STUDIES ON THE EMBRYOLOGY, ECOLOGY AND EVOLUTION OF SEA TURTLES IN THE EASTERN MEDITERRANEAN

by

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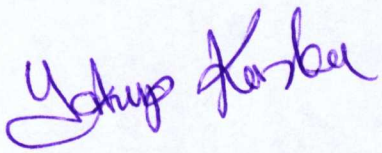
AUTHOR'S DECLARATION

I declare that the work recorded in this thesis is entirely my own, unless otherwise stated, and that it is of my own composition. No part of this work has been submitted for any other degree.



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SUMMARY

1-) The temperature of sea turtle nests in the Eastern Mediterranean was between 24 and 35 °C and rose by up to 10 °C during incubation.

2-) The mean incubation temperature can be used for estimating the incubation period but provides a poor prediction of sex ratio.

3-) The mean temperature during the middle third of the incubation period was closely correlated with the percent sex ratio.

4-) There was a female dominated sex ratio among the 22 nests and only one loggerhead turtle nest showed less than a 50 % female sex ratio.

5-) There was a consistent temperature difference within the nest with top eggs warmer, bottom eggs cooler and middle ones intermediate. Therefore the majority of hatchlings from the top level in nests were females and those from the bottom level were predominantly males.

6-) Temperature differences within the nest also influenced the rate of development; the greater the difference in temperature between top and bottom the longer the time required to complete hatching of all embryos of the nest. The hatching intervals of green turtle nests were shorter than those at loggerhead turtle nests. Temperature variation between top and bottom of nests was low within green turtle nests. In general, a 1 °C temperature difference within the clutch caused a 4 day range in both hatching and emergence of hatchlings.

7-) Since the temperature within the nest and between the nests was so variable, sand air or sea water temperatures gave a poor prediction of the temperature of a nest and therefore the sex ratio.

8-) Although the predation pattern of sea turtle nests varied in relation to nest age, this predation can be reduced by screening the nest with mesh grids.

9-) Inundation was one of the main abiotic factors lowering the hatching success on the beaches. Hatching success can be increased by relocating the nests to a safer area on the night of laying.

10-) The mean grain sizes of sand ranged from 0.49 to 2.20 phi(ϕ) on 10 beaches but hatching success was not related to mean grain size of sand on the beaches.

11-) Simple embryonic staging of Mediterranean sea turtles was developed after measuring a set of selected morphological characteristics. The frequency of gross abnormalities among the samples was also calculated. Most common abnormalities were supernumerary and subnumerary scutes, albinos, head and jaw abnormalities and twinning.

12-) The heavy metal concentrations in the tissues (yolk, liver and eggshell) of loggerhead turtle eggs and hatchlings were analysed. The concentrations of mercury, cadmium, lead, iron and copper were highest in the liver, while zinc concentrations were highest in the yolk. The concentrations of metals were similar on different beaches, except for lead concentrations in the eggshells, which varied between sites.

13-) The genetic structure of loggerhead turtle samples from Cyprus exhibited haplotype B and green turtle samples haplotype XIII. No additional haplotypes were found. The presence of only single haplotypes suggests little variation in genetics within the Mediterranean and that these population were recently established by a small number of immigrants from the Atlantic.

Chapter 1. General Introduction:

This chapter presents a review of sea turtle biology and status in the Mediterrranean, and finishes with an outline of the content of the rest of the thesis.

1.1. Sea turtles in the Mediterranean:

Five of the seven species of sea turtles have been recorded in the Mediterranean but only two of them nest regularly on Mediterranean beaches: the loggerhead turtle *Caretta caretta* and the green turtle *Chelonia mydas*. Non-nesting leatherback turtle *Dermochelys coriacea*, hawksbill turtle *Eretmochelys imbricata*, and olive ridley turtle *Lepidochelys olivacea* occur, having been reported irregularly by fishermen who have found dead ones (Sella, 1982; Groombridge, 1990). All five are recognised as globally threatened species; the loggerhead is ranked "Vulnerable", the remainder "Endangered" (IUCN, 1988). Hathaway (1972) stated that there are more turtles in the Mediterranean than in any other sea, but Groombridge (1990) noted that no nesting population of marine turtles in the Mediterranean is large by world standards. According to investigations made so far, there may be on average some 2000 female *Caretta caretta* and 300-500 *Chelonia mydas* nesting annually in the Mediterranean (Groombridge, 1990).

The major nesting beaches identified for *C. caretta* were in Greece and Turkey, with smaller numbers recorded in Cyprus, Libya, Tunisia, Israel and Italy. The distribution of nesting *C. mydas* was found to be much more localised, the only substantial nesting areas being Turkey and Cyprus, with a few nests also recorded in Israel. Recent surveys in Libya, Egypt and Syria have added these countries to the list of minor nesting areas for *C. caretta* (Kasparek, 1993, 1995; Laurent *et al.*, 1997).

1.2. Previous studies in Northern Cyprus and Turkey:

In 1988, Whitmore and Groombridge conducted a survey of a large proportion of the nesting beaches in N. Cyprus between 18th June and 15th July (Groombridge and Whitmore, 1989). They counted recent tracks and nest pits during day-time surveys of the beaches and estimated that there had been 96 *C. mydas* and 122 *C. caretta* nests during the period of study.

Since 1992, Glasgow University Turtle Conservation Expeditions (GUTCE) have been carried out each year and the results of these expeditions give much more detailed data on turtles in N. Cyprus. Up to 461 green turtle and 519 loggerhead nests were recorded in any seasons from 1992 to 1995 (Broderick and Godley, 1996).

On the mainland, using my own unpublished data (1988-1993) and published sources (Geldiay *et al.*, 1982; Baran and Kasparek, 1989; Canbolat, 1991; Baran *et al.*, 1992; Kaska, 1993; van-Piggelen and Strijbosch, 1993; Baran *et al.*, 1994, 1996; Baran and Turkozan, 1996), there are 1200-1700 *Caretta caretta* nests and 400-600 *Chelonia mydas* nests on 17 different beaches (with a total length of 140 km.) in Turkey annually. It is estimated that each female lays an average of 3 nests in any season with each female nesting every 2-3 years (Groombridge, 1990). From these figures it can be estimated that approximately 400-570 *Caretta caretta* and 130-200 *Chelonia mydas* females nest annually on the beaches of Turkey.

1.3. Threats to Mediterranean sea turtles:

It seems that sea turtles in the Mediterranean, are mainly threatened by degradation of their nesting habitat through beach development and tourism. The life cycle of sea turtles and negative effects to the population in the Mediterranean are shown in Figure 1.

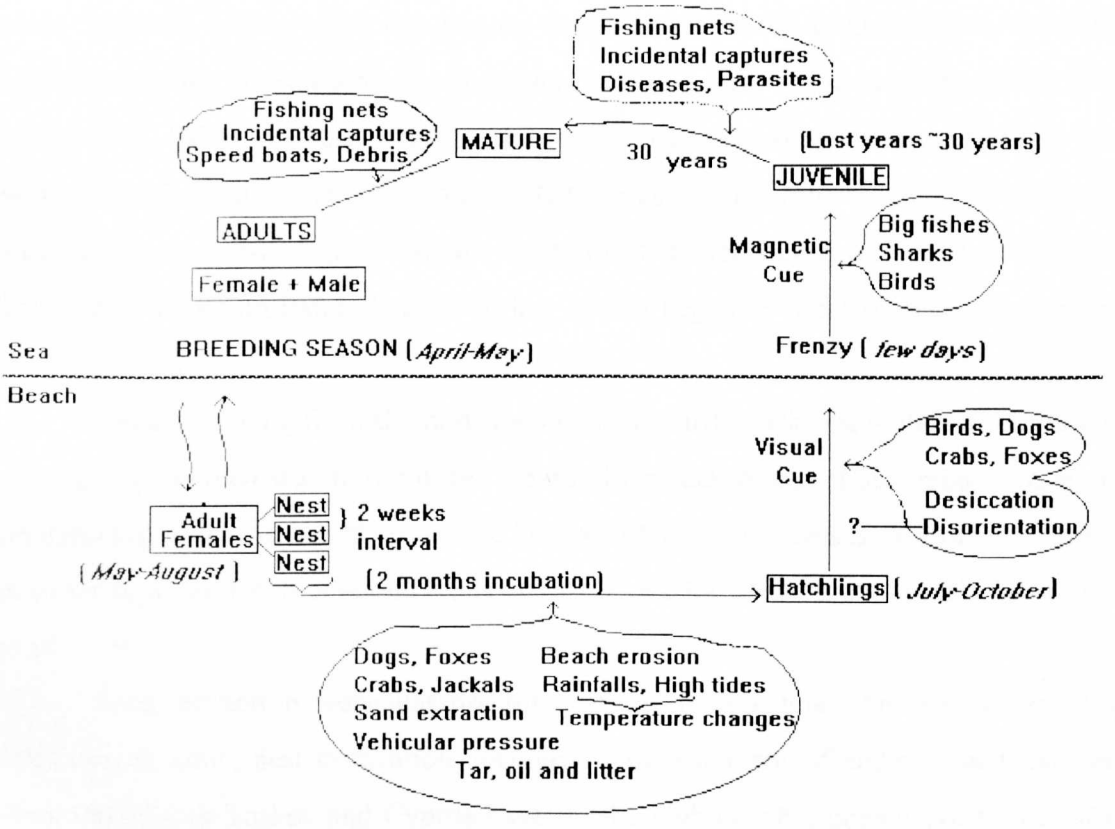


Fig. 1.1. Life cycle of sea turtles and negative effects to the population in the Mediterranean.

