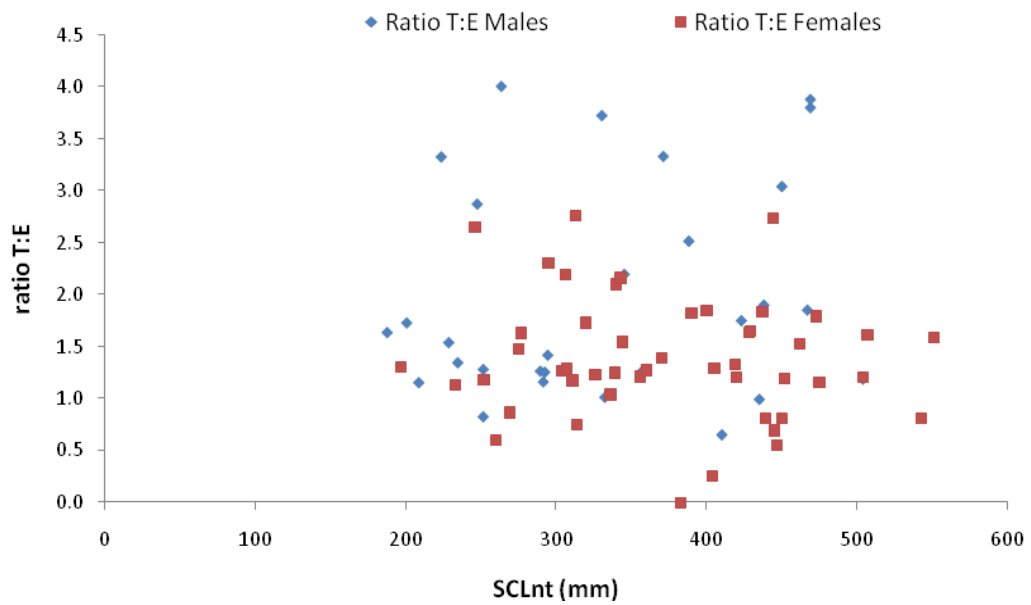
Fig. 19 Ratio Testosterone:Estradiol (T:E) across size classes in juvenile males and females.

Sex steroids and SST: is steroids RIA temperature-dependent?

To evaluate possible temperature effects, sex steroids were contrasted with SST. A significant correlation between testosterone levels and SST was found (T=3.65*SST-55.803, n=73, r^2=0.276, F=27.394, p<0.0001) (Fig. 20); however, this correlation was not significant for estradiol (T=1.72*SST-19.084, n=63, r^2=0.083, F=5.63, p=0.021) (Fig. 21) and for the ratio T:E (T=0.0139*SST+0-909, n=62, r^2=0.003, F=0.202, p=0.655) (Fig. 22).
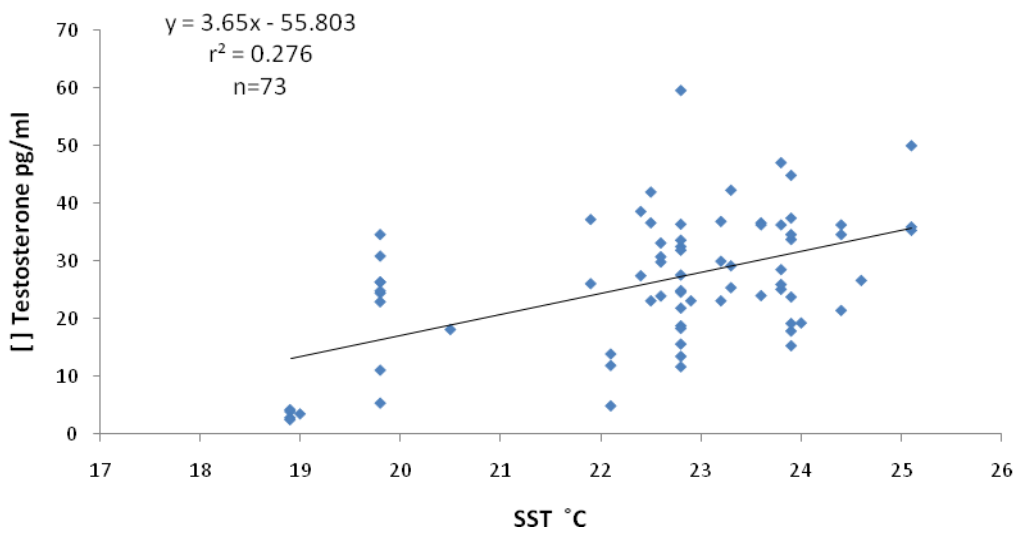
Fig. 20 Juveniles' testosterone levels as a function of SST (p<0.0001).
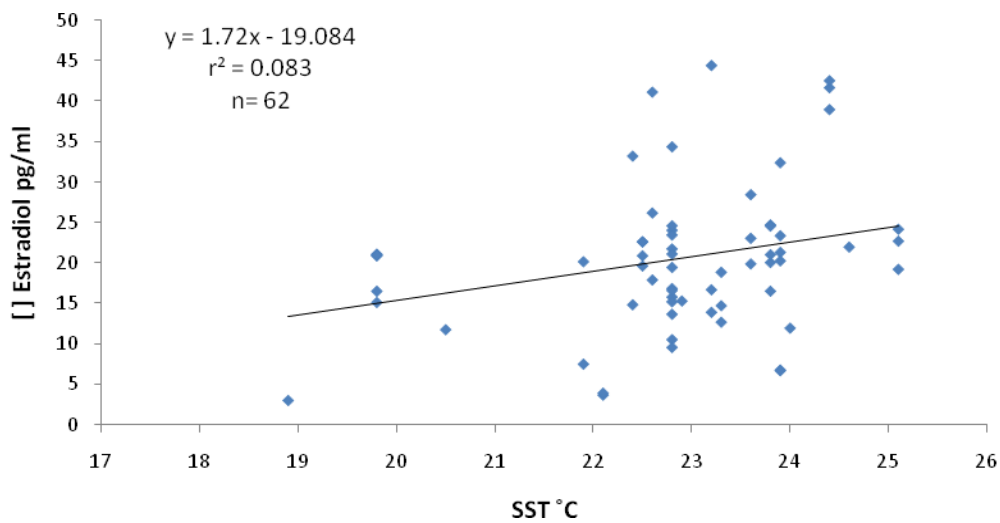
Fig. 21 Juveniles' estradiol levels as a function of SST (p=0.021).
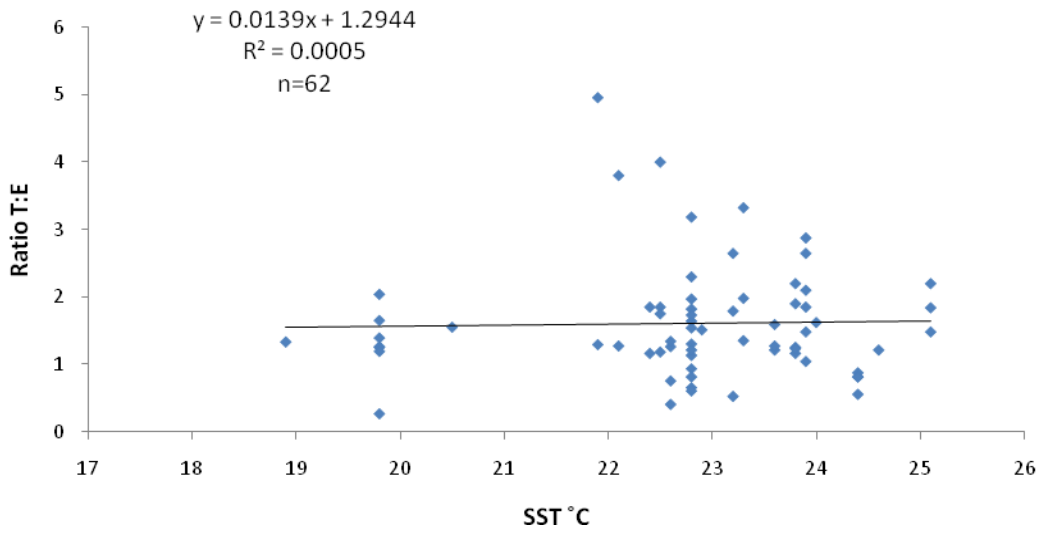
Fig. 22 Juveniles' ratio T:E as a function of SST (p=0.655).

Circulating hormones and oocyte size

The oocyte diameter found for juvenile females was compared to testosterone and estradiol (Fig. 23) but no relationship was found (testosterone: F=0.942, r^2=0.025, n=37, p=0.338; estradiol: F=0.521, r^2=0.017, n=31, p=0.476).
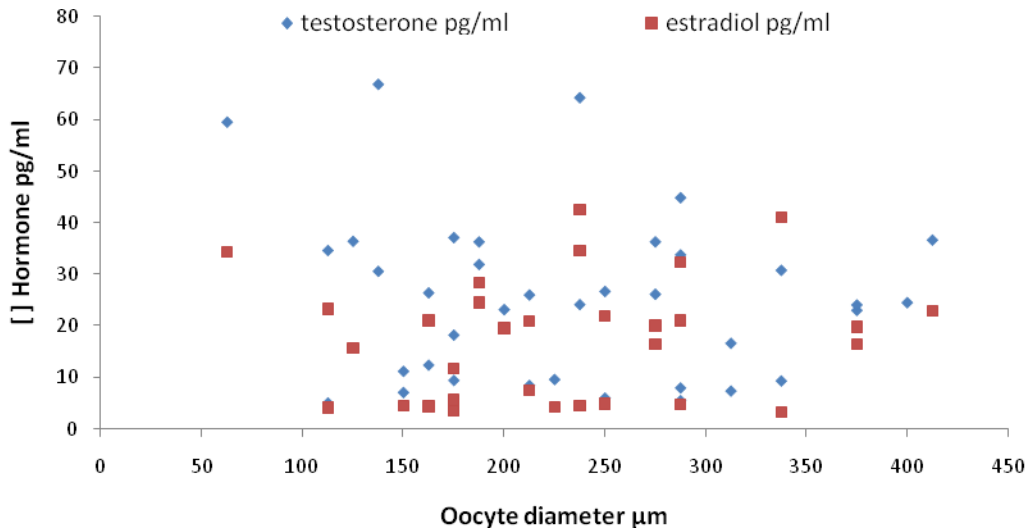
Fig. 23 Relationship between oocyte diameter and testosterone and estradiol levels in juvenile females.