Different strategies for the conservation of sea turtles have been reviewed by Pritchard (1980). These strategies are; 1-) The passage of laws to prevent sea turtles from featuring in international commerce, 2-) The protection of nesting female turtles

from poaching by the establishment of beach patrols, 3-) The movement of eggs to beach hatcheries or to artificial incubators such as styrofoam boxes with release of hatchlings as they emerge, 4-) Maintaining hatchling turtles in captivity for a period of time until they have grown sufficiently to be deemed safe from the majority of hatchling predators (head-starting), 5-) The distribution of hatchlings (or eggs) from a healthy breeding population to areas where the turtles have disappeared. Pritchard's list is, however, incomplete. For example, artificial light can seriously disturb the breeding behaviour of adult nesting females and the water finding behaviour of the hatchlings (Frazer, 1986), but protection of nesting beaches against photopollution is not included in the five categories of sea turtle conservation plans discussed by Pritchard (1980). The installation of artificial light should be considered as habitat destruction and is likely to affect adversely the local sea turtle population. Declines in nesting populations of loggerheads in Florida are attributed by Worth and Smith (1976) to urban development, artificial lights and human activities. Bustard (1972) considered coastal development and construction in nesting areas the greatest threat to sea turtles in Queensland, Australia. Since sea turtles depend strongly on optical cues for finding the sea (Ehrenfeld, 1968; Mrosovsky, 1978), artificial lights in the vicinity of the nesting beaches disorient both nesting females and hatchlings. Sea turtle hatchlings can be protected against lights of an adjacent highway by strips of scrub vegetation between beach and road (McFarlane, 1963), but fences on Patara beach, Turkey have a negative effect on beach topography (Kaska, 1993).

Upon emerging from the nest, the hatchling turtles risk encounters with numbers of predators on their way to the water. Foxes, dogs, jackals and ghost crabs are common predators of nests and hatchlings on the beaches of Turkey. Cages over nests can be used in order to avoid fox predation (Canbolat, 1991; Kaska, 1993; Baran *et al.*, 1994; Yerli *et al.*, 1997).

Sand extraction, vehicular pressure, beach erosion, litter, tar and oil, and high tides can all affect nest excavation, incubation, development of embryos and hatching. Most beaches in Turkey and Cyprus have on the high beach a conspicuous strip of oil and tar, carried by storms. Tar bars are also present under the surface of the sand. It has been suggested that turtles may mistake marine debris for edible items: plastic bags appear like jellyfish in the water, and jellyfish are more common in the diet of some marine turtles (Gramentz, 1988). That is why sublethal effects of debris ingestion have an unknown but probably negative effect on the demography of sea turtles. Recently,

Bjorndal *et al.* (1994) found that debris ingestion was significantly affected by the sex of the turtle. Frequency of occurrence of debris was significantly higher in females, but the mass or volume of ingested debris were not significantly different between the sexes on the coasts of Florida.

Solomon and Baird (1977) studied fungal penetration of green turtle eggs and concluded that a heavy infiltration of fungus may impair gas exchange across the eggshell, posing a hazard to the development of the embryo. Thus high moisture levels in the sand around incubating eggs should be avoided, as these conditions are favourable for fungal growth that may cause egg mortality.

Excessive rainfall and / or inundation can indirectly affect natural turtle nests by lowering the ambient sand temperature, thereby increasing the incubation period. Inundation can also harden upper sand layers, slowing the digging efforts of emerging sea turtle hatchlings (Hendrickson, 1958).

Fishing nets, speed boats and lines cause trouble to adult turtles in the sea as they may become entangled and drown. In the whole Mediterranean, an estimated 50,000-100,000 mature and young turtles are caught each year on longline hooks and in nets set to catch fish (Groombridge, 1990). There is clearly a need for better data on fishing related mortality, especially concerning adults. In the absence of reliable data, it would be sensible to minimise risks especially to adult turtles, by restricting fishing close to known turtle feeding grounds, and nesting beaches.

Among the newer threats to turtles is the increasing incidence of fibropapilloma disease. Affected turtles exhibit large external tumours which may impair movement or grow across the eyes or mouth inhibiting feeding, breathing and vision. This disease is documented mainly in the green turtle in which it is common and is thus commonly known as "green turtle fibropapilloma disease" (GTFP). Glazebrook *et al.*(1993), in a study of the diseases of the green and the loggerhead turtles, found what they termed the 'five disease complex' (ulcerative stomatitis-branchopneumonia; ulcerative stomatitis-obstructive rhinitis; ulcerative stomatitis-obstructive rhinitis-branchopneumonia; obstructive rhinitis-branchopneumonia and ulcerative stomatitis-focal pnemonia) and observed that it was equally distributed amongst hatchlings and juveniles. They also suggested that the best way to treat ulcerative stomatitis in green turtles was to individually isolate affected animals and administer the appropriate antibiotics (streptomycin topically and chlortetracycline IM with a vitamin C supplement for at least 2 weeks). Evidence is presented that spirorchidiasis is prevalent in sub-adult loggerhead

sea turtles, is responsible for extensive lesions, and may be responsible for significant debilitation and mortality (Wolke *et al.*, 1982). They suggested that a drug (amoscanate), used to treat schistosomiasis in man and lab animals, can be given as a single oral dose, to treat female turtles during nesting. There is no report of disease in Mediterranean sea turtles to my knowledge.

1.4. Population genetics:

Turtle species tend to exhibit relatively low levels of genetic variation and differentiation (Avise *et al.*, 1992; Karl *et al.*, 1992), therefore the maternal mode of inheritence makes mitochondrial DNA (mtDNA) especially appropriate for studies of female natal homing in marine turtles because the effects of male migratory behaviour do not confound mtDNA phylogeographic patterns.

Norman *et al.* (1994) used the method for the detection of population-specific genetic markers in mtDNA to analyse population structure of *Chelonia mydas* in North Australian waters and found out that only two of the 10 rookeries surveyed could not be differentiated, and they indicated that the Indo-Pacific *Chelonia mydas* include a number of genetically differentiated populations, with minimal female-mediated gene flow among them. Recent studies of mtDNA restriction fragment length polymorphism (RFLPs) have revealed a characteristic pattern of low but geographically structured variation in the green turtle (Bowen *et al.*, 1992; Norman *et al.*, 1994) to the extent that fixed haplotype differences were commonly observed among populations. Meylan *et al.* (1990) attribute this pattern of geographic variation to the propensity for female turtles to return to their natal site to breed.

In terms of the distribution of mtDNA lineages, the various Atlantic and Mediterranean nesting populations are significantly different (Bowen *et al.*, 1993a,b). In the Mediterranean, habitats that were too cold to support nesting and feeding 12000 years ago are now utilised extensively by loggerhead turtles (Bowen *et al.*, 1993b). They suggested that loggerhead turtles are active colonizers that can occupy newly opened habitat over relatively short evolutionary time scales.

Recent mtDNA analyses have provided evidence that the beaches to which turtles return are the same ones they hatched on years before (Bowen *et al.*, 1993a). Bowen *et al.* (1993b) analysed mtDNA variation from four nesting beaches in the Northwestern Atlantic Ocean and from one nesting beach in the Mediterranean Sea, and they found significant differences in haplotype frequency between nesting populations in Florida and in Georgia/South Carolina, and between both of these assemblages and the

Mediterranean nesting colony, and hypothesed that this indicates substantial restrictions on gene flow between regional populations, and therefore a strong tendency for natal homing by females.

Baker *et al.* (1990) used a distribution of mtDNA restriction types among geographical isolated sub-populations of the humpback whale (*Megaptera novaeangliae*) to infer the pattern of seasonal migration of animals between summer (feeding) and winter (breeding) habitats. Their study indicated that whales inhabiting the feeding habitat were from two genetically discrete breeding populations.

The supposition that juvenile loggerheads from North Atlantic nesting beaches occur on Mediterranean feeding grounds is based on three lines of evidence. First, Carr (1987) noted that more juvenile loggerheads occupy Mediterranean feeding grounds than could be generated by the Mediterranean rookeries alone. Second, a loggerhead tagged in the Azores subsequently was recovered in the Mediterranean (Bolten *et al.*, 1993), and two tagged females in Dalyan beach (Turkey) have been recovered in the gulf of Gabes (southest of Tunisia) and another at sough Dugi Otok near Miglia (Italy) (Erk'akan, 1993). Third, the North Atlantic current system (believed to passively transport juvenile loggerheads) branches into the Mediterranean. Groombridge (1990) speculated that surface currents and oceanic topology may trap pelagic stage (juvenile) loggerheads in the Mediterranean Basin, and that some of these may stay there for breeding. The mtDNA data (Bowen *et al.*, 1993b) indicate that female mediated gene flow between the Northwestern Atlantic and Mediterranean rookeries is limited. Recently, Schroth *et al.* (1996) found that colonies of loggerhead turtles separated by only 10s of km are genettically distinct. In green turtles, restriction fragment analysis of nDNA loci demonstrate that nesting populations are less structured with respect to these biparentially-inherited markers than is the case for maternally-inherited lineages (Karl *et al.*, 1992).

Several researchers have speculated that North Atlantic turtles may enter the Mediterranean system via currents of the North Atlantic gyre and become trapped in the Mediterranean Basin by strong currents at the Straits of Gibraltar (Carr, 1987), and eventually recruit to Mediterranean nesting colonies (Groombridge, 1990).

1.5. Nesting and nest environment:

1.5.1. Nesting:

Sea turtles mate in coastal waters during the day, at some distance from the nesting beach or in the lagoon (e.g. Alagol in Dalyan). Only the females emerge to lay their eggs

in the sand at favourable nesting grounds. Breeding females come ashore on beaches where they deposit their eggs and subsequently return to the water. Hatchlings later emerge from these sandy nests primarily at night and immediately move toward the sea. In both adult and hatchling sea turtles, correct seaward orientation is critical. Individual females, newly arrived for purposes of nesting, may show these phases: approaching the beach, emerging on to the beach, wandering(or resting) on the beach, digging the body pit, digging the egg chamber, egg laying, covering the nest, returning to the sea. Similar phases have been described for all sea turtles by different authors (Hendrickson, 1958; Bustard and Greenham, 1968; Ehrenfeld, 1979; Hailman and Elowson, 1992). Marine turtle nesting behaviour is influenced by such variables as beach accessibility, site preference, lighting on nesting beaches, and disturbance (human or predator) while selection of nesting place is occurring. If beachfront lighting could be designed to emit light visible to humans, but outside the spectral range that significantly affects the seaward orientation of sea turtles, the sea turtles could be protected without jeopardizing human safety. Marine turtles show a strong site tenacity which, in the multiple nesting of a given season, may bring a turtle back to the same short section of a twenty-mile beach, or to one of many similar little coves around an island (Carr and Hirth, 1962); or to one among many small islands in a close knit system (Hendrickson, 1958). Geldiay *et al.* (1982) claimed that this is related to water temperature (the water of the eastern Mediterranean coast is warmer than the western, i.e., Alanya= 28 °C and Dalyan= 25 °C), but there are some beaches which are used by both species. On the other hand, the beach shifts of sea turtles demonstrate the degree to which there is opportunity for gene flow-assuming that copulation takes place off the nesting beaches (Bjorndal *et al.*, 1983). If the temperature is important for nest site selection, such kind of small changes can not be effective. Loggerheads can raise body temperatures 3.8 °C above ocean temperatures by basking in water and green turtles can elevate 5 °C above ocean temperatures by basking on the land (Spotila and Standora, 1985). The factors influencing nest site selection by marine turtles in the Mediterranean are still unknown.

When the female is ready to nest, she starts with the digging of the body pit. For digging the body pit, the sand is swept by turning the anterior edge of a flipper down into the sand and moving the limb rearward so that the ventral part of the foot sweeps against the sand. Digging the body pit, which involves sweeping movement of the flippers to push away the dry sand, is related to nest temperature, because if she starts to dig the nest chamber without digging the body pit, even in a wet area, that depth's temperature

may not be suitable for development of the eggs. Females may dig body pits apart from the nests while they are emerging on to the beach or returning to the sea. These extra body pits may be either for searching for a suitable nest place or camouflaging the real nest from predators. After the brief preparation of the body pit, the female begins excavating a cavity for the eggs. The female makes a few inital flicks of sand. The hindflippers are brought alternately to the mid-posterior position and then move outward and forward with a rapid movement that flicks sand with the dorsal surface of the flipper. The turtle digs as deeply as she can and flicks the bottom of the nest hole before starting to lay eggs, so that eggs are not damaged by the hard bottom of the nest hole during laying. If a female is disturbed during excavation of the egg chamber, she usually abandons the nesting attempt. The egg chamber lies directly under the turtle's extended cloacal tube, so that the animal does not have to reposition herself for laying. During laying, her mass is completely supported on her plastron, with the hindlimbs resting on the sand, and the foreflippers dug into the sand, which may help prevent the turtle from sliding backwards in the tilted body pit. Just before an egg-drop, flippers rise noticeably at their distal ends and maintain the extension. Females at this stage are not very sensitive to lights. After egg laying, she fills the egg chamber by pulling and then packing the sand round and over the egg chamber. The female begins covering the body pit after filling the egg chamber. She starts to throw sand in a manner similar to the digging of the body pit. All throwing is done by digging the anterior edge of the flipper into the substrate and moving sand backward with the flipper's ventral surface. While the female is covering the nest she rotates to throw more sand on the nest. This also affects the temperature of the eggs and camouflaging the nest's place because predators and humans may think that the nest is under the hole which is the last body pit made during the covering of the nest. *Chelonia mydas* throws more sand on the nest than *Caretta caretta*, due to large body size or different nest temperature.

1.5.2. False crawls:

Many turtles turn-around after ascending the beach and go back to the sea without nesting. Human disturbance, barriers to ascent and excessive illumination on the beach may cause a false crawl (non-nesting emergence of female). On the other hand, this behaviour might be either for searching the beach for future nesting or to make sure to herself that she is ready for laying, because on the beach, she can feel a pressure on her plastron that helps movements of the eggs in the oviduct. Dean and Talbert (1975) observed that loggerhead nesting activity in South Carolina was lowest in areas where

beach homes were present even if the beach appeared ideal for nesting. Although Patara Beach (for location see Fig. 2.1) is an undeveloped beach, and there are no visible light sources on the beach, due to the special protection, beach-back vegetation and fences, it held fewer turtle nests [52 (31.90% of the total emergences)] than Kizilot Beach (for location see Fig. 2.1) and Kizilot Beach has many hotels just 20-100 meters from beach, but held many more turtle nests [143 (48.47% of the total emergences)] (Kaska, 1993). In addition to that, I found that one of two emergences resulted in a nest on Kizilot Beach but one of three emergences resulted in a nest on Patara Beach (Kaska, 1993).

1.5.3. Eggs:

In eggs of turtles, the eggshell consists of two parts: a fibrous shell membrane adjacent to the albumen and a calcareous layer attached to the outer surface of the membrane (Packard *et al.*, 1982). The calcareous component of turtle eggshell is calcium carbonate which occurs as aragonite in chelonian eggs (Baird and Solomon, 1979). Flexible-shelled eggs laid by turtles are characterized by a well-defined calcareous layer that usually is about the same thickness as the shell membrane to which it is attached (Solomon and Baird, 1976; Ewert, 1979).

Eggs can be distinguished as fertile or infertile by the presence of a visible circle on the outside of the eggshell. This circle indicates the adherence of the shell membranes to the shell that occurs during early incubation of fertile eggs (Hirth and Carr, 1970; Ewert, 1979; Blanck and Sawyer, 1981; Thompson, 1985). Signs of a white circle or patch on the outside of the eggshell appears on the shell at the site of adherence between the vitelline membrane and the shell membrane within approximately 24h of oviposition in fertile eggs and this enlarges as incubation progress, wheras infertile eggs generally remain a creamy colour (Blank and Sawyer, 1981). The white patch is a regional drying of the shell, influenced by the developing embryo and related to its respiratory requirements.

1.5.4. Embryonic development:

Early studies of the embryology of turtles included a large number of experimental studies on descriptive developmental anatomy or on physiological ecology and immunology as well as on experimental embryology (Rathke, 1848; Agassiz, 1857; Parker, 1880; Mitsukuri, 1890, 1894; Allen, 1906; Jordan, 1917a, b). Although the development of marine turtles was considered in a few studies (*Chelonia mydas*, Rathke, 1848; Parker, 1880; Mitsukuri, 1894; Miller, 1982; *Carette caretta*, Agassiz, 1857; Jordan, 1917a, b; Miller, 1982; *Eretmochelys imbricata*, Miller, 1982; *Dermochelys*

coriacea, Rathke, 1848; *Lepidochelys olivecea*, Crastz, 1982; Miller, 1982; *Natator depressus*, Miller, 1982), the emphasis was on the more accessible nonmarine species (Rathke, 1848; Agassiz, 1857; Mitsukuri, 1891, 1893). Most of the workers made simple descriptions and illustrations of marine turtle embryos except Miller (1982) and Crastz (1982). They described a complete development series of marine turtles. Complete embryological series are available for only two other turtles (*Chelydra s. serpentina*, Yntema, 1968; *Chrysemys picta belli*, Mahmoud *et al.*, 1973), both of which are from freshwater, and the results are applicable to the development of marine turtles only in a general way. Development studies which involve incubating the marine turtle embryos at different controlled temperatures, or transfering eggs from one temperature regime to another during incubation, require a convenient and reliable means of identifying specific embryonic stages so that effective comparison can be made among embryos developing at different rates.

Miller (1982), using 6 species of marine turtle, has defined 31 stages of development from measurements of 18 morphological characters and substantial descriptive records. However this work does not include any descriptions of organogenesis. Morphological characters including somite number, eye development, pharingeal clefts, tail length, shell formation, development of scales and pigmentation were used to identify each stage of development and Straight carapace length (SCL), fore-flipper length (FFL) and wet weight (WW) for the rate of development for whole embryos. Wet weight is the most reliable indicator of embryonic growth as it can be measured at all stages of development and reflects changes in the organism as a whole (Harry and Williams, 1991).

In some studies (Fox, 1977; Ewert, 1985; Raynaud and Pieau, 1985) the development of the urogenital system in marine turtles is mentioned. Gonadogenesis has been described for several species of fresh-water turtle and origin and migration of the germ cells has been traced for these turtles (Allen, 1906; Risley, 1933) and for *C. caretta* (Jordan, 1917a).

1.5.4.1. Development differences:

Domontay (1968) reported that green turtle eggs had completed the early stages of gastrulation by the time of deposition. Many females emerge for several nights before they successfully nest (Parmenter, 1980). This is especially so when the beach is not suitable for nesting or if there is a disturbance on the beach. Thus, it would be predicted that the eggs deposited in these delayed nests should have a shorter than average

incubation time, since they would have developed further in the oviduct, but Parmenter (1980) claimed that embryonic development is suspended while the eggs are held in the oviduct.

Packard *et al.* (1987) found, in a study of incubation of eggs of painted turtle (*Chrysemys picta*), that hatchlings are heavier from eggs incubated in hydric conditions than from the eggs in dry settings. Eggs of snapping turtle (*Chelydra serpentina*) in dry environments hatched sooner, but produced smaller young than those in wet environments (Packard and Packard, 1984).

Viable eggs incubated on a wet substrate absorb water from the environment and increase in mass during incubation (Packard *et al.*, 1983, 1987). Under these conditions, embryos may have greater survivorship at thermal extremes (Packard *et al.*, 1987), but there may be little or no effect at optimal temperatures though, they take more time to develop (Packard *et al.*, 1980) and have greater total metabolism (Gettinger *et al.*, 1984; Miller and Packard, 1992) than embryos incubated at the same temperature but on drier substrates.

The larger size of turtles from eggs incubated on wet substrates might be due to a more efficient utilization of their yolk (Miller and Packard, 1992). That is embryos in eggs on wet substrates might gain a greater increment of growth per unit O_2 consumed compared with embryos in eggs on dry substrates. A relatively slow increase in the concentration of urea inside large eggs may reduce the potential for inhibition of metabolism by this substance and may thereby promote a higher rate of embryonic growth (Packard *et al.*, 1984), and so may delay stimulation of pipping and thereby prolong incubation (Packard *et al.*, 1987).

Hatchlings from a wet substrate are larger and have a smaller residual yolk than those hatched from dry conditions (Tracy *et al.*, 1978; Gettinger *et al.*, 1984; Miller *et al.*, 1987; Packard *et al.*, 1987); and the composition (percentage of lipids and proteins) of hatchlings differs depending on the water availability of the incubation substrate (Packard *et al.*, 1987). Packard *et al.* (1987) also reported that embryos incubated at lower temperatures require a greater length of time to hatch than those incubated at higher temperatures.

1.5.4.2. Embryonic mortality:

Dead loggerhead eggs and hatchlings have been reported from nests inundated by rainfall and sea swells, with mortality attributed to lack of respiratory oxygen (Kraemer and Bell, 1980). Incubation in sand moistened by water with salinities equal to 75% and 100% sea

water prevented normal metabolism and caused subsequent failure in green turtle eggs (Bustard and Greenham, 1968). Embryonic mortality is observed during early development, before pigmentation of the eye, and supports the belief that the first three weeks of incubation is a critical period in embryonic sea turtle development (Blank and Sawyer, 1981; Whitmore and Dutton, 1985).