Adult Females

Nesting females were sampled in 2003 and 2004 nesting seasons both in Ervatão and Ponta Cosme beaches in south-eastern Boa Vista Island. From the 89 females sampled blood sex steroids levels were obtained for 75 individuals (27 in 2003 and 48 in 2004). Turtles' size ranged from 680-872 mm (Mean=754.23mm; SD=38.30).

Two sex steroids were analyzed: testosterone ranged from 3.9-144.5 pg/ml (Mean= 41.2 pg/ml; SD=34.6; n=69) and estradiol 2.7-38.6 pg/ml (Mean=13.1 pg/ml; SD=9.7; n=73). Statistical outliers were excluded from de analysis (5 for testosterone and 1 for estradiol).

Corticosterone levels ranged from 0.05-0.79 ng/ml (M=0.32 ng/ml; SD=0.19; n=36).

Madeira Island juveniles and Boa Vista Island nesting females

The sex steroids plasma levels in pelagic juvenile females and in nesting females were compared for absolute levels and between juveniles and nesting females regarding the ratio T:E.

Due to the non-normal data distribution a Mann-Whitney *U* test was performed to compare data between female juveniles and adult females.

Estradiol did not differ significantly between juveniles and adult females (U=1458.5, n1=49, n2=74, p=0.067), but testosterone differed significantly (U=1449.5, n1=58, n2=70, p=0.005) (Fig. 24). The ratio T:E was significantly higher in adult females than in juveniles (U=1206.0, n1=77, n2=73, p<0.0001) (Fig. 25).

Corticosterone levels were significantly higher in juveniles than in adult females (U=2.0, n1=22, n2=36, p<0.0001).

Fig. 24 Testosterone and Estradiol levels in juveniles and adult females. Juveniles correspond to the left group (SCLmin<600 mm); adult females correspond to the right hand group (SCLmin>680 mm).

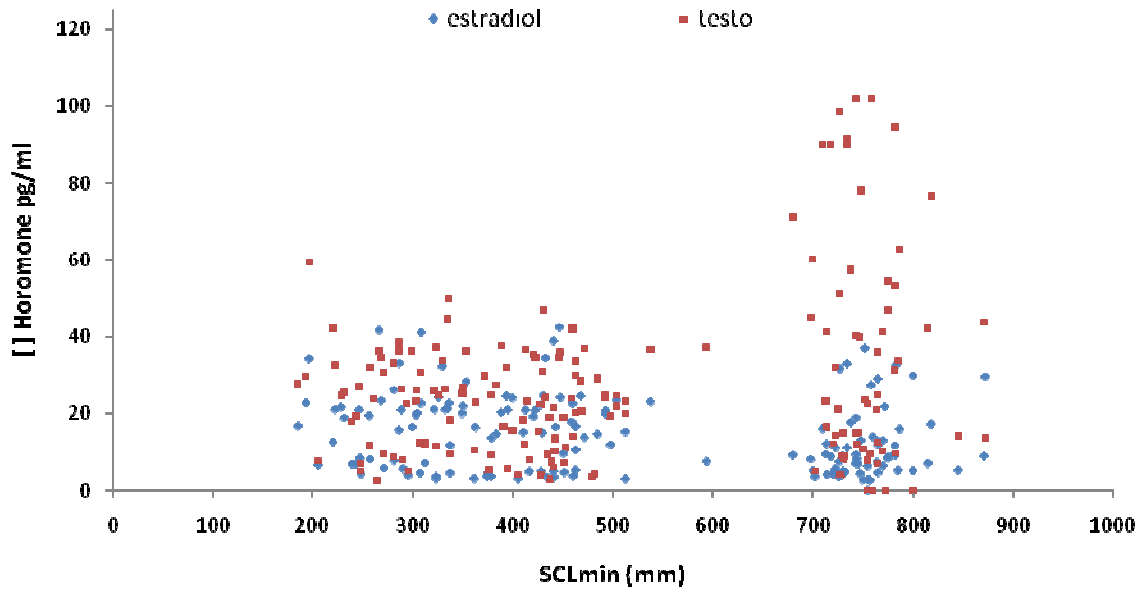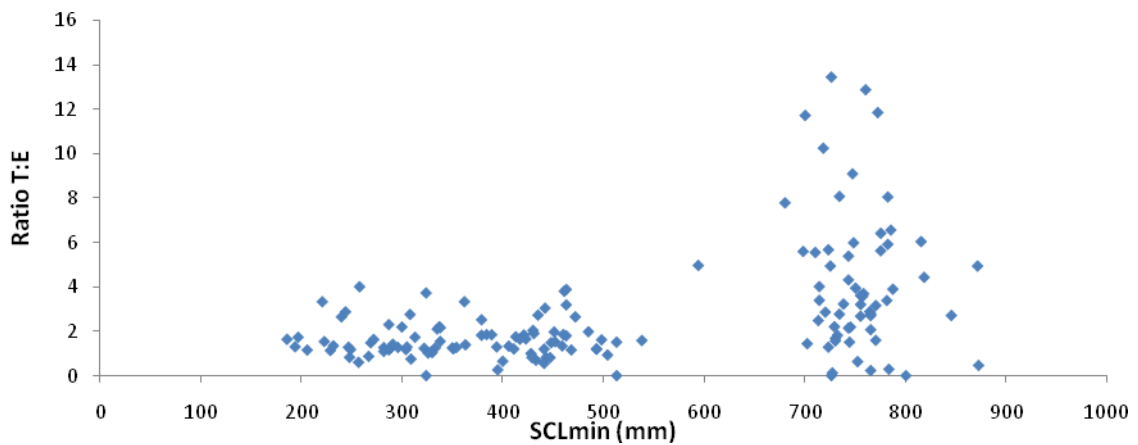Fig. 25 Ratio Testosterone:Estradiol in juveniles and adult females. Juveniles correspond to the left group (SCLmin<600 mm); adult females correspond to the right hand group (SCLmin>680 mm).

Discussion

Sex steroids levels for Madeira Island juveniles and Boa Vista Island nesting females

Pelagic juveniles' and nesting females' plasma samples were analyzed by a standard radioimmunoassay to determine circulating T and E_2. Therefore, this study reports the sex steroids levels for juvenile loggerheads offshore Madeira Island, and compares those levels with those of nesting females in Boa Vista Island (Cape Verde).

The absolute levels reported for juveniles do not differ significantly between the sexes. This lack of a sex-specific pattern may explain the lack of any secondary sexual dimorphism in this reproductively immature phase. Since testosterone is a known precursor of estradiol and subsequent estrogens, this fact may account for the high mean testosterone levels shown in juvenile females.

This lack of sex-specific pattern was also found in plasma samples analyzed using HPLC, from post hatchlings approximately 6 months of age and weighing at least 120g, which were being reared for laparoscopic examination. Interestingly, males exhibited higher maximum amounts of estradiol than females, reaching 0.3 µg/100µl, while females averaged only 0.2 µg/100µl. Conversely, females had higher maximum levels of testosterone (up to 10 µg/100µl) than their cohort males (5 µg/100µl), and thus the hormonal profiles gave no reliable indication of turtle sex (Botterill, 2005).

The absolute levels reported for juveniles appear to be very low. This may be due to the fact that blood sampling took place the day following capture. This procedure was elected in order to avoid the effects of handling stress as reported for turtles immediately after capture (Gregory et al., 1996; Gregory and Schmid, 2001), since plasma corticosterone levels are considered a stress indicator in reptiles (Guillette Jr. et al., 1995). In fact, animals may be able to acclimate to a repeated or prolonged stressor (e.g. confinement), such that plasma corticosterone levels fall despite continued exposure to that stress. In such cases, plasma corticosterone levels drop to baseline levels after 2 hours in lizards (*Eulamprus heatwolei*) (Langkilde and Shine, 2006), or 6 hours in loggerheads (Gregory et al., 1996). Since sex steroids such as testosterone are known to be stress-labile (Owens, 1997), our results may in fact

represent the most proximate to basal levels. Furthermore, our dip-netting capture method is relatively rapid, and probably not as stressful as capture methods used in other studies where turtles were captured in pound nets or 'turtle-rodeo' and blood for hormonal analysis was sampled 15-30 minutes after capture.

Corticosterone (B) levels found for juveniles in this study were much higher than the ones found for nesting females, suggesting a strong stress response in juveniles. However, straightforward comparisons between these two groups are misleading, since marine turtle females of at least 4 species – including loggerheads – have a physiological ability to downregulate or desensitize their corticosterone stress response (Gregory et al., 1996; Jessop, 2001; Jessop et al., 1999; Valverde et al., 1999) in order to maximize their reproductive output (Hamann et al., 2003). Additionally, in contrast with the findings of Whittier et al. (1997) no relationship between T and B was found, suggesting that stress is not affecting sex steroid levels in our pelagic juveniles. Nevertheless, stress response in these cohorts should be further investigated.

Comparisons between the juveniles and the nesting females cannot be straightforward and should be done cautiously, since nesting females are at a very particular physiological stage, where hormones show particular 15 days cycles (Owens, 1997; Owens and Morris, 1985). Moreover, sampling of adult females coincided with oviposition, when hormones other than testosterone and estradiol also play an important role. In contrast, juveniles are at a 'neutral' physiological stage regarding reproductive events. A more straightforward and logical comparison would be with non-nesting females during the 2-3 years interval reproductive period, which can be regarded as a more 'neutral' physiological period regarding reproduction, but this is logistically difficult to achieve. Even though, and although juveniles' and female adults' levels do overlap, results show that absolute sex steroids levels in both juveniles' sexes are significantly lower than the ones found for nesting females, and females show a wider range in sex steroids levels. Moreover, nesting females show distinctly much higher T levels than juvenile males. This may not be surprising since it is known that circulating testosterone is high in the reproductively active female (Owens, 1997; Wibbels et al., 1987a), and steadily drops throughout the nesting season till it reaches a minimum at the end of the nesting season (Wibbels et al., 1990b). Due to the high atmospheric temperatures registered in Boa Vista Island, some degradation of female

adult blood samples may have happened since refrigerating conditions at the campsite were very limited and blood samples could only be refrigerated instead of frozen. Moreover, the portable 12 V centrifuge available was compatible with cars only, and a car was not always accessible readily. Some samples were thus frozen only 24 hours after withdrawal. Higher levels for female adults may in fact be expected given proper sample freezing conditions.

Overall, and although direct comparisons between assays should be taken cautiously, as absolute values may vary among laboratories and RIA's (Wibbels et al., 2000), our study reveals very low levels for both steroids assessed when compared to other studies.