1.5.4.2.1. Inundation:

Inundation of nests by sea water leads to egg mortality from suffocation (Whitmore and Dutton, 1985) and/or chloride toxicity (Bustard and Greenham, 1968). The survival of eggs to hatching may therefore be higher in nests laid further from the sea where inundation is less likely. Counterbalancing this selective pressure favouring nests laid further from the sea it has been suggested that hatchlings emerging from nests sited further inland may take longer to reach to sea or be unable to find the sea at all (Mrosovsky, 1983), and may therefore have a higher risk of dying or desiccation, heat stress or predation on the beach. In addition, a nest excavated amongst supra-littoral vegetation, may suffer high egg mortality as a result of roots invading the egg chamber or may stay deep under the sand because of the beach erosion and redeposition of sand (Kaska, 1993).

The diffusion of gas may be slow and may be even be insufficient in artificial nests, and this may cause the death of emerging hatchlings and embryos within the nests (Ackerman, 1977). Simon (1975) reported that sea turtle egg mortality increased when the spaces between the eggs were filled with sand. This may be due to impeded gas exchange by the eggs (Ackerman, 1980). Gas transfer between the atmosphere and a clutch of buried eggs occurs by diffusion through the sand or mound material and the rate of diffusion depends on gas pressure gradients developed by egg metabolism and organic decomposition in the sand (Seymour and Ackerman, 1980). Ackerman (1980) proposed that the requirement for optimal gas tensions in the nest might limit clutch size in marine turtles and account for i) the remarkable selectivity of the females for certain nesting beaches with suitable gas transport properties, ii) the similarity of clutch mass between species of very different body weights, and , iii) the requirement for multiple layings within a year. He found that the gaseous environment of natural nests resulted in the shortest incubation and greatest hatching success of *Chelonia mydas* and *Caretta caretta*.

1.5.4.2.2.Predation:

Snow (1982) examined the relationship between nest age and predation rate in painted turtles (*Chrysemys picta*) and found that new nests (less than 72 h old) do not appear to have a greater risk of predation than older nests. A relatively short incubation, such as would be induced by exposure to either high temperature or a dry substrate, could be advantageous to the extend that eggs would be at lower risk to predation in the nest (Snow, 1982).

The location of nest, while important with regard to abiotic factors, did not appear to be important in determining if a nest was eaten by raccoons, *Procyon lotor*. Most of these nests were taken by raccoons during the first two or three nights (Hopkins *et al.*, 1978). Raccoons destroy over 95% of the loggerhead turtle nests laid on some South Carolina beaches (Stancyk *et al.*, 1980). Foxes and dogs are the main predators in the Mediterranean (Baran and Kasparek, 1989; Canbolat, 1991; Kaska, 1993; Broderick and Godley, 1996).

Production of a greater number of small clutches would increase the probability of at least one clutch surviving the many sources of mortality (e.g., predation, inundation, destruction by other nesting females), but each nesting emergence is costly to the female, both in energy (Bjorndal, 1982; Hays and Speakman, 1991) and risk of predation (Stancky, 1982).

Larger hatchlings have been suggested to have a selective advantage, relative to smaller conspecifics, due to increased competitive ability or improved predator evasion, simply as the result of being larger (Packard *et al.*, 1981). Miller *et al.* (1987) showed that the impact of embryonic water exchanges on the physiology of developing turtles extends beyond hatchling and affects the locomotor performance of young turtles.

Ghost crabs, in their younger stages, must remain nearer the water to keep their gills wet. With each succeeding molt, the crab is able to spend less time near a source of water, and digs its burrows higher in the dunes. Therefore, because of this life cycle, the ghost crab appears to be more prevalent near nests later in the season.

1.5.4.2.3. Reducing the mortality:

Increased hatching success can be attained by protection (caging) and/or artificial incubation (hatchery) of turtle eggs. The successful conservation of sea turtles will demand a through underestanding of the biology of incubation (Ackerman, 1980).

If there is a high mortality of large numbers of eggs in their natural nests and it is considered necessary to relocate them, it is important that artificially made nest sites be

chosen to provide the same environmental temperature ranges as the natural nests. This will be easier to achieve by having several well separated hatcheries rather than one large central hatchery. In this way the natural sex ratio is more likely to be maintained without it having to be measured.

Incubating the turtle eggs in styrofoam boxes in an above ground hatchery, usually a shed on or near the nesting beach was found to be causing a bias in the hatchling sex ratio towards males (Mrosovsky, 1982; Morreale *et al.*, 1982; Dutton *et al.*, 1985). Once the seasonal profile of the natural sex ratio is known, it may be possible to use styrofoam boxes for clutches laid during the period when the natural sex ratio is all male.

By using either of these methods hatching success can be increased or the predation can be decreased. Changing the place of a nest has great importance for the protection of sea turtles in dangerous places. In order to produce natural sex ratios, care is taken to duplicate as closely as possible the depth, the amount of shading, and the egg chamber dimensions of natural nests. There is a critical period of from less than 12 hours to about 14 days after laying during which the hatching success of loggerhead eggs is significantly decreased by movement of eggs (Limpus *et al.*, 1979). That is why relocating a clutch is a delicate process and should be done within a few hours of it being laid i.e. before the embryonic membranes have begun to develop. Relocation should be done on the same beach, and by copying the parents behaviour, working on the assumption that natural selection has dictated that parents produce the optimum egg chamber. We do not know whether an adult sea turtle relocates the beach of its birth by means of imprinting in the period from hatching to entering the sea, but if this early period is important in this way, any deviation from natural activities could be a threat to the population (Pritchard, 1980). Egg relocation is an effective conservation method because eggs in undisturbed natural nests can have lower hatching success than relocated eggs (Wyneken *et al.*, 1988). Eggs can be protected by relocating eggs for incubation under natural conditions (protected areas where eggs are reburied in the sand above the anticipated spring high tide level) or artificial conditions using expanded polystyrene incubators or other nonmetal containers (Styrofoam boxes). In the absence of data on temperature-dependent sex determination and a thermal transect of a beach, artificial hatcheries should not be used. Rather, efforts should be directed to marking natural nests as they are made and to enclose them in wire mesh fences as soon as possible (Morreale *et al.*, 1982).

1.5.5. Temperature, gaseous and hydric environment:

Under natal beach conditions, sea turtles' eggs incubate at temperatures between 24° and 33°C (Hendrickson, 1958; Caldwell *et al.*, 1959; Bustard, 1972; Ewert, 1979) and hatchlings emerge from the nest 45 to 75 days after laying. Incubation period may vary because of temperature, moisture, clutch size, depth of the nest (Yntema, 1978). Developing turtle embryos and hatchlings continually require O_2 (Lynn and von Brand, 1945). Gas diffusion through the sand and the egg shell usually allows adequate exchange for sea turtle eggs (Prange and Ackerman, 1974; Ackerman, 1980). However, gas diffusion may be modified by particle size and water content of the sand (Ragotzkie, 1959; Prange and Ackerman, 1974; Ackerman, 1980, 1981a, b; Kraemer and Bell, 1980). In artificial nests, inadequate gas exchange prolongs incubation by slowing growth and reduces hatching success (Ackerman, 1981b). A large mass of eggs may not get enough oxygen to develop properly (Ackerman, 1980). Temperature influences the duration of incubation and the rate of development (Miller, 1982). Low temperatures increase the duration of incubation and slow the rate of development, whereas high temperatures decrease the duration and increase the rate (Miller, 1982). For example in *Chelonia mydas*, incubation lasted 94 days at 23-25°C (Ackerman and Prange, 1972) and 47-49 days at 32°C (Bustard and Greenham, 1968). During incubation the temperature within the egg mass increases 2-4°C above that of surrounding sand in nests of *C. mydas* (Hendrickson, 1958; Bustard, 1972). This increase is attributed to metabolic heat generated by the later-stage embryos (Yntema, 1960; Bustard, 1972; Maloney *et al.*, 1990; Godfrey *et al.*, 1997), but this increase which occurs later in incubation is unlikely to play a significant role in sex determination. In general, eggs incubated at warm temperatures grow faster and hatch earlier than those incubated at cool temperatures (Kam and Lillywhite, 1994). High temperatures, however, such as 31°C, have been shown to reduce the hatching success from the optimum in snapping turtles (Packard *et al.*, 1987). Kam and Lillywhite (1994) suggest that high temperatures may result in critical oxygen tensions in the nest due to the eggs' increased metabolic rate, and hence increased oxygen demands. Maloney *et al.* (1990) have shown that, within any one clutch, there is a gradual fall in temperature from the top to the bottom of the clutch, thus influencing fluid uptake by the eggs. They also suggest that the daily movement of the water table may influence the temperature toward the bottom of the clutch.

Temperature both influences sexual differentiation and rate of development of *Caretta caretta* (Yntema and Mrosovsky, 1980; Mrosovsky, 1980, 1982; Mrosovsky and

Yntema, 1980), and *Chelonia mydas* (Morreale *et al.*, 1982). As it does with many other chelonian species, within the limits of embryonic tolerance cooler temperatures produce male hatchlings (26 and 28°C, *C. caretta*; 26°C, *C. mydas*), and warmer temperatures produce female hatchlings (32°C, *C. caretta*; 29 and 32°C, *C. mydas*) and the temperatures between these produce both sexes. The phenomenon of temperature dependent sex determination has important consequences in conservation practices. Studies on marine turtles (Bull and Vogt, 1979; Bull *et al.*, 1982; Yntema and Mrosovsky, 1982; Mrosovsky and Provancha, 1992; Wibbels *et al.*, 1993; Godfrey *et al.*, 1997) and nonmarine turtles (Crews and Bergeron, 1994) indicate that the critical period for such influence is the second quarter of incubation just preceding and during gonadal differentiation. It is not useful to collect embryos earlier than stage 16 as their biomass is insuffient for subsequent biochemical analyses. The middle one-third of the incubation period (stages 22-27) is the sex determining period for *C. caretta* (Yntema and Mrosovsky, 1982). Prior to stage 21, the embryonic kidney is not differentiated into a functional mesonephros and there is no visible gonad (Harry and Williams, 1991). Harry and Williams (1991) reported for *C. caretta* that the urogenital system of male (26 °C) embryos initally weighs significantly more than the female (32 °C) system. Different growth patterns for the male and female urinogenital systems are found during the sex determining period (stage 22-27) of embryogenesis. Thus, within this critical period, incubation temperature has a particular influence on the growth of urogenital tissue. The rate of embryonic development is faster at the warmer temperatures. During development female embryos consistently weigh less than male embryos at the same development stage, but at hatching there is no significant difference in weight of male and female loggerhead turtles (Harry and Williams, 1991).

Marked diel cycles in nest temperature have been reported for freshwater turtles (Packard *et al.*, 1985) and at shallow depths on marine turtle nesting beaches (Mrosovsky and Provancha, 1989). Similarly in green turtle nests in Costa Rica, Standora and Spotila (1985) reported that diel temperature variations were very small (<0.5 °C). Presumably, since green turtle nests are very deep, diel temperature variations occuring near the surface are very much reduced at nest depths. Reported temperature rises in natural nests are between 2 and 7 °C (Carr and Hirth, 1961; Bustard and Greenham, 1968; Hendrickson, 1968; Godfrey *et al.*, 1997).

Intra-beach thermal variation on turtle nesting beaches may be large. For example, nest temperatures may vary depending on the extent of nest shading by

vegetation (Morreale *et al.*, 1982). Beach colour also affects temperature of the sand with darker beaches being warmer than lighter coloured beaches (Limpus *et al.*, 1983).

Egg temperatures of leatherback, green and ridley turtles, nesting in Surinam and French Guiana, averaged 30.6 °C, 29.7 °C and 28.7 °C repectively (Mrosovsky and Pritchard, 1971). Carr and Hirth (1961) on Ascension Island recorded the temperatures of green turtle nests as at 27.8- 28 °C, with an average gain of 2.3 °C during incubation. Hendrickson (1958) in Malaysia gives the range 28 °C to 30.4 °C but recorded approximately 6 °C rise in temperature during incubation for the same species. Carr and Hirth (1961) noted that a significant temperature gradient existed between the clutch and the surrounding sand, but this would probably not be more than 2-4 °C.

Nests of sea turtles show small daily variations in temperature (Bustard, 1972; Mrosovsky, 1982); this variation may, neverthless, cross threshold boundaries and may cover a significant range (>8 °C) over the course of incubation (Morreale *et al.*, 1982).

The uppermost eggs will experience greater fluctuations each day than the lowest eggs (Wilhoft *et al.*, 1983; Thompson, 1988; Godfrey *et al.*, 1997). Georges *et al.* (1994) suggested that daily proportion of development is occuring at a temperature is important for sexual differentiation rather than time spent at that temperature. During incubation a temperature differential will set up between the centre and edge of the nest that will cause a redistribution of water within the clutch, water moving from eggs in the centre and being absorbed by eggs on the periphery (Ackerman, 1980).

The sand in which the eggs are buried helps prevent desiccation and hides the eggs from predators; it must also impede the gas exchange between the developing egg and the atmosphere (Prange and Ackerman, 1974). The eggs of *Chelonia mydas* absorb water during the first 48 hours and become turgid; both conditions depend upon the moisture conditions of the sand (Bustard and Greenham, 1968; Bustard, 1971). Under natal beach conditions, eggs tend to increase slightly in weight during incubation (Miller, 1982). The percentage hatch of loggerheads is greatest at 25% moisture and significantly less at higher and lower levels of moisture (McGehee, 1990). Hatchling plastron length and carapace length are greatest at 25% moisture and significantly smaller in other levels of moisture. The average moisture content of the sand in natural nests of *Caretta caretta* is 18% on Merritt Island, Florida (McGehee, 1990). High moisture levels caused by heavy rains and high tides can destroy entire turtle clutches (Ragotzkie, 1959; Kraemer and Bell, 1980; Kaska, 1993). Gas exchange is impeded when the eggs are in a moisture saturated environment, and oxygen diffusion between the atmosphere and the eggs in a

turtle clutch may affect the rate and success of embryonic development (Ackerman and Prange, 1972; Ackerman, 1980; Ackerman *et al.*, 1985). Embryonic turtles exposed to wet conditions during development had longer incubation periods and grew larger than the embryos incubated in drier settings (Hendrichson, 1958; McGehee, 1990). Most sea turtles nest above the high tide line, thus avoiding emplacement of nests in areas that are normally inundated by rising tides. Kraemer and Bell (1980) emphasized the potential importance of nest site selection by parent turtles in relation to higher hatchling success associated with proper moisture conditions and subsequent hatchling survival. Lengthening the incubation period increases the chances that a predator will find a nest. Since nests, which the incubation period is long due to the hydric environment, produce more batches of young than the nests have short incubation period. Sea turtle eggs consume O_2 throughout their incubation period and the pattern of O_2 uptake appears sigmoidal (Ackerman, 1981a). The rate of O_2 uptake increases rapidly during the second half of incubation, slowing slightly just before hatching (Ackerman, 1981a). The loggerhead egg consume much less O_2 than green eggs during incubation, this may be related to loggerhead turtle eggs have shorter incubation periods than green, therefore grove more rapidly (Ackerman, 1981a).

Oxygen concentration in the sand, away from developing nest, is about the same as in free air and the rate of diffusion must vary with the particle size and dampness of the sand (Prange and Ackerman, 1974). An additional parameter that may be important in altering the hydric environment of the clutch is the water table level. This action will directly influence gas conductance through the sand (Prange and Ackerman, 1974). Maloney *et al.*(1990) speculated that the movement of the water table, up and down, along with the daily variations in level may act as a pump to "ventilate" these clutches and improve gas exchange and effect water transport within the clutch.

Bustard *et al.* (1969) have reported that turtle embryos remove calcium from the eggshell. This activity, in addition to supplying the animal with calcium, may also improve gas exchange as development process.

Significant lengthening in total incubation time of eggs of the green turtle was produced by alterations of the gaseous environment (O_2 and CO_2 simultenously). In artificial nests with respiratory environments similar to those in natural nests, *C. mydas* embryos had faster growth rates, shorter incubation times, and higher hatching success, and yielded larger hatchlings than embryos from similar nests with lower oxygen and carbon dioxide conductances (Ackerman, 1981b). Etchberger *et al.* (1992) investigated

the influence of gaseous carbon dioxide on the development and physiology of embryonic *Trachemys scripta*. Altered levels of oxygen alone produce small, but significant, changes in total incubation time in *Trachemys scripta* and the elevated levels of carbon dioxide significantly hindered post-hatching yolk utilisation and marked changes in the length of incubation with only slight increased mortality (Etchberger *et al.*, 1991). The much larger shifts in total incubation time in *C. mydas* might thus reflect changes due largely to carbon dioxide. During the most of development, loggerhead sea turtle nests are typically subjected to oxygen concentrations ranging from ca. 14-19%; only near the time of hatching oxygen concentrations fall to extremely low levels (e.g., 5%, Ackerman, 1977). An increase in carbon dioxide generally accompanies the low gas exchange through natural sea turtle nests (Ackerman, 1981b).

An oxygen shortage prolongs incubation more in sea turtle nests than in freshwater turtle nests (Ackerman, 1981b; Etchberger *et al.*, 1991). The lengthened incubation periods allow the embryo to convert a higher proportion of the egg into hatchling (Packard and Packard, 1984). However, smaller hatchlings were always produced when incubation time of *T. scripta* lengthened with low level of oxygen (Etchberger *et al.*, 1991 and 1992). Total incubation time and temperature during the incubation period gave good prediction of sex ratios for *T. scripta* hatchlings (Etchberger *et al.*, 1992).

1.5.5.1. Temperature determined sex determination:

In 1966 M. Charnier published a two page paper, published in French, showing that the temperature at which eggs from the rainbow lizard, *Agama agama*, were incubated affected the sex ratio of the hatchlings (Charnier, 1966). From then on, different researchers from different countries started to work on temperature-dependent sex determination in reptiles. Yntema (1976) showed the egg incubation temperature also determined the sex of snapping turtle, *Chelydra serpentina*, embryos.

A variety of factors (maternal behaviour in choosing a nest site, the zygote's response to temperature in becoming male or female, environmental effects (temporal or spatial temperature of the nesting area) interact to determine the sex ratio.

Laboratory and field experiments have shown that sex in many turtle species is determined by egg incubation temperature, usually during the middle third of development (Yntema, 1979; Bull and Vogt, 1979, 1981; Yntema and Mrosovsky, 1980, 1982; Moreale *et al.*, 1982; Pieau, 1982; Vogt *et al.*, 1982; Janzen and Paukstis, 1991; Wibbels *et al.*, 1994; Mrosovsky, 1994; Viets *et al.*, 1994). Temperature dependent sex

determination (TSD) has been reported in at least five families of Chelonians (7 genera, 72 species).

General patterns of temperature effects on gonadal differentiation have been described by Bull (1980) as;

-Type A; females produced at lower temperatures, males produced at high temperatures (most crocodilians and lizards).

-Type B; females produced at high temperatures, males at lower temperatures (many turtles).

-Type C; females produced at low and high temperatures, males at intermediate temperatures(three crocodile, one lizard, and three turtle species).

Sex allocation theory suggests that sex ratios within a population should conform to 1:1 (Fisher, 1930; Charnov, 1982). The sex ratios of turtles with TSD appear to threaten this theory. Almost all biased sex ratios reported for sea turtles have been biased in the female direction (Mrosovsky, 1994).

The thermosensitive period is a time period when the gonads are making a transition from sexually undifferentiated to the inital stages of sexual differentiation. If temperature during this critical period (middle third of the incubation period, stages 16-22 (in *Emys orbicularis*; Pieau and Dorizzi, 1981 and same for *Chremys picta* and *Graptemys ouachitensis*; Bull and Vogt, 1981) 15-19 (in *Caretta caretta*; Yntema and Mrosovsky, 1982) and reported as 22-27 for *Caretta caretta* by Miller (1985)) correlated well with the total incubation duration, it might still be possible to estimate the approximate sex ratio produced by a population of turtles. The critical period tends to lie between about stages 14 and 20 (Janzen and Paukstis, 1991). The critical periods for sea turtles turn out to be relatively early in incubation, before metabolic heating becomes important. This means that temperatures of sand adjacent to nests, rather than those within the actual egg mass itself, can be used to discover the environmental temperature of eggs in a given place or season at the time of gonadal differentiation (Mrosovsky and Yntema, 1980).