The closest values reported to date are the ones found for the corresponding juvenile neritic population (Bolten et al., 1998) in the Core and Pamlico Sounds, North Carolina, USA, and confirmed under laparoscopy. The population sampled (41.4-75.4 cm) showed T levels for females of 6.7-128.0 pg/ml and for males 372.0-1884.0 pg/ml (Braun-Mcneill et al., 2007).

Despite some animals showing very high values (>1000 pg/ml) on a study on the Mediterranean loggerhead population with a population size range (29-65 cm SCCL) equivalent to the Madeira's juveniles, testosterone titers revealed a bimodal distribution, with a lower mode at ~50 pg/ml and a higher mode at ~400 pg/ml that should be attributable to females and males respectively (Casale et al., 1998). However, those samples were collected from animals accidentally caught in fisheries and that were presumably under great stress, and the authors themselves were concerned that stress levels may have affected the results (Casale et al., 1998).

In an in-water study dealing with immature loggerheads (76 cm maximum length) and validated by laparoscopy, immature females showed testosterone values up to 31.0 pg/ml, whereas immature males ranged from 76.4 to 545.0 pg/ml (Wibbels et al., 1987a). However, these values were reviewed later due to a problem with the standard testosterone and should be increased 10-fold (Braun-Mcneill et al., 2007; Lee and Owens, 2005). In this same study seasonal changes in the serum titers of adult loggerheads were detected, with mean monthly values peaking in August and the lower in March for all males population. In contrast, reproductively active males appeared to have higher titers during February through April, and decreased in May (Wibbels et al., 1987a). Adult females had testosterone titers ranging from <41.4 pg/ml to 1209.1 pg/ml (but these values were reviewed and should be increased 10-

fold), but females sampled at the nesting beach (and thus reproductively active) had higher values than the females offshore (Wibbels et al., 1987a). In any case, the methods used to capture individuals were either by trawling, tangle or pound net, which should be quite stressing for the individuals, and were all sampled within 30 minutes up 6 hours after their removal from the nets, but post-hoc tests for stress effects revealed no significant differences.

When sexing immature loggerheads along the Atlantic Coast of the United States Wibbels (1987b) found that serum testosterone titers were an accurate indicator of sex, whereas neither tail length or straight carapace width/length ratio were accurate sex indicators. Mean concentrations found were 13.9 pg/ml for females and 149.7 pg/ml for males, but again these values were later corrected 10-fold (Braun-Mcneill et al., 2007; Lee and Owens, 2005). Nevertheless, the ranges did not overlap between males and females, providing an accurate sexing technique as validated through laparoscopy in a subset of turtles.

In another study on loggerheads in Queensland (Australia) values reported for reproductively inactive females referred relatively low levels of E_2 (<81.4 pg/ml) and T (<56.0 pg/ml); no seasonal changes in gonadal steroids were detected in these non-breeding females (Wibbels et al., 1990b). That same study followed one nesting female throughout the season, and reported mean levels of E_2 of 168.8 pg/ml and T 528.2 pg/ml, sequentially along the nesting season, with levels for both steroids dropping along the season, and at the last nesting event were <50 pg/ml; a parallel study performed in Melbourne Beach, FL, detected a similar trend in gonadal steroids along the season, but once again these values were corrected 10-fold (Braun-Mcneill et al., 2007; Lee and Owens, 2005).

More recently, a small pool of loggerhead males were screened for reproductive activity in the Cape Canaveral, Florida (Blanvillain et al., 2007). A total of 11 adult males showed T levels ranging from 2.4 ng/ml to 221.9 ng/ml, far higher than the values reported here for juvenile males. Furthermore, two distinct groups were detected with 4 animals having T levels less than 10 ng/ml, and 7 animals with T levels above 150 ng/ml. All turtles with high T levels showed signs of reproductive activity according to laparoscopy and testis biopsy.

Sex steroids and SST: are steroids temperature-dependent?

Sea surface temperature clearly affected testosterone levels in the juveniles studied, as well as estradiol levels although with a much weaker effect. This is not surprising since environmental temperature directly influences some sea turtle endocrine systems through the pineal complex, and other environmental factors such as photoperiod and nutritional history also appear to be capable of regulating reproductive cycles in these marine reptiles (Owens and Morris, 1985). Owens (1997) speculated that juveniles may respond more to temperature environmental clues, while adults may respond more to photoperiod as the large pineal system begins functioning at the reproductive level during puberty.

Interestingly, Braun-Mcneill et al. (2007) found that the T sexing technique was reliable only for turtles sampled during the summer months (July and August), when water temperatures are between 24-28°C – which do not overlap with SST observed offshore Madeira –, and also found a significant positive relationship between temperature and log-transformed T concentrations. In their study to evaluate a possible temperature SST or seasonal effect with immature neritic loggerheads the T sexing technique was not reliable for animals sampled during winter months

(Braun-Mcneill et al., 2007). In our study, all animals were sampled during the warmer months, but SST was usually below the 24°C lower limit suggested by Braun-Mcneill et al.'s study. Thus, we can only speculate that the RIA sexing technique did not work in this study for two main factors: the SST range did not allow a reliable RIA or the sex steroids levels are in concordance with the lack of sexual dimorphism in juveniles and thus explain the lack of bimodal sex steroids distribution. If possible, sampling during the winter months, when SST drops to 16-17°C, would help to better clarify seasonal effects.

Circulating sex steroids and oocyte size

Although a positive significant correlation was found between oocyte diameter and individual's size in juvenile females (see Chapter III), which suggests that oocyte development is an ongoing process, there is no correlation between oocyte diameter and sex steroids circulating levels (Fig.

23). This unexpected fact, on the other hand, suggests that other clues rather than the sex steroids analyzed are triggering the gonad development.

Interestingly, in a study with post-hatchlings captive reared up to 6 months or 120 g weight, Wyneken reported a seasonal effect on gonadal macroscopical appearance (Wyneken et al., 2007), which most likely should be mediated through sex steroids, but no correlation was found between hormones and sex in that same study (Botterill, 2005).

Ratios T:E

Despite the absence of differences on the sex steroids plasma absolute levels between sexes in our study, the results indicate that males as a group, have a slightly higher ratio of T:E (Fig. 19); however, these values still overlap to a great extent with the females' and thus the ratio T:E cannot be a reliable predictor of sex. A larger sample size may show probable differences between sexes. Furthermore, probable increasing differences at larger size classes could become evident, as sexual dimorphism becomes apparent at larger size classes. If these higher T:E values are reflecting the physiological difference between sexes and eventually age classes, this could be attributed to an increased requirement for the somatic and reproductive differentiation processes to occur during ontogenesis.

The ratio T:E for the adult females analyzed is significantly different from the juveniles'. This difference may sound surprising, however it is not indicative of a sex-specific pattern since it is known that circulating testosterone rises while estrogen drops at the time of the nesting migration in the reproductively active female (Owens, 1997). Moreover, at the time of nesting and until after 48 hours, E lowers in C. *mydas* (Owens and Morris, 1985), which explains increased T:E in nesting females.

Interestingly, in a study also addressing ratios between sex steroids in loggerheads – but using the inverse ratio – E:T was found to be a reliable predictor of hatchling sex (Gross et al., 1995). Instead of measuring sex steroids in the hatchling blood, the authors used the chorioallantoic/amniotic fluid from recently hatched eggs. In that case, the ratio of estrogen to testosterone was greater in the fluids from hatchling females than from males and it could be used as a non-invasive technique to generate the sex ratio from freshly hatched clutches.

Those results contrast with the ones found in this study, where no meaningful differences were found. The significant differences found in that study for the chorioallantoic/amniotic fluid might be explained by the close proximity in time to the critical thermo-sensitive period, when the gonad's fate was determined and gonad differentiation took place (Yntema and Mrosovsky, 1982), in contrast to the juveniles, where no relevant gonadal activity seems to be occurring, as indicated by the very low levels of gonadal steroids found in this study. On the other hand, during embryonic development the embryo is still under the influence of maternal hormones inherited via the vitello (Crews et al., 1989).

Relevance of the Sex steroids analyzed

The sex steroids addressed (testosterone and estradiol) are believed to be the target ones, as estrogens are involved in normal female development of many organisms with TSD, including turtles (Sheehan et al., 1999), and testosterone is the main androgen related to development of male sexual dimorphism in marine turtles (Owens, 1997). Estradiol levels in marine turtles have been addressed in several studies (Gross et al., 1995; Guillette Jr. et al., 1991; Lance et al., 1979; Licht et al., 1979; Owens, 1974; Wibbels et al., 1990b) but it is possible that estradiol is not the major circulating estrogen (Owens and Morris, 1985). In fact, estrone was identified as the major estrogenic steroid in marine turtle plasma, including C. *caretta* (Coufal and Whittier, 2003), with circulating levels ranging from 29.63 pg/ml to as high as 2.0 ng/ml in vitellogenic females (Coufal et al., 2003) and we could suppose we might have missed much higher levels of this slightly different but apparently important molecule in our oceanic juveniles.

On the other hand, estrone was not found in the allantoic fluid of recently hatched loggerhead eggs which are known to still contain steroids from the embryo and was found only sporadically in the plasma of one week old hatchlings, and only sparingly in the plasma of captive reared loggerheads 5 months old, all in the same study. In contrast, estradiol was found in the majority of the plasma and fluid samples (Botterill, 2005), suggesting a more important role for estradiol for younger turtles.

In future studies, both estradiol and estrone should be screened in our pelagic juveniles in order to clarify any different patterns or key-roles in sex differentiation for pelagic juveniles. Analysis

of steroidogenic pathways in juvenile gonads using radioactive precursors is a requirement to clarify this aspect.

In any case, estrogen circulates at low levels in all sea turtles, and specially so in green turtles (Wibbels et al., 1992).

The fact that the levels of steroids other than free steroids, such as sulphates and glucoronides, were very low or below the assay's detection limit allows us to be confident that the physiological key levels were in fact measured, since excretion metabolites were not neglected.

Sex steroids levels and population sex ratio

As no bimodal distribution was found for both testosterone and estradiol levels, as well as for the ratio T:E, this juvenile pelagic population's sex-ratio cannot be extrapolated using a RIA, neither the individual's sex can be predicted.

At least one study using testosterone titers to assess sex ratios in loggerheads also failed to achieve it (Bolten et al., 1994). However, the actual values found were not reported. A second study in C. *mydas* (Schroeder and Owens, 1994) was also not able to detect sex differences in steroid levels. In both cases no bimodal distribution was obtained for the pool of animals sampled, i.e., there was no clear division between males and females, and thus no sex-ratio was extrapolated for the populations under study. Wibbels et al. (1987b) speculated that variables such as temperature, time of year and the time period between capture and blood sampling could affect testosterone titres, increasing the number of turtles with intermediate, overlapping values. This hormonal overlap between sexes was also found in loggerheads' both plasma and allantoic fluid (Botterill, 2005).