Past studies of reptiles with TSD have shown that admistration of exogenous estrogens, and to a lesser extent testesterone, can induce embryos to develop as females even if eggs are incubated at male-producing temperatures (Raynaud and Pieau, 1985; Gutzke and Bull, 1986; Bull *et al.*, 1988; Crews *et al.*, 1989, 1991, 1994; Janzen and Paukstis, 1991; Wibbels and Crews, 1992; Wibbels *et al.*, 1992, 1993, 1994; Crews and

Bergeron, 1994). Temperature may be regulating the production of steroid hormone as an integral part of the sex determination cascade.

1.5.5.2. Threshold temperature:

Bull (1980) introduced the term "threshold temperature" to describe the incubation temperature at which the shift in sex ratio occurs. Mrosovsky and Yntema (1980) used the term "pivotal temperature" to describe the temperatures at which the ratios between the sex changes rapidly. Limpus *et al.* (1983) used SDT_{50} which stands for sex determining temperature, 50% female.

When eggs are incubated at constant temperatures there is a narrow range of temperatures over which both males and females will be produced, and wider ranges above and below this threshold at which only one sex results (Bull, 1980). Most of the turtles have a single threshold temperature (e.g., map turtles (*Graptemys* sp.), and sea turtles) but Painted turtle (*Chrysemys picta*), Mud turtle (*Sternotherus odoratus*) and Snapping turtle (*Chelydra serpentina*) embryos have two threshold temperatures, one high (27-30 °C) and one low (<24 °C) at which females are produced (Yntema, 1976; Vogt *et al.*, 1982; Bull *et al.*, 1982; Gutzke and Paukstis, 1984; Mrosovsky, 1994). For marine turtles, population survival is therefore dependent on the occurrence of both sexes. The question of how this necessary range of incubation temperatures is realised is therefore of great scientific and conservation interest (Janzen and Paukistis, 1991; Mrosovsky and Provancha, 1992).

Pivotal temperatures for sea turtles are conserved within a 1 °C range(28.6-29.7 °C) and lack clear geographical variation (Mrosovsky, 1994). However, geographical trends, while seemingly slight, do occur in freshwater species (reviewed by Ewert *et al.*, 1994). The variety of relationship between pivotal and beach temperatures suggested that diversity of sex ratios in different populations should be expected (Mrosovsky, 1994). Choice of nesting sites also corresponds to pivotal temperature within a single region (Ewert *et al.*, 1994). Pivotal temperatures within the best-documented species (few freshwater turtle) and genera tend to increase with both latitude and longitude across central and southern North America (Ewert *et al.*, 1994).

The combined pivotal temperature from North Carolina, Florida and Georgia of 29.0 °C (Mrosovsky, 1988) is below the 30.0 °C reported previously for loggerhead turtle eggs from Little Cumberland Island, Georgia (Yntema and Mrosovsky, 1982).

The overall estimate of 29.0 °C for the threshold temperature of North American loggerhead turtles (Mrosovsky, 1988) is close to the overall value of 28.6 °C given by

Limpus *et al.* (1985) for Australian loggerhead turtles. The pivotal temperature of Surinam green turtles is 28.75 °C (Mrosovsky *et al.*, 1984) and of Costa Rican green turtles, estimated from monitoring sand temperatures in the field, is 28.5 °C (Morreale *et al.*, 1982).

1.5.5.3. Sex ratio:

The sex ratio of loggerhead eggs, incubated in the laboratory, changes from 100% female to 100% male over a 4 °C span (Yntema and Mrosovsky, 1980). Three potential factors interact to influence hatchling sex ratios in turtles; maternal nesting behaviour, environmental effects on the temperature of the nesting area, and the zygote's response to temperature in becaming male or female (Bull *et al.*, 1982). There may be marked seasonal variations in sand temperature. For example, for loggerhead turtles nesting in Georgia and South Carolina, USA, Mrosovsky *et al.* (1984) found that more females were produced from nests laid at the start and the end of the nesting season when the sand was relatively cool, while more females were produced during the warmer interim period. Table 1.1 summarise the sex ratio data on loggerhead and green turtles.

Table 1.1. The temperature and sex ratio data on turtles

Species	Temperature- % ratio	Reference
Chelonia mydas	28.8 °C - threshold	Mrosovsky *et al.*, 1984b
Chelonia mydas	28.0-30.3 °C- threshold	Standora and Spotila, 1985
Chelonia mydas	30.5 °C- 94-100% female 28 °C - 90-100% male	Morreale *et al.*, 1982
Chelonia mydas	26 °C - 85.7 % male 14.3 % intersex 29 °C - 90.2 % female 9.8 % intersex 33 °C - 85.7 % female 14.3 % intersex	Miller and Limpus, 1981 (in Standora and Spotila, 1985).
Caretta caretta	>,= 32 °C- 100% female <,= 28 °C- 100% male 30 °C - threshold	Yntema and Mrosovsky, 1982
Caretta caretta	29.0 °C- threshold	Mrosovsky, 1988
Caretta caretta	28.6 °C- threshold	Limpus *et al.*, 1985
Caretta caretta	29.7 °C- threshold	Maxwell, 1987 (in Mrosovsky, 1994)

Mean incubation temperatures may be adequate to predict sex ratios only in sea turtles that have deep nests which experience little temperature fluctuation (Bull, 1980; Morreale *et al.*, 1982). Sex ratios in sea turtles are a function of nest beach characteristics, as vegetated nesting beaches appear to produce more males than open areas (Mrosovsky *et al.*, 1984; Vogt and Bull, 1984). At Tortuguera, the sex ratio of hatchlings in a nest is affected by the zone in which the clutch is deposited (Spotila *et al.*, 1987). This is also reported by Mrosovsky and Provancha (1992) as there might be male-producing areas in the north and female-producing areas in the south. Recently, Marcovaldi *et al.* (1997) estimated the sex ratios of loggerhead turtles in Brazil from pivotal incubation durations and found that 82.5% of the loggerhead hatchlings produced were female.

1.5.5.4. Sexing:

Common techniques for sexing sea turtles hatchlings include histological examination of gonads (e.g., Paukistis *et al.*, 1984) and direct examination of gonadal morphology by glycerine clearing (e.g., van der Heiden *et al.*, 1985; Mrosovsky and Benabib, 1990), dissection (Mrosovsky, 1982), or laproscopy (e.g., Bull, 1987). Recently Gross *et al.* (1995) developed a noninvasive technique for sexing hatchling sea turtles by analysing sex steroid concentrations in egg chorioallantoic/amniotic fluid. Turtles with complete Mullerian ducts were classified as females, and those in which ducts were interrupted or missing were judged to be males (Bull and Vogt, 1981). Specimens were microscopically sexed as males using the differentiated into distinct presumptive seminiferous tubules with associated germ cells, while the cortex was reduced to an investing squamous epithelium. With females the cortex was well developed containing numerous germ cells and a columnar epithelium, while the medulla showed no development of seminiferous tubules.

An organ culture study of the sea turtle *Lepidochelys olivaceae*, which has TSD, revealed that isolated gonads differentiate according to the egg incubation temperature prior to excision and sexual differentiation of isolated gonads was not influenced by culture temperature, which suggests that temperature is acting on the gonads via an extragonadal mechanism (Merchant-Larios and Villalpando, 1990).

Adult sea turtles can be sexed using external secondary characteristics and internally. Male turtles have very elongated tails which extend more than 25 cm beyond the carapace and have a pronounced recurved claw on each flipper. Their paired gonads are abdominal. They are attached to the peritoneum either side of the dorsal midline just

anterior to the pelvic girdle, and are immediately ventral to the kidneys (Rainey, 1981). A testis is a smooth surfaced elongate organ in which the seminiferous tubules can be seen through the investing tunica albuginea. An ovary has a granular surface and varies from a compact mass of oocytes and previtellogenic follicles up to 3 cm diameter. The oviduct varies from a thin straight white duct on an unexpanded mesovarium lying lateral to the ovary to a broad convoluted pink duct suspended into the body cavity on an expended mesovarium (Limpus and Reed, 1985).

1.5.5.5. Incubation period and temperature of nest:

Bustard (1972) reported the optimal temperature range for sea turtle egg incubation as 27-32 °C; overall range was 25-37 °C. Dimond (1965) reported the hatching time of loggerhead was 54-55 days (30 °C) and Bustard (1972) reported 55 days incubation for green turtles at the same temperatures.

When turtle eggs are kept at constant temperature, incubation duration is longer at cooler temperatures; over the range of 26-32 °C, a 1 °C change decrease adds about 5 days to incubation (Mrosovsky and Yntema, 1980). In natural conditions, it has been estimated that a 1 °C decrease adds about 8.5 days and 4.4 days in different populations (see Mrosovsky, 1982).

Incubation temperature may vary with the position of the nest on the beach. For example, for green turtles nesting at Tortuguero, Costa Rica, Morreale *et al.* (1982) reported that nests at the back of the beach tended to be cooler due to shading from supra-littoral vegetation and thus produced a greater proportion of male hatchlings than nests on the open unshaded beach. The mean sand temperature may also vary with depth, providing the potential for depth-related differences in the hatchling sex ratio. Nests may also undergo marked cooling following periods of prolonged heavy rain (Morreale *et al.*, 1982). Hot exposed nests produce female turtles and cool shaded nests produce males (Bull, 1985). Clutch size may also affect the temperature of the nest and incubation period, but Frazer and Richardson (1985) found no significant monotonic increase or decrease in clutch size over the course of the season for loggerhead nesting in Georgia.

Factors such as eggshell thickness affecting the amount of evaporative cooling could have led to different temperatures within the eggs of one clutch (Wibbels *et al.*, 1994). The eggshell is approximately 0.33 mm thick for sea turtle eggs (Ackerman and Prange, 1972). Clutch temperature is known to increase in nests of marine turtles (Hendrickson, 1958; Bustard and Greenham, 1968) due to internal metabolic heating (Godfrey *et al.*, 1997). Nests may experience wide daily fluctuations in temperature (up

to 12 °C) and nest temperatures are higher later in the season than earlier. Top eggs experience generally warmer conditions than bottom eggs within a nest (Thompson, 1988). Incubation time in the field depends on which part of the season oviposition occured and on the degree of shading of the nest. Egg temperatures in the whole nest may rise above the temperature of the nesting beach (Hendrickson, 1958; Carr and Hirth, 1961; Bustard, 1972; Godfrey *et al.*, 1997) and a temperature gradient of this magnitude may exist between the center and periphery of the nest toward the end of incubation (Bustard, 1972).