The lack of correlation between anatomical sex and sex steroids should not be attributed to individual's sex, since the stress response is not different between males and females, at least in green turtles C. *mydas* (Jessop and Hamann, 2005).

Although this study failed to exhibit a bimodal endocrine response between sexes, it puts in evidence that the lack of morphological sexual dimorphism might be reflecting the lack of endocrine distinction between sexes at this young size classes. The very low sex steroids levels compared with other populations of the same species plus the histological data for this

population (see Chapter III) suggests that reproductive development is quiescent or at least very low at this size classes.

These results represent the first quantitative analysis of sex steroids in juvenile oceanic loggerheads and form a basis for further studies aimed at the understanding of the endocrine physiology of free-ranging juvenile marine turtles.

CHAPTER V

Sexual Dimorphism and Sex ratios in Juvenile Marine Turtles

Introduction

Ideally, a sexing technique should be accurate and logistically feasible for sexing large numbers of individuals (Wibbels, 2003). Since marine turtles have TSD, several molecular markers have been tested as a sexing tool, but until now have not proven useful. Physiological differences such as sex-specific hormone levels, which rely in little-invasive techniques like blood sampling, seem to be reliable only for the adult and sub-adult life stages and even at those size classes it is still restricted to sea surface temperatures SST above 24°C (Braun-Mcneill et al., 2007).

At adult life stages phenotypic sex differences are evident, with males showing a long tail and developed claws (Owens, 1997). In pelagic juveniles phenotypic sex differences have never been tested statiscally for any marine turtle species.

Information on the sex ratio of wild marine turtle populations is useful for population modelling and provides baseline data for conservation strategies (Mrosovsky, 1994). Thus, assessing sex ratios of marine turtles both on nesting beaches and foraging grounds is of interest for wildlife conservation.

TSD raises numerous questions which are of ecological, evolutionary and/or conservational significance, such as what are the natural sex ratios in sea turtle populations, if sex ratios do vary between and among populations, what effect does sex ratio have on the reproductive rate of a population, or if certain sex ratios are optimal for the survival of a population (Wibbels, 1999).

Once a population's sex ratio has been generated, it is useful to compare it with existing sex ratios for different cohorts within a population or among different sub-populations. Balanced 1:1 Fisherian sex ratios are the most common sex ratios (among vertebrate species at least), and are usually advantageous (Kraak and Pen, 2002), hence systems of sex determination tend to be most stable when they lead to balanced sex ratios (Bull, 1983).

However, skewed and dynamic sex ratios have been documented for marine turtle species (Wibbels, 2003; Wibbels et al., 1987b), and are fairly common among TSD species (Bull and

Charnov, 1989; Charnov and Bull, 1989a). Under some conditions mechanisms that bias the sex ratio are actually favoured (Kraak and Pen, 2002).

Sex ratio evolution plays an important role in the evolution of sex determining mechanisms, and because sex determining mechanisms control the inheritance of sex, they also determine the sex ratio among offspring (Kraak and Pen, 2002). Sex ratio selection is also thought to explain the evolution of ESD (Bull, 1983).

The main source rookeries for the Azorean-Madeiran population are primarily the southeastern United States (Bolten et al., 1998), with the northwestern Atlantic beaches hosting one of the largest nesting aggregates in the world, and the Atlantic coast of Florida hosting the vast majority of nesting effort in the region (Murphy and Hopkins-Murphy, 1989). Based on hatchling productivity from the rookeries in southeastern U.S. the expected female to male sex ratio of hatchlings entering the ocean is 6:1 (Hopkins-Murphy et al., 2003). However, the sex ratio of loggerhead hatchlings leaving south Florida beaches is highly female-skewed (Mrosovsky and Provancha, 1989) when compared with U.S. northern beaches. Although the overall sex ratio produced in the eastern U.S. nesting beaches is biased towards females, the northern subpopulation originating in North Carolina, Virginia and northern Florida (37.5°-29°N lat.) is balanced (reviewed in Hopkins-Murphy et al. (2003)).

On the other hand, the sex ratio of benthic immature loggerheads foraging along the eastern United States is biased towards females with sex ratios reported for these rookeries being consistently 2F:1M (Owens, 1997; Wibbels et al., 1991c; Wibbels et al., 1987a).

Once sex ratio data have been collected from a population, in addition to examining pooled data from a population, it may be advantageous to subdivide the data based on such factors as size classes of turtles, time of year when sampled, and sampling location (Wibbels, 1999).

Objectives

The objectives of this chapter are to evaluate potential biometrical sex-specific parameters and compare the sex ratios obtained for this juvenile population and the ones known for the other life stages of the same population.

Materials and Methods

This chapter analyses the data obtained in the previous chapters, regarding both biometrical data and sex ratio data.

Individual's sex was diagnosed morphologically through laparoscopy (juveniles) (Chapter II) and by gonad histology (hatchlings) (Chapter III). Nesting adults are obvious females.

Concordance for the different sexing methods was compared: laparoscopy (Chapter II), gonad histology (Chapter III) and sex steroids (Chapter IV).

Sexual Dimorphism

Biometrical parameters were taken to the nearest mm for potential secondary characteristics and were compared among the 3 different life stages: hatchlings, juveniles and adult (Table 5).

Table 5 - Biometrical parameters compared for potential sexual dimorphism (all measurements were taken in mm).

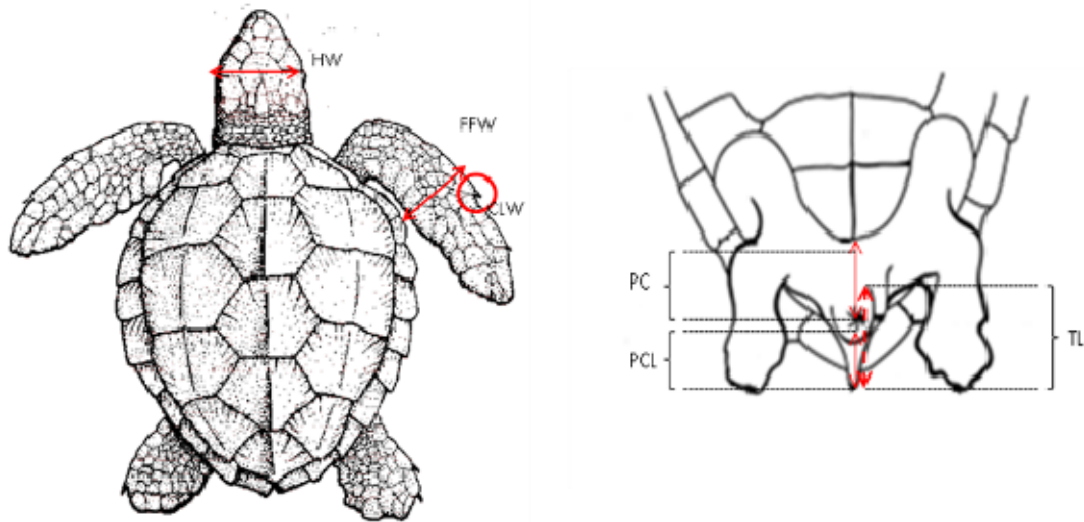| Biometrical parameters	Description
Post-cloacae tail length	distance from mid-cloacae opening to tip of Tail
Plastron-Cloacae	distance from the midline of the posterior margin of the Plastron to the mid-cloacae opening
Tail Length	distance from tip of tail to furthest point between tail and carapace
Head Width	distance across the widest part of the head
Fore Flipper-Width	flipper width measured just before 1st claw
1st claw length	measured on right fore-flipper

Fig. 26 Diagram of biometrical parameters compared for sex dimorphism: HW: head width, FFW: fore-flipper width, CLW: 1st claw length, PC: plastron-cloacae distance, PCL: post-cloacae to tip of tail distance and TL: tail length (measured between carapace and tail).

Sex ratios

Sex ratios were generated following laparoscopy and according to histological validation of the technique. Sex ratios distributions for the juvenile pelagic population were tested against an expected outcome of 2F:1M found for the overall population under study, as well as against an even sex ratio and a 6F:1M sex ratio. Chi-square analysis was employed to evaluate sex ratio differences among cohorts and among-years across the capture period range (2000-2006). Comparisons with the sex ratios known for the western north-Atlantic rookeries were also performed.

Due to the small number of animals sexed in the years 2002 and 2003 (6 and 7 turtles respectively) these cohorts were pooled together for sex ratio analysis. Unknowns were excluded from the analysis.

Overall sex ratio for the juvenile population was also compared with the sex ratios known for the Western North Atlantic rookeries.

Sex diagnosis and correlation among methods

Three sexing methods were employed in this study: laparoscopies, gonad histology and measurement of sex steroids (testosterone and estradiol).

A 96.3% validation of the laparoscopic technique (n=207) was obtained following histological validation of 36.4% (n=82) of the animals submitted to laparoscopy. No bimodal distribution was found for both sex steroids measured (testosterone and estradiol), as well as for the ratio T:E.

Biometrical parameters and correlation with sex

Biometrical parameters were taken for the three different life stages studied (Table 6). No significant sex-specific differences were found for any parameter.

Table 6 - Biometrical parameters compared for sex differences among the 3 life stages.

Parameter	Hatchlings			Juveniles			Adult Females		
	Mean	SD	n	Mean	SD	n	Mean	SD	n
SCLnt	42.08	1.406	61	352.08	103.525	197	NA		
SCLmin	41.91	1.418	57	345.67	99.135	196	752.84	36.648	69
SCW	32.94	1.754	64	305.27	89.442	197	604.30	45.148	69
HW	14.93	0.629	61	78.98	20.952	197	NA		
TCA1	-3.61	0.847	64	21.54	8.407	197	3.35	24.373	69
TCA2	-3.55	0.872	64	12.93	6.735	196	15.66	50.386	68
PTL	11.81	1.271	64	31.76	26.880	188	183.46	35.101	69
PC	7.86	1.207	64	46.63	17.325	188	149.88	26.378	69
TL	8.38	0.941	26	43.45	14.252	197	118.68	19.905	69
FFW	11.02	0.975	63	66.23	15.224	197	NA		
CLW	2.59	0.496	63	11.90	3.059	196	NA		

Note: NA (not available).