1.5.6. Sand grain size:

Only a fraction of the volume of the beach surrounding the sea turtle egg clutch is occupied by gas. Thus the quantity of gas which moves through the beach, primarily by diffusion (Prange and Ackerman, 1974), is related to the volume of gas present per volume of sand. This gas fraction is related in turn to the size distribution of sand grain particles and sand water content. Finer grained beaches contain greater quantities of gas than more coarsely grained beaches when dry, but hold more water, due to greater capillarity when wet (Hillel, 1971 in Ackerman, 1980). Since only the top layer of beach is dry, water content and other variables, such as rainfall or flooding must be considered when assessing the gas diffusivity of nesting beach.

1.6. Emergence of hatchlings:

Hatchling sea turtles generally start to emerge at night from underground nests, then immediately crawl to the sea and then begin swimming towards open water. Not all the hatchlings of a nest travel to the surface at the same time. Most commonly, a large batch one night is followed by a smaller batch the following night. Some nests were recorded as producing small batches of young on as many as five different nights. Nests were excavated 7 to 10 days after hatching (Kaska, 1993), because the climbing of hatchlings to the surface requires from 3 to 7 days after hatching (Quiescence of hatchlings). The period of quiescence results from the hatchlings being confined in a subterranean chamber where their carapaces are flattened. During this period, the yolks are resorbed and the muscles are prepared for a long swim. Alternatively quiescence may be obligatory when the oxygen demand of the emerging hatchling exceeds the rate of oxygen diffusion into the chamber. An inhibition of many activities by warmth might help explain why it takes turtles several days to reach the surface after hatching (Hendrickson, 1958; Carr and Hirth, 1961). The problems of activity deep within the nest and the

possible role of social factors among the hatchlings have been discussed in some studies(Carr and Hirth, 1961; Bustard, 1967).

1.6.1. Nocturnal emergence:

Upon encountering temperatures much above about 30 °C., the hatchlings cease to avoid coming to the surface during the day. Bustard (1967) stated that occasionally during the day topmost hatchlings are pushed out of the sand by the activity of those below. Hatchlings which reach the surface have a dampening effect on the activity of those below, because experimental removal of individuals at the surface led to activity within the nest and emergence of the turtle brood within minutes (Bustard, 1967; Kaska, pers. obs.). Hendrickson (1958) has suggested that, during the day, heat inhibits activity and keeps the turtles below the surface until the cool of the night. A tropical beach often becomes extremely hot by day and there may be little or no shade available to the hatchlings; exposure to the intense heat of day could easily be fatal to them. There exists therefore a direct pressure for the development of thermal inhibition of activity. Emerging under cover of darkness also provides better protection from terrestrial and airborn predators. The longer the time to reach the sea the more chance predators have of making a catch. Daytime temperatures slow the hatchlings down, if they can not reach the sea; either they are caught by predators or killed under the sun (Hendrickson, 1958; Dimond, 1965; Bustard, 1967; Mrosovsky, 1968).

1.7. Hatching success:

Hatching success is influenced by several abiotic factors including temperature (Mrosovsky *et al.*, 1984), oxygen levels (Ackerman, 1980), chloride levels (Bustard and Greenham, 1968) and moisture content (McGehee, 1990; Mortimer, 1990) in the nest. Hatching success (the ratio of hatchlings successfully emerging to the clutch size) is apparently influenced by local sand conditions and external pressures of vehicles in Torres Strait, Australia (Parmenter, 1980). He stated that hatchling mortality within the nest was greater on soft beaches with coarse-grained sand than on firm, fine-grained beaches. The success of the clutch of eggs depends upon the interactions of a number of factors, such as salinity, humidity, temperature, gas flow, rainfall, tidal inundation, erosion and predation (Hendrickson, 1958; Ragotzkie, 1959; Bustard and Greenham, 1968; Prange and Ackerman, 1974; Ackerman, 1977, 1980, 1981a, b; Fowler, 1979; Kraemer and Bell, 1980; Mrosovsky, 1980; Parmenter, 1980; McGehee, 1990). In conservation efforts, improper handling of eggs during movement to hatcheries may increase mortality (Limpus *et al.*, 1979; Parmenter, 1980; Blanck and Sawyer, 1981).

High species diversity of bacteria inside eggs or the occurrence of the same bacteria in both females and their eggs was correlated with lower hatching success (Wyneken *et al.*, 1988).

1.8. Heavy metals in tissues of sea turtle eggs:

The heavy metal concentration of loggerhead eggshells differs significantly between Florida and Georgia/ South Carolina rookeries (Stoneburner *et al.*, 1980), a distinction that is further supported by differences in the epibiota and heavy metals that accumulate during non-nesting intervals, since these environmental markers indicate some level of segregation on feeding grounds or migratory routes.

The presence of significantly different mean heavy metal burdens in the eggs collected from these nesting beaches would indicate the existance of groups of female loggerhead turtles and support the hypothesis for the existence of distinct groups of female turtles present in the Mediterranean.

Sea turtles and their eggs have been analysed for traces of heavy metals (Hillestad *et al.*, 1974; Stoneburner *et al.*, 1980; Witkowski and Frazier, 1982; Davenport and Wrench, 1990; Rybitski *et al.*, 1995; Bishop *et al.*, 1995; Sakai *et al.*, 1995).

1.9. Aims of the Thesis;

1. To determine the natural temperature regimes of the nests of *Caretta caretta* and *Chelonia mydas* on the beaches in the Mediterranean.

2. To determine the effects of ecological conditions(i.e., temperature, moisture level in the sand and grain size) on the development of the embryos, incubation period, hatching success and nest site selection of females. The most common cause of mortality in developing eggs and hatchlings is nest predation, therefore to study the efficiency of a caging programme in protecting nests. Combining all the information obtained above helps to develop a programme of nest relocation. It should always be remembered that the ultimate reason for examining the factors affecting the development of *Caretta caretta* and *Chelonia mydas* is the hope that through understanding the cause of mortality we may be able to reduce them, especially under artificial conditions.

3. To obtain material from nests destroyed by predators to compare developmental stages of Mediterranean sea turtles with the other published works and also to study embryological aspects of failure to hatch in turtle eggs.

4. To obtain tissue samples from dead hatchlings and sacrificed hatchlings to determine the genetical diversity of the population level by comparing mt-DNA sequences.

5. To document the heavy metal burden in the eggs of loggerhead sea turtles nesting along the Southwest coast of Turkey, with regard particularly to the possible use of heavy metal profiles as indication of population distinctness.

Chapter 2. Materials and Methods:

In this Chapter, general methods used during the field work will be described. Specific methods used for each chapter will be described in each chapter.

2.1. Study sites:

This work was carried out over three summer seasons. In the summer of 1994, some embryological material was collected from the beaches of Northern Cyprus and Turkey. In the summer of 1995, detailed work was carried out at the west coast of Northern Cyprus and some embryo samples were also collected from other beaches. In the summer of 1996, detailed work was carried out at the southwest beaches of Turkey and some embryo samples and temperature data collected also from Cyprus. All these beaches are shown in Figure 2.1.

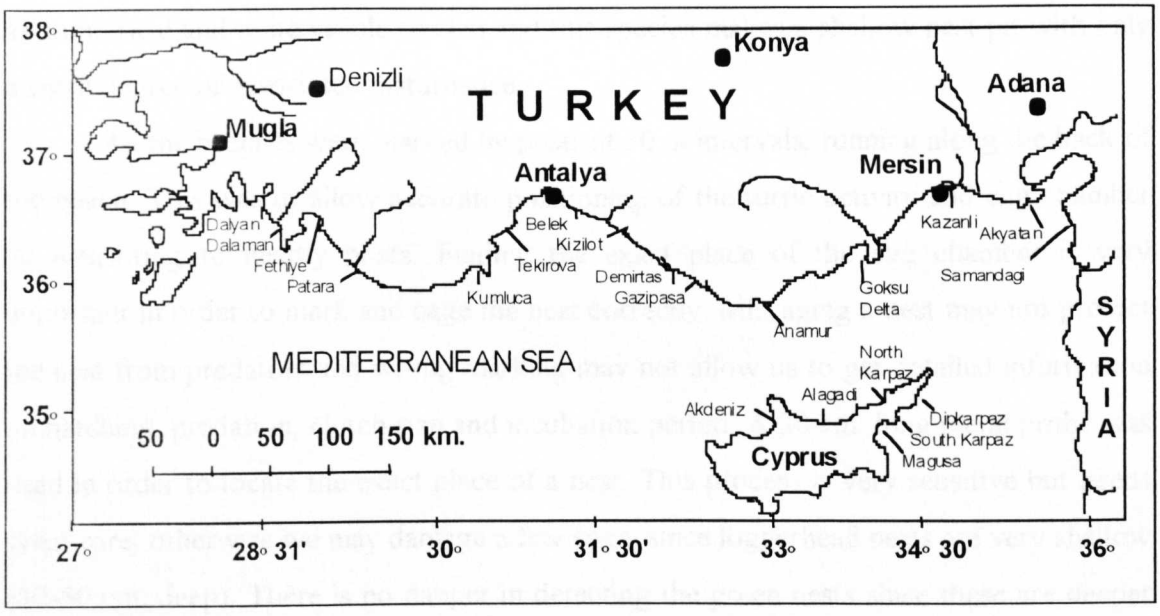


Figure 2.1. The study beaches in the Eastern Mediterranean.

2.2. Beach patrols and recording nesting activities:

The beaches were patrolled during daylight hours at the west coast of Northern Cyprus and all the turtle activities on the beaches were recorded. In order to place the temperature recorders during oviposition, beaches were also patrolled during the nights of laying. Each activity was assessed and classified into the following categories:

a) Nest: The nest was recorded when a crawl track was visible leading to an area of disturbed sand where digging and covering had occurred.

b) False Crawl: This was either recorded when some digging in the sand, if only slight, occurred but no covering up was apparent (i.e., an attempt to dig a body pit and/or egg chamber by the female) or recorded when a turtle made no nesting or digging attempts, simply a crawl on the beach and back to the sea.