Population Sex-ratios

Sex Ratio comparison between Eastern and Western North Atlantic

The overall population sex ratio found was 2F:1M according to laparoscopy. Statistical differences were tested between the juveniles' sex ratio and the sex ratios known for the hatchlings (6:1) and for the benthic immature (2:1) using χ^2 tests, as well as for an even sex ratio. Results show that hatchlings' sex ratio is significantly different from the juvenile pelagic sex ratio ($\chi^2=61.3$, df=1, p<0.0001), but not from the benthic immatures. The overall sex ratio is significantly different from a 1:1 sex ratio ($\chi^2=23.0$, df=1, p<0.0001).

Sex ratio variation on a temporal scale

The sex ratios across years showed a consistent predominance of females (Fig. 27). χ^2 results comparing each year's cohort against a 2:1 sex ratio known for the whole population were not significant at $\alpha=0.05$, but were significantly different from a 6:1 sex ratio (Table 7).
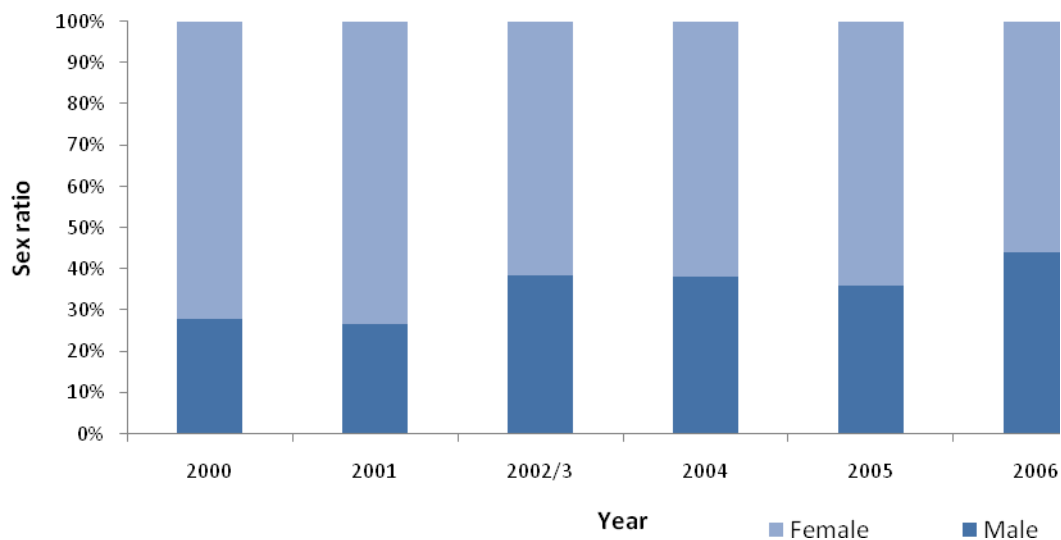
Fig. 27 Sex ratios across years (2000-2006).

Table 7 - Sex ratios for the period 2000-2006 and significance values compared for a 1:1, 2:1 and 6:1 F:M sex ratios using χ^2 tests (* depict significant results at α=0.05).

Year	N (total)	N females	N males	Sex ratio	α (1:1)	α (2:1)	α (6:1)
2000	43	31	12	2.58	0.004*	0.450	0.011*
2001	56	41	15	2.73	0.001*	0.617	0.008*
2002/3	13	8	5	1.60	0.405	0.695	0.013*
2004	34	21	13	1.61	0.170	0.544	<0.0001*
2005	36	23	13	1.78	0.096	0.724	<0.0001*
2006	25	14	11	1.27	0.549	0.258	<0.0001*

Sex ratio among size/age classes

In order to assess possible age/size-dependent sex ratios, the sample was subdivided into 50 mm size classes (SCLnt) as usual in the literature. Results are shown in table 8.
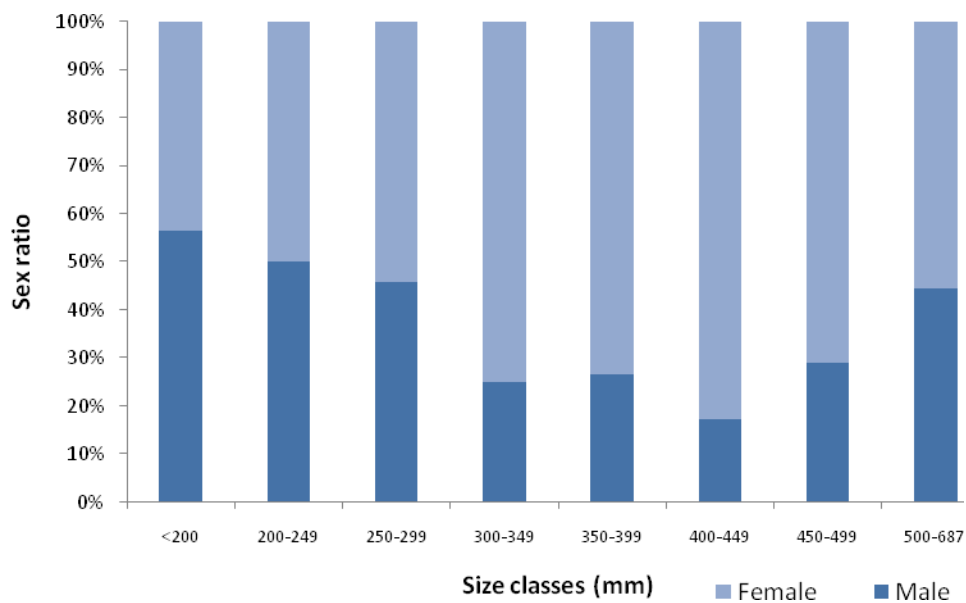

Fig. 28 Sex ratios across size classes (SCLnt).

Table 8 - Sex ratios across size classes and significance values compared for a 1:1, 2:1 and 6:1 F:M sex ratios using χ^2 tests:1M (* depict significant results at $\alpha=0.05$).

Size Class	N (total)	N females	N males	Sex ratio	p (1:1)	p (2:1)	p (6:1)
<200	23	10	13	0.77	0.532	0.018 *	<0.0001*
200-249	22	11	11	1	0.835	0.140	<0.0001*
250-299	23	13	11	1.18	0.683	0.194	<0.0001*
300-349	28	21	7	3	0.008*	0.350	0.105
350-399	30	22	8	2.75	0.011*	0.439	0.053
400-449	46	38	8	4.75	<0.0001*	0.022	0.547
450-499	24	17	7	2.43	0.041*	0.665	0.037*
500-687	9	5	4	1.25	0.739	0.480	0.010*

Discussion

Sex identification and correlation among methods

According to the high rate of agreement between the laparoscopy and histological technique, we may conclude that future studies in order to monitor sex ratio populations will need only to use the laparoscopy technique, with minimum damage to the animal. This correlation provides strong evidence that laparoscopy is an acceptable and valid method for sexing juvenile animals. For the hormonal evaluation, studies relating sex steroids and capture stress should be performed so that any stress-related effects can be evaluated. The possibility that other steroids (e.g. 5α-dihydrotestosterone or others not yet identified) could be important to diagnose sex at this stage should be investigated.

Since oocyte diameter did not correlate with hormones, a comprehensive analysis of whole gonads from dead turtles should be performed in order to detect any sampling bias and to fully understand the variations between and within the gonads.

Biometrical parameters and correlation with sex

The sizes of marine turtles are thought to be affected by age, genetics and environment (Kamezaki, 2003). All the biometrical parameters tested in this study for potential sexual dimorphism did not show any sex specific variability in our juvenile population. Since secondary sexual characteristics are induced by elevated levels of testosterone (Owens, 1997), and in this study we found very low levels of this androgen for both males and females juveniles, we may conclude that testosterone levels in these size classes are still not high enough to induce or trigger secondary sexual characteristics.

No threshold value for tail length or ratio between tail and body length was found in juveniles that could suggest sexual dimorphism, such as the ones applied for adults. Adult males and females differ mainly in tail length. In a study by Wibbels et al. (1987a), animals with tail lengths less than 25 cm were considered females and males with tails over 40 cm the difference being even clearer when tail length is compared controlling for body size (Wibbels et al., 1987b). Although Deraniyagala (1939) and Pritchard and Trebbau (1984) mentioned that Atlantic loggerhead males present larger heads than females, no sex differences in head size were found for Madeira pelagic juveniles.

Population Sex-ratio

The importance of obtaining accurate estimates of sex ratios and sex ratio trends cannot be overstated: this parameter is key-stone to obtain accurate population models and population trends. In the current study the sex-ratios estimated were obtained on the basis of the most reliable sexing method, i.e., direct examination of the gonads (laparoscopy), as gonads exhibit sex-specific differences in both external morphology and histology (Wibbels, 2003; Wyneken, 2001; Yntema and Mrosovsky, 1980).

Once sex ratios have been generated, appropriate statistical analyses can be conducted. The sex of a sea turtle represents a qualitative rather than a quantitative variable, and a sex ratio is a derived variable. To appropriately compare sex ratio data, i.e., to compare observed frequencies of males and females in a population to a predicted value, the chi-square goodness of fit test is appropriate when working with moderate to large sample sizes (Wibbels, 1999).

Sex Ratio on the Eastern vs. Western North Atlantic

A comprehensive understanding of this population of juvenile loggerheads also requires a comparison with the corresponding hatchling and adult populations. Results show that the pelagic juveniles' sex ratio is similar to the adults' one, but is significantly different from the hatchlings sex ratio.

Causes for these differences can be attributed to possible differential mortality and/or behaviour-related differences between the sexes such as different migratory routes or developmental areas. There is also the possibility that the assessments of hatchling sex ratios do not accurately reflect the sex ratios entering the ocean.

Other causes include different population sources, such as a population input from the Cape Verde Archipelago or from the Mediterranean population. This would not be surprising since one individual tagged in Madeira island as a juvenile was seen nesting in Boa Vista Island (Dellinger and Ferreira, 2005). The Cape Verde source-rookery was not assigned in the work by Bolten et al. (1998) since at the time that rookery was poorly known. Therefore, its contribution was not estimated for the mixed-stock analysis and the U.S. nesting beaches may have been overestimated as the primary source for the Madeiran population; therefore, the origin of this population might be re-opened for debate. More thorough genetic analysis of this population, both across years and around year as far as logistically feasible are needed to resolve management units of these migratory marine animals. In fact, new research is showing the Cape Verdean contribution for Mediterranean and Madeiran loggerheads (C. Carreras, pers. comm., unpublished data).

On the other hand, the male-biased sex ratio reported for the Mediterranean Sea would account for the significant difference observed between the source-hatchling population and the juvenile population in Madeira. Migratory movements in and out of the Mediterranean have been reported for loggerheads (Camiñas, 1995; Carreras et al., 2007).

In fact, a mixed stock population may explain a large part of this sex ratio, since mixed stock analyses of loggerhead juveniles indicate that cohorts from genetically distinct rookeries extensively mix on oceanic habitats. For instance, surveys of pelagic stage juveniles indicate no population structure among locations across the North Atlantic, and loggerhead turtles have

progressively greater population structure as they advance in age and developmental stage (Bowen and Karl, 2007).

According to Bowen and Karl (2007) the type of DNA addressed on genetic analysis influence the conclusions regarding populations, since either nDNA or mtDNA alone can provide incomplete and misleading conclusions about population structure. In fact, the mtDNA surveys of North Atlantic juvenile turtles, taken alone, would indicate a single panmictic population, obscuring the true structure of subadults and nesting adults (Bowen and Karl, 2007). In any case, samples for genetic analysis for the vast majority of the animals sampled since 1999 at Madeira already exist, including the ones sexed for this study, and hopefully will help clarify this aspect.

Sex ratio variation on a temporal scale

Results of this study show an overall sex ratio for juvenile loggerhead sea turtles of 2F:1M (n = 207) spread over 7 sampling seasons. This sex ratio is consistently significantly different from an even sex ratio for every year examined. Some variation among years is observed, and although a slight trend for an increasing number of males can be seen, this is not statistically significant. This minor sex ratio variation is likely due to yearly fluctuations in hatchling sex ratio production. Since juvenile pelagic loggerheads represent a condensation of many years of hatchling sex ratios, the variability of sex ratios observed at the hatchlings cohort is 'buffered' at larger size classes such as juveniles', and sex ratios from hatchlings at beaches may not reflect the sex ratios of large juveniles about to enter their reproductive phase (Wyneken et al., 2006).

Although sex ratios reported for marine turtles are commonly strongly biased towards females, some exceptions have been documented. The North Carolina and Virginia hatchling subpopulation produces a balanced sex ratio, in contrast to the southern Florida female-skewed sex ratios (Turtle Expert Working Group, 1998). At higher size classes balanced or male-skewed sex ratios were found, namely for the eastern Australian population, with male to female sex ratios of 1:0.41 in the Great Barrier Reef and 1:0.54 in Moreton Bay (Limpus and Limpus, 2003), and a ~1:1 for the Mediterranean population (Casale et al., 2006).

Long-term assessments of sex ratios are desirable in order to detect significant trends and mitigate confounding inter-annual effect, since wide variation in sex ratios are common (Wyneken et al., 2006).

Sex ratio among size/age classes

The analysis of the data concerning sex ratios within and among the different size classes show that a sex ratio of ~2:1 female to male is already observed at the smallest cohorts arriving to Madeiran waters. Despite the smaller number of individuals sampled at the smaller and larger size classes, it is possible to conclude that sex ratios are already significantly different from the source rookery, which presents a 6F:1M sex ratio. Moreover, the sex ratio in the <200 mm SCLnt size class suggests that a differential mortality towards females is likely to happen within the first months of life, although other factors may interfere, such as different geographical distributions between sexes.

Sex ratios across size classes show a steady tendency for an increase in the surplus of females until the 450 mm SCLnt size class (Fig. 28). Hence, the percent of females present in Madeiran waters seem to start to decrease at the size classes larger than 450 mm SCLnt which coincides with the size ranges that these animals are supposed to start leaving to neritic habitats. Therefore, we may speculate that females may start the ontogenetic shift earlier than males.

Causes for Dynamic Sex Ratios

Reasons for skewed and/or dynamic sex ratios are diverse. Plausible causes that may explain sexually biased sex ratios and dynamic sex ratios as well the differences found between the sex ratios found for the eastern and western Atlantic populations include:

- annual hatchling sex ratio variation,
- different geographic distribution,
- sex reversal,
- differential survival/mortality rates between the sexes.

Since annual hatchling sex ratio variation is beyond the scope of the present study, only the last three causes are discussed.

Different migratory routes and/or developmental areas

When investigating the sex ratio of juveniles it should be taken into account that different populations may share a common foraging area. Hence, the observed sex ratio at a given place might actually represent that of different contributions of mixed populations (Casale et al., 2006).

Sex-specific developmental areas or migration patterns are not known for the Atlantic juveniles and thus it is unclear whether males and females utilize or not the same habitats in open ocean. Although a dispersal of females is not expected into the Mediterranean sea since the sex ratio of Atlantic specimens entering the Mediterranean is probably male-biased (Casale et al., 2002), an input of juveniles from the Cape Verde population into Madeiran waters cannot be excluded. A Cape Verdean contribution would likely balance the Madeiran population sex ratio, since the sex ratios found for Boa Vista hatchlings are in the order of ~2F:1M (Abella et al., 2007; Delgado et al., 2005).

A complicating factor for the understanding of pelagic sex ratios is the recently demonstrated pattern of migration from oceanic to neritic foraging grounds for Atlantic loggerheads. It has been assumed that this transition from an oceanic to neritic existence is a discrete ontogenetic niche shift. However, satellite tracking data demonstrated that this shift is both complex and reversible, with some individuals moving back into coastal waters and then return to the open ocean, sometimes for multiple years (McClellan and Read, 2007). Hence, more than addressing dynamic sex ratios within the oceanic environment, we may be addressing a truly dynamic population.

Sex reversal

Sex change could also be an explanation for the 2F:1M unexpected sex ratio when compared to the corresponding source-rookery, which exhibits a 6F:1M sex ratio (Hopkins-Murphy et al., 2003). Since marine turtles have TSD, phenotypic sex is not dependent on genetic determination, but rather depend on environmental conditions. In TSD species, sex reversal can be induced by exogenous compounds that disrupt or mimic some steroidogenic pathways that normally facilitate

sex differentiation in the developing embryo (Crews et al., 1995a; Guillette Jr and Crain, 1996).

A variety of natural and synthetic chemicals known as endocrine disrupting compounds (EDCs) mimic or interfere with the mechanisms that govern reproductive development and function (Crews et al., 2000). There is increasing evidence that some EDC's such as polychlorinated biphenyl compounds (PCB's) are capable of disrupting reproductive and endocrine function in fish, birds, and mammals, including humans (Crews et al., 1995a), as well as reptiles (Bergeron et al., 1994; Guillette Jr. et al., 2000; Milnes et al., 2004; Sheehan et al., 1999).

Reproductive disorders resulting from exposure to these xenobiotic compounds may include reductions in fertility, hatch rate and viability of offspring, as well as alterations in hormone levels or adult sexual behaviours, all of which have further implications in wildlife population dynamics. Furthermore, certain PCB's are synergistic in their effect at very low concentrations (Crews et al., 1995a). Over 70,000 man-made chemicals are found in food, water, air or soil (DeRosa et al., 1998).

Although a laboratorial experiment showed that the environmental contaminant DDE failed to influence the normal sexual differentiation in the marine turtle *Chelonia mydas* (Podreka et al., 1998), these contaminants can still potentially create shifts in populations sex ratios compared to the natural ones.

Sex reversal leading to reproductively capable females can have adverse demographic consequences, and clearly, at high rates of sex reversal, the reproduction of populations will be compromised. Lower rates could also have adverse effects, depending on whether other events such as habitat loss, predation, global warming (which is particularly applicable in TSD animals), or other stressors occur at the same time (Sheehan et al., 1999). Studies show that environmental contamination of reptiles is associated with population declines due to lethal and reproductive effects of the contaminants in embryos, juveniles, or adults, as well as developmental abnormalities of embryos, including major teratogenic effects in turtles and more subtle effects on the development of the reproductive system of alligators, and abnormalities of the endocrine system (Guillette Jr and Crain, 1996).

Although concentrations of EDC's within the open ocean are not expected to be high, and thus an impact on phenotypic sex is unlikely to occur, the bioaccumulation of these compounds on high trophic level organisms could be relevant, since marine turtles are TSD species.

Differential mortality

Since highly skewed sex ratios towards females are reported at hatching, and this bias is decreased at the older life stages, differential mortality is one of the possible explanations for the decreased sex ratio found for pelagic populations, and later on for the adult populations at neritic foraging grounds. Differential mortality may occur for a variety of reasons: genetic, behaviour-related, different sex-specific fitness, etc.

Since size distribution of the turtles sexed during this study was representative of the pelagic population, we have reasons to believe that the sex ratio found do not reflect any size-related bias. On the other hand, sex ratios derived from dead stranded turtles within the same region, mainly from by catch in black-scabbard fisheries, has provided a 4F:1M sex ratios (unpublished data). This, together with the sizes distribution found, suggests that the 2:1female biased sex ratio found for the live animals does in fact reflect the true sex ratio and that a sampling bias towards females does not explain the sex ratio bias found. Moreover, the 4F:1M sex ratio found for the by-catch animals is complementary to the one found for the live animals, and suggest that the differential mortality does not happen only at the post-hatchling size classes but continues throughout the juvenile phase.

Since no differential distribution patterns for juvenile males and females within the oceanic realm are known, a differential mortality due to different home ranges, where females could be more susceptible to increased mortality due to human-related factors, is not a plausible explanation either.

However, females could have different physiological needs, namely in terms of food intake, that might make them more prone to take risk and get caught in fisheries, as indicated by the 4:1 sex ratio obtained in turtles from black-scabbard fisheries in Madeira. Mortality rates in oceanic waters seem to be low however: mortality rates for pelagic turtles reported in the waters of the

Azores are in the order of 0.094% (Bjorndal et al., 2003b). Unfortunately, these estimates did not address differences between sexes.

Differential fitness between sexes could also be one reason. In a study designed to test fitness-related attributes in turtle hatchlings Booth et al. (2004) found that in green turtle C. *mydas* hatchlings warmer incubation temperatures significantly influenced hatchling fitness and post hatch mortality in this species. Moreover, incubation temperature significantly influenced size and amount of yolk material converted to hatchling tissue as well as swimming performance during the 24 hours frenzy swimming period that occurs within 48 hours of hatching, besides the hatchlings' sex. In another study Burgess et al. (2006) found that clutch of origin did influence hatchlings' size rather than incubation temperature. However, hatchlings from eggs incubated at 25.5 and 26°C had a lower stroke rate frequency and lower force output than hatchlings from 28 and 30°C, but clutch of origin did not influence swimming performance.

These two studies suggest that hatchlings incubated at lower temperatures have reduced swimming ability which promotes rapid dispersal to deeper offshore water, and which may affect their survival when entering the ocean and trying to escape the predators on near-shore waters, prior to reaching the relative safety of the open sea (Burgess et al., 2006). Since females are produced at warmer incubation temperatures (Yntema and Mrosovsky, 1980; Yntema and Mrosovsky, 1982), one may expect that female hatchlings in general have a higher fitness than their cohort males. This finding, however, is in direct conflict with the finding in this study that a main part of differential mortality seems to occur at the earliest life stages.

On another level, the duration of incubation varies inversely with temperature, i.e., while at lower incubation temperatures embryonic development takes longer, at the upper extremes of temperature development is rapid and the possibility of developmental abnormalities is enhanced (Miller et al., 2003). Higher rates of kidney malformations are common in reptiles incubated at temperatures closer to upper extremes of incubation temperature (J. Wyneken, pers. comm..). Hence, we may speculate that female incubation temperatures are more likely to produce hatchlings with abnormalities, but this was not tested in the wild. Whether the effects of these supposed higher rates of abnormalities in females are lethal or sub-lethal, and when in the post-hatchling phase that mortality would take place, remains to be determined.

Finally, the hatchlings from the Florida and the North Carolina rookeries may incur differential mortality rates when entering the ocean, and thus the predicted contributions for the pelagic sex ratios become altered. The highly female-biased Florida population is probably more prone to higher mortalities when entering the Gulf Stream Current since they spend more time in near-shore waters and thus are more susceptible to capture and mortality till they reach safer oceanic open waters (Hopkins-Murphy et al., 2003). This neritic differential mortality would make the contribution from the northern U.S. rookeries become more expressive at the oceanic pool of animals.

Differential mortality is therefore the most likely reason for the sex ratios observed in loggerheads within the North Atlantic, both for hatchlings and pelagic stage loggerheads. The reasons for this higher mortality rates, whether behaviour or physiological related, should be further investigated.

Since 'optimum' sex ratios of 1.28F:1M have been pointed as optimal to potentiate population growth in *Lepidochelys kempi* (Coyne, 2000), the adaptive significance of the sex ratios produced at the nesting beaches in contrast to the ones observed at higher size classes needs clarification. One reason could be that the species would be producing a surplus of one of the sexes in order to compensate for posterior higher mortality rates in that sex.

CHAPTER VI

Conclusions and Further Research Needs

Marine turtles are endangered species in need of conservation management. However, species conservation can only be achieved with a detailed knowledge of their biology during the whole life cycle, especially in relation to reproductive development. Much work and international effort is aimed at understanding the role and advancing conservation of these marine reptiles in the marine ecosystems. Due to the difficulty in addressing the pelagic stage of any sea turtle species the role and relevance of turtles within the oceanic environment is far from being understood.

Undoubtedly, the early pelagic stage that occurs in most sea turtle species is the poorest known life-stage and the location of this stage is only known for three populations – the Mediterranean, the North Atlantic and the North Pacific loggerhead populations. High priority must be given to studies of these pelagic populations (Bjorndal, 1999).

Therefore, this study focused on the poorly known populations of loggerhead sea turtles within the North Eastern Atlantic and relied on the capture of pelagic juveniles around Madeira Island and hatchlings and adult females at Boa Vista Island (Cape Verde archipelago) nesting beaches. Prior to this work there was no knowledge on the gonad development and on the sex ratios of Madeiran waters pelagic loggerheads, indeed of the sex ratios of any pelagic population. Only embryonic development has been addressed before as well as reproductive aspects at mature stages (Miller, 1985; Miller and Limpus, 2003), and very recently, in captive-reared post-hatchlings (Wyneken et al., 2007). Furthermore, the relationship between gonad development and individuals' sex with sex steroid hormones had never been described previously.

Sexing techniques and gonad development

This study provided the first description of gonad morphology – both histologically and macroscopically – and the profile of plasma sex steroids for juvenile sea turtles. Notably, these advances were achieved without the sacrifice of the animals.

Laparoscopy proved to be an effective and reliable sexing tool in pelagic juvenile loggerhead turtles, as validated through gonad biopsy histology. However, the sex steroids analysed did not show any sex-specific differences, and thus did not provide an effective sexing method in juvenile loggerheads.

Therefore, in future studies addressing juvenile sex ratios on pelagic loggerheads only laparoscopy should be used, with no further invasive techniques.

The lack of correlation between individual's sex and sex steroids (T and E_2) can be attributed to several factors: on the one hand the low titre levels suggest that circulating hormones are not a useful tool in these loggerheads. This might be due to the immature reproductive status of the animals sampled, which do not allow using hormones to predict sex differences, i.e., they may not be old enough to show a sex-specific hormone profile. Reproductive maturity (or a stage approaching to reproductive maturity) most likely explain the better outcomes of hormone studies in neritic juvenile and adult loggerhead turtles (Wibbels et al., 1991c; Wibbels et al., 1987b). In fact, this lack of sex specific differences was also found for small Kemp's ridleys (Wibbels, pers. comm., unpublished data). On the other hand, according to Braun-Mcneill et al. (2007), the hormone technique is reliable only at sea surface temperatures (SST) above 24°C. Since water temperatures observed during this study were usually below that threshold level, this also might account to the lack of correlation between testosterone levels and phenotypic sex. Nevertheless, a significant correlation between testosterone levels and SST was detected at these young size classes, which indicates a hormonal seasonal effect already at these young size classes. Besides ambient temperature, photoperiod may also play a role on seasonal hormones variation and should be addressed in future studies. The lack of correlation between phenotypic sex and sex steroids cannot be attributed to the RIA assay sensitivity, since both hormone levels analyzed were above the RIA detection limits and the percent bound between the hormone and the tritium labeled marker was high.

Although stress-related factors cannot be discarded, it most probably does not explain the absence of sex specific differences between males and females, since no correlation between corticosterone levels and testosterone were found. However, when compared to nesting females, juveniles did show higher corticosterone levels, suggesting higher stress levels, and thus this factor should be further investigated in future studies. Whether the high corticosterone levels are characteristic of these size classes is not known, but the low corticosterone levels are known to be characteristic of the nesting episode in sea turtles (Owens, 1997; Whittier et al., 1997). A full understanding of the stress profile and its correlation with testosterone levels in juveniles should

examine both testosterone and corticosterone at the moment of capture and at several moments until a 24-48 hour captivity period. Moreover, seasonal effects should be addressed as far as logistically feasible, since testosterone is subject to seasonal variation in many species, and its activity appears to facilitate or enhance responsiveness to stressors both directly and indirectly (Greenberg et al., 2002). Additionally, other hormones should be investigated in future studies, such as estrone (Coufal and Whittier, 2003) and dihydro-testosterone (DHT), an active metabolite of testosterone in mammals.

Although the laparoscopy sexing technique proved valid and efficient it is still logistically complex and implies a high degree of manipulation for the animal. Other sexing techniques rather than the ones studied in this work should be attempted, namely the measurement of vitellogenin (Vtg) levels in blood. Vitellogenin is a follicular precursor protein characteristic of females only and thus could be an indicator of individual sex. Due to the species-specificity of the Vtg molecule, a specific quantitative assay for sea turtle Vtg is currently being developed at the Department of Biological Sciences in South-Eastern Louisiana University, with whom the author has established a collaboration protocol. The analysis of 48 plasma samples of juveniles whose sex was verified through laparoscopy will take place during the first semester of 2008, for which a grant by FLAD (Luso-American Foundation) was awarded.

Vtg has been described as a biomarker of endocrine disruption in several oviparous species (Crain and Guillette Jr, 1998) in contaminant-impacted environments, since males exposed to endocrine disruptor substances in the wild may present elevated levels of female hormones. Therefore, such data could also be used to identify possible endocrine disruptor mechanisms and be measured concurrently with the presence of xenobiotic compounds such as pesticides accumulating in the different tissues, including in the juvenile gonadal tissues. Since marine turtles are upper-level predators, have a long average life span and reach sexual maturity late in the life cycle, the potential for bioaccumulation of environmental contaminants is wide, making these species potential biomarkers or models for addressing contamination of the pelagic environment (Bergeron et al., 1994; Crain and Guillette Jr, 1998). Vtg assessments could therefore be used in monitoring programs within the oceanic environment.

The possibility of identification of a DNA marker would be ideal, although no sex determining gene has been found until now.

The gonad development and its relationship with hormone titres were addressed in this study. Although no correlation was found between ovarian development and hormone levels, a positive correlation between maximum oocyte diameter and body size was found for females, suggesting that reproductive development is an on-going process. Intersex frequency was very low and within expected values (Limpus et al., 1982).

Due to constraints of working with live animals belonging to an endangered species some aspects of gonad histological morphology could not be further explored. In order to better understand gonad morphological development, future studies using dead turtles gonads should be attempted in order to circumvent the gonad biopsy limitations. Furthermore, analyses of steroidogenic pathways in juvenile gonads using radioactive precursors would help clarifying which molecules play a key-role in juvenile gonad development.

Sex ratios

The sex ratios generated from this study are of importance for demographic modelling, since conservation management for marine turtles is based on basic biological data and demographic trends for a given population and need to know the sex ratio of the various stages of a population.

An overall 2:1 female to male sex ratio was found for the juvenile oceanic loggerheads offshore Madeira Island. Female biased sex ratios are common among TSD species such as marine turtles. The adaptive significance of TSD was reviewed and discussed by Janzen and Phillips (2006), but much work is still needed in order to understand the adaptive importance of TSD and highly female-skewed sex ratios observed in certain populations at hatching as opposed to closer to even sex ratios observed at older cohorts. The highly skewed sex ratios female-biased observed could be an adaptive species response to differential mortality striking females more heavily right after hatching or elsewhere during the life cycle, i.e., the species would produce an optimal sex ratio species-specific in order to compensate for posterior higher mortality rates in one of the sexes.

Although sex ratios presented in this study span over a 6 year period, it would be valuable to continue sex ratio assessments in order to monitor sex ratios trends, since it is best to characterize the sex ratio of a region based on many years of sex ratio data (Wyneken et al., 2006). Moreover, since these juveniles represent a composite of multiple years of sex ratios that contribute to the population's functional sex ratio(Wyneken et al., 2006), the sex ratios observed at the juvenile pelagic stage can give a more accurate prediction of the future functional sex ratios that will contribute for reproductive rate. Furthermore, sex ratios predicted at the hatchling phase are commonly extrapolated from data such as incubation temperature, duration of incubation, number of clutches hatched throughout the whole nesting season and along several beaches. However, pivotal temperature from laboratory experiments do not necessarily apply to the wild nor does the postulate that TSP (i.e., the temperature-sensitive period for sex determination) occurs during the middle third of the incubation period in time. With increased temperature variation during incubation, TSP may be shifted. Hatchling sex ratios are usually estimated based on laboratory data using few validation in the field and extrapolate this to large geographic areas. Thus published hatchlings sex ratios are prone to error.

Sex ratios generated for the pelagic stage using a definite method such as laparoscopy do not require any data extrapolation and thus are much more accurate in predicting any major population trends. Furthermore, pelagic juveniles sex ratios databases can give a more appropriate indicator of sex ratios trends since these cohorts will soon recruit to the neritic feeding grounds and will contribute to the reproductive active cohorts much sooner than the hatchlings.

TSD sex ratios and climate change

A naturally occurring female biased sex ratio seems a good strategy to potentiate a populations reproductive output. In fact, artificially female biased sex ratios have been proposed as a conservation management tool in TSD turtle species to aid in the recovery of threatened or endangered species (Girondot et al., 1998; Vogt, 1994). Furthermore, multiple mating and multiple paternity observed in marine turtles, along with the concomitant gene flow by males, would alleviate the concern that males may be a limiting resource for reproduction (Bowen,

2003). However, sea turtle sex ratio dynamics are still poorly understood and their manipulation may lead to undesirable consequences and threaten species conservation (Lovich, 1996; Morreale et al., 1982; Mrosovsky and Godfrey, 1995). Even a change of 1 to 2°C in incubation temperature can make a considerable difference to the sex ratio of the hatchlings (Mrosovsky and Yntema, 1995), and a temperature change of 3°C or less could potentially shift sex ratios from all male to all females or vice-versa (Wibbels, 2003), making global warming a major conservational concern for TSD species.

In fact, with a forecast scenario of rising temperatures, some populations are predicted to become 'naturally' female ultra-biased with as little as 1°C of warming and experience extreme levels of mortality if warming exceeds 3°C (Hawkes et al., 2007) and males may in fact become scarce enough or disappear on the long term (Abella et al., 2007). Such scarcity of males would not ensure fertilization of enough reproductive females, despite multiple fertilization in marine turtles, or produce a genetic 'bottleneck' effect due to low rates of genetic flux.

These concerns over the possible impact of global temperature change upon offspring sex ratios have already been confirmed for a population of the painted turtle *Chrysemys picta*. Consistent with theoretical predictions, annual offspring sex ratio was highly correlated with mean July air temperature, validating the expectations about the effect of climate change on population demography in a TSD species (Janzen, 1994).

Some adaptive changes in turtle behaviour may come into play as a species-response for a changing environment: earlier nesting in the season, i.e., shifting nesting season to relatively cooler months of the year (Weishampel et al., 2004) is one possibility, and moving nesting to other beaches to compensate for climate effects as in the TSD lizard *Physignathus lesueurii* (Doody et al., 2006) is another. Changes in species distribution could also be a response (McMahon and Hays, 2006).

These changes in marine turtle behaviour are theoretically possible since there is no absolute, exact geographical, natal homing in marine turtles as nesting habitats are ephemeral ecosystems over evolutionary time, continually arising and disappearing with changes in physical environment (sea level, geography, beach characteristics), climate (glacial intervals) and biotic environment (nest predation or competition for nesting space) (Bowen, 2003). Over the 100-

milion-year history of this group, absolute natal homing would be a strategy for extinction, i.e., the habitats that were appropriate in the Cretaceous, Eocene, or Miocene are not the same ones that are appropriate today (Bowen and Karl, 2007). However, at the current rate of climate change it is doubtful that marine turtle species could adapt at a relatively fast rate. Evolutionary rapid changes in pivotal temperature are unlikely since this parameter has been shown to be heritable in a few turtles with TSD (Bull et al., 1982; Janzen, 1992). In fact, quantitative genetic analyses and behavioural data suggest that species with TSD may be unable to evolve rapidly enough to counteract the negative consequences of rapid global temperature change (Janzen, 1994). This adaptive inertia was demonstrated by Reece (2005), that found a coincidence between major historical climate change events and subsequent severe population contractions in loggerheads, hawksbills, and green turtles, demonstrating how sensitive marine turtles are to global climate change.

Therefore, monitoring and understanding the spatio-temporal variations in sex ratios in TSD species in an era of global climate change can be a major contribution for species management and conservation. Moreover, populations of TSD species may serve as ideal indicators of the biological impact of global temperature change (Janzen, 1994).

Summary

Madeiran waters hold a remarkable loggerhead juvenile oceanic population, mainly originated from the southeastern U.S. rookeries (Bolten et al., 1998), but probably also from other population sources such as the Cape Verde Islands and the Mediterranean.

Although it can be considered as a management unit for conservation purposes, this population depends entirely on its source populations, and thus any management programs need to be multinational in scope. Even complete protection in one region may not be sufficient to save a population if excessive exploitation or mortality occurs in other geographic areas (Bolten et al., 1998).

Assessment of the population and conservation status of the loggerhead turtle population foraging offshore Madeira Island is, however, hindered by the lack of a reliable and feasible in-water census methodology and, thus, there are no quantitative census data for this population.

Only relative abundance data extrapolated from capture-per-unit-effort in fisheries exist (Ferreira, 2001), and are the most accurate monitoring that can be obtained within an oceanic environment.

Population census and demographic models can be useful tools for decision makers because they can quantify the relative effectiveness of different management options (Heppell et al., 2000).

Causes for the differential mortality towards females suggested by the analysis of the sex ratios found in this study and for sex ratios for turtles accidentally caught in fisheries should be addressed in future studies. Protection on nesting beaches is not sufficient as it seems that the differential mortality is occurring within oceanic environments. Long-line fisheries are known to incidentally catch oceanic juvenile loggerheads globally, and the cumulative effect of multiple long-line fisheries that capture sea turtles from several life stages may be very high (Heppell et al., 2003). Furthermore, the differential mortality between sexes should be incorporated into demographic models developed to monitor responses of Atlantic loggerhead populations to management policies and threats (Crouse et al., 1987; Crowder et al., 1994).

Sex ratios are important drivers in population dynamics and different sex ratios can have profound effects on the population estimates. Therefore, it would be of the utmost importance to continue a comprehensive and long term monitoring assessment of sex ratios in the pelagic stage that should incorporate periodic assessments of current sex ratios on the different size/age classes in order to ensure accuracy of population models. Moreover, it should integrate a combination of genetic data for rookery-source assignment and subpopulation distribution (Bowen and Karl, 2007; Casale et al., 2006) as well as survivorship rates for the different age classes.

Similar sex ratios assessments would also be important on the geographically close archipelagos of the Azores and the Canary islands, as well as for the important foraging grounds off the Great Banks of Canada, the so called North Atlantic Long Distance Waters, for a more clear perspective of demographic dynamics within the Atlantic Ocean basin.

Despite the present paucity of marine turtle species, they have great economic value as well as extreme vulnerability to mankind, both while nesting and to accidental fisheries, and their inclusion on most lists of threatened or endangered species is a reflection primarily of past

overexploitation and current need for better management rather than to inherently poor adaptation to post-Pleistocene conditions (Pritchard, 1997). The long generation time also make them especially vulnerable to depletion and their vast cartographic ranges make them especially difficult to manage, since decades are the units by which sea turtle recovery can be measured (Bowen and Karl, 2007).

Only such baseline data on population parameters will allow and enhance the ability to make informed management decisions. Otherwise, in times of rapidly changing environments we will not have the necessary information to assess possible and probable impacts on sea turtle populations and apply early and appropriate management practices (Hamann et al., 2003).

Since resources for endangered species research and conservation are limited, they need to be used effectively. Therefore, representative populations should be selected for intensive studies and long-term monitoring and used as 'index' populations (Bjorndal, 1999).

The juvenile loggerheads within Madeiran waters can be used as an index population providing the baseline information for the pelagic-stage in order to meet the current and future challenges of conservation and management of marine turtles.

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Appendices

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Histological procedure for marine turtle gonads

A. Tissue fixation

Place tissue sample in Bouin's solution or 10% buffered neutral formalin. Tissues are fixated at a volume ratio 1:10 (tissue:fixative) for 24-48 hours and then transferred to 70% EtOH or left in the fixative.

B. Embedding in paraffin mounting media

1. 90% EtOH _____ 1 hour
2. 95% EtOH _____ 1 hour
3. 100% EtOH I _____ 1 hour
4. 100% EtOH II _____ 1 hour
5. 100% EtOH III _____ 1 hour
6. Toluene I _____ 30 min.
7. Toluene II _____ 30 min.
8. Toluene III _____ 30 min.
9. Paraffin/toluene (1:1) overnight at room temperature
10. Paraffin/toluene (1:1) in a warm oven (50°C) _____ 4 hours
11. Paraffin in a warm oven (50°C) _____ 4 hours
12. Final embedding in paraffin blocks (60 °C).
13. Leave paraffin blocks to dry (± 2 days).

C. Sectioning with a microtome

Tissue sections are cut with a rotary microtome (6 μm) and floated on a warm water bath (41-43°C); then they are picked up on a glass microscopic slide. The glass slides are then left to dry on a tray for 2 days (35-40°C).

D. Hematoxilin and Eosin staining procedure

Deparaffinize:

1. Xylene I _____ 5 min.
2. Xylene II _____ 5 min.
3. Xilene III _____ 3-5 min.

Hydrate to water:

4. 100% EtOH I _____ 5 min.

5. 100% EtOH II _____ 5 min.

6. 100% EtOH III _____ 5 min.

7. 95% EtOH _____ 5 min.

8. 90% EtOH _____ 5 min.

9. 70% EtOH _____ 5 min.

10. 50% EtOH _____ 5 min.

11. Running tap water (rinse) _____ 10 min.

12. Distilled water

13. Harris Hematoxilin _____ 10 min.

14. Running tap water (blueing) _____ 5 min.

Destain:

15. Acid alcohol _____ 2-3 quick dips

16. Running tap water (re-blueing) _____ 10 min.

17. Scott's tap water substitute _____ 5-10 min.

18. Running tap water _____ 2 min.

19. Eosin 1% _____ 5 min.

20. Running tap water _____ quick rinse 1-2 min.

Decolourize and dehydrate:

21. 70% EtOH _____ 3-4 quick dips

22. 90% EtOH _____ 3-4 quick dips

23. 95% EtOH _____ 3-4 quick dips

24. 100% EtOH I _____ 3-4 quick dips

25. 100% EtOH II _____ 3-4 quick dips

26. 100% EtOH III _____ 3-4 quick dips

27. Xylene I _____ 5 min.

28. Xylene II _____ 5 min.

E. Coverslipping

The stained section on the slide is covered with a glass coverslip and glued with Entellan.

F. Drying the slides

Keep slides at 60 °C for 3-5 days.