It is very apparent if an adult turtle has been on a beach from the sand disturbance and tracks left. Species identification was possible using the criteria of track and nest pit morphology (Groombridge, 1990). *Chelonia mydas* makes a symmetrical crawl track (the fore-flippers' tracks are quite visible and the back-flippers' tracks are almost connected to fore-flippers' track) and nesting attempts are usually associated with a large deep pit and large amount of sand disturbance. With successful nesting, a great deal of covering up activity is evident. *Caretta caretta* makes a markedly asymmetrical crawl track (the fore-flippers' tracks are almost invisible or small tracks and the backflippers' tracks are asymmetrical and quite visible tracks) and this species makes a shallow nest pit with only a small degree of associated disturbance.

All the beaches were marked by posts at 50 m intervals, running along the back of the beach. This was to allow accurate positioning of the turtle activity and egg chamber by measuring to nearby posts. Finding the exact place of the egg chamber is very important in order to mark and cage the nest correctly. Miscaging a nest may not protect the nest from predators and wrong marking may not allow us to get detailed information on hatching, predation, clutch size and incubation period. A 30 cm. long metal probe was used in order to locate the exact place of a nest. This process is very sensitive but needs great care, otherwise we may damage a few eggs, since loggerhead nests are very shallow (30-50 cm. deep). There is no danger in detecting the green nests since these are deeper but we have to distinguish the nests of both species. Simply by sticking this probe into the sand we can find the location of the egg chamber, since the sand where it has been dug is softer than in the surrounding area. Once a person gets experience of using this method, it is safe, and an experienced person can often find the egg chamber first time. After finding the egg chamber, caging and marking the nest is very easy. Location of any activity was measured to the nearest marker posts and strand line. A bamboo cane, with date and which species' nest written on it, was put behind the nest and another near the vegetation in line with the nest. Tracks were then raked over to avoid double counting.

2.3. Predation, relocation and caging:

The number and distribution of nests and predation on the beaches at the west coast of Northern Cyprus was determined by patrolling the beach for turtle crawl tracks and

evidence of nesting activity and predation in order to investigate factors which might affect this pattern and to determine when the majority of predators occurs. The relationship between number of nests and amount of predation, ratio of predators to nests on different beaches, temporal distribution of predation, relationship between predation and nest age and predation pattern differences between turtle species were investigated. Beaches on the west coast were divided into ~250 meters zones and a number was given to each zone (Figure 2.2.)

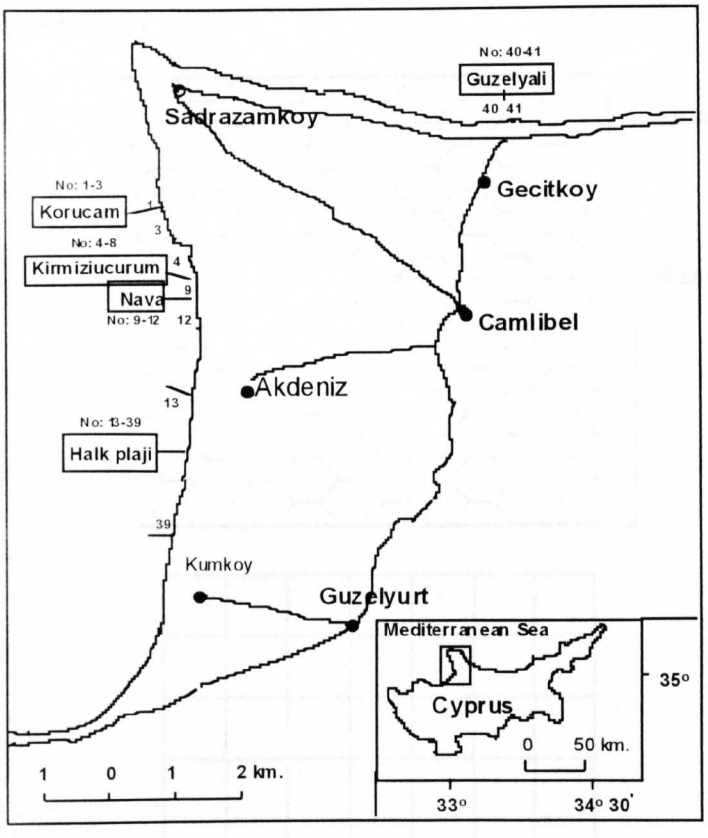


Figure 2.2. The beaches and zone numbers at the west coast of Northern Cyprus.

Recently predated nests often had tracks leading to the nest, allowing identification of the predator as dog or fox. Fox prints are smaller than dogs with a different arrangement of paw pads. Dogs have larger pads that are closer together than a fox's (Bang and Dahlstom, 1990). The tracks of any other potential predators (usually ghost crabs, scavenging birds and hedgehogs) were noted and identified where possible.

After any predation, the sand column surrounding the nest was checked for eggs left by the predator. Any damaged or unviable eggs were taken for determination of the stage of development. Any eggs which were considered viable were sometimes relocated and sometimes covered again and then caged.

Some of the predated nests were covered well again but not caged. This was done mainly to test ideas on predator behaviour. After a few nests had been predated again on consecutive nights, we started caging every predated nest. To test the same idea, intact eggs were translocated to another place and caged. All of the nests from which temperature meaurements were taken were also caged. As mentioned above some of the nests after predation and some of them after laying early in the morning were relocated into a safer place and then caged. One of two nests laid in the zone where 7 meters to sea was also relocated and caged.

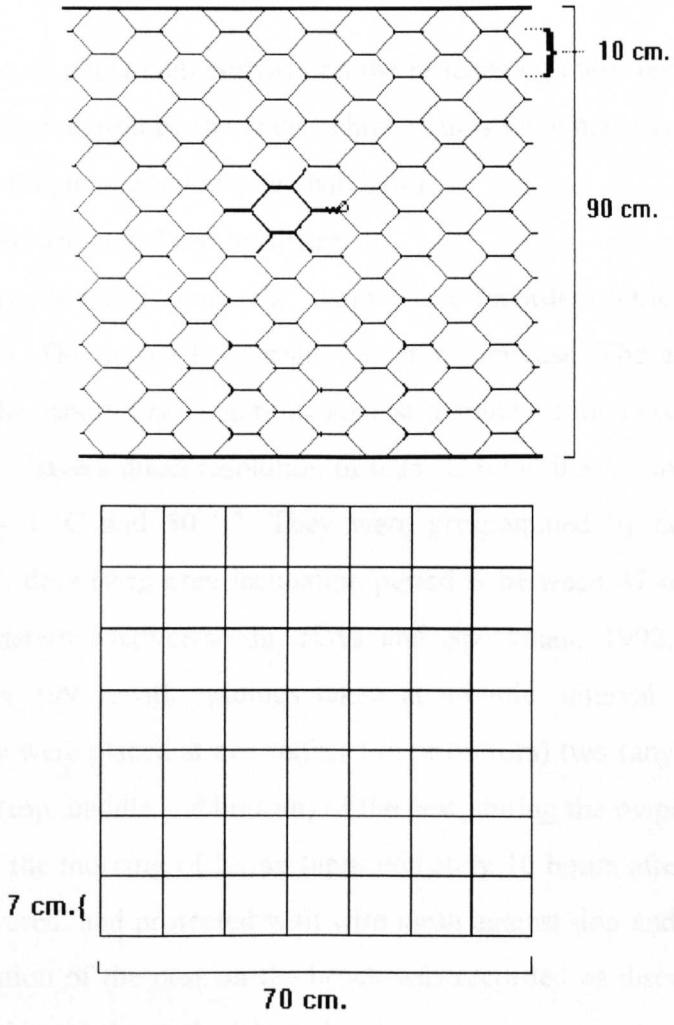


Fig. 2.3. Two types of cage used against fox and dog predation.

(Top figure shows a cage made of wire mesh and the lower one a cage made from rigid metal wires)

The cage was placed at depths of 20-40 cm (depending on the depth of the egg chamber and the species) below the sand surface so that the hole in the middle of the cage was located on top of the egg chamber. This is important because if the cage was misplaced, then predators could reach the egg chamber from the side of the cage. Each

cage was marked and held in position by 4 stakes, one at each corner. Two types of cages were used against predation (Figure 2.3.). One was made of wire mesh (with 10 cm mesh size) and another was specially made as a metal grid from rigid metal probes, with a 7 cm grid size.

These caged nests were checked for signs of attempted predation and for evidence of emerged hatchlings during the surveys and cages were removed after the nests had completely hatched. Some of the nests were caged at different times after incubation began, and before hatching, in order to test the relationship between predation and nest age.

The distribution of ghost crab burrows on the beaches was also recorded in order to understand hatchling predation by the crabs. Three counts were made around the same nest during incubation (beginning, middle and hatching).

2.4. Recording the nest and sand temperature:

Temperature was measured using "Tiny talk" temperature recorders (Orion Components (Chichester) Ltd., UK). The device fits within a 35 mm film case. The accuracy of the device was tested under laboratory conditions against a standard mercury thermometer, and they were found to have a mean resolution of 0.35 °C (min. 0.3 °C, max. 0.4 °C) for temperatures between 4 °C and 50 °C. They were programmed by computer for a recording period of 60 days (Reported incubation period is between 47-60 days for sea turtle nests in the Eastern Mediterranean; Hays and Speakman, 1992; Kaska 1993; Broderick and Godley 1996) with readings taken at 48 min. interval. This gave 30 readings per day. They were placed at one (either top or bottom) two (any two levels) or three different depths (top, middle and bottom) of the nest, during the oviposition or after excavating the nest in the morning of laying (approximately 10 hours after oviposition). The nest was then covered, and protected with wire mesh against dog and fox predation (Figure 2.4). The position of the nest on the beach was recorded as distance (m.) from vegetation and sea, and in relation to beach marker posts.

A few days before anticipated date of hatching these temperature recorders were taken from the nest and the information offloaded to a computer. 5-6 eggs were taken (CITES permit nos 81772, 81773, 94207, 94208) from each level together with the Tiny talks. These eggs were incubated in moist sand for a few days until they hatched. Hatching times of these eggs were also recorded.

Temperature measurements were also taken on different parts of the beach which have a different sand type by means of temperature probes and tiny-talks in order to

understand the temperature profile of the beach. Initially sand temperatures were measured every two meters from the sea and from these measurements it was found that sand temperatures changed about every 7 meters from the sea. Therefore, later in tthe season, the sand temperature measurements were only taken at 7 meter inttervals from the sea.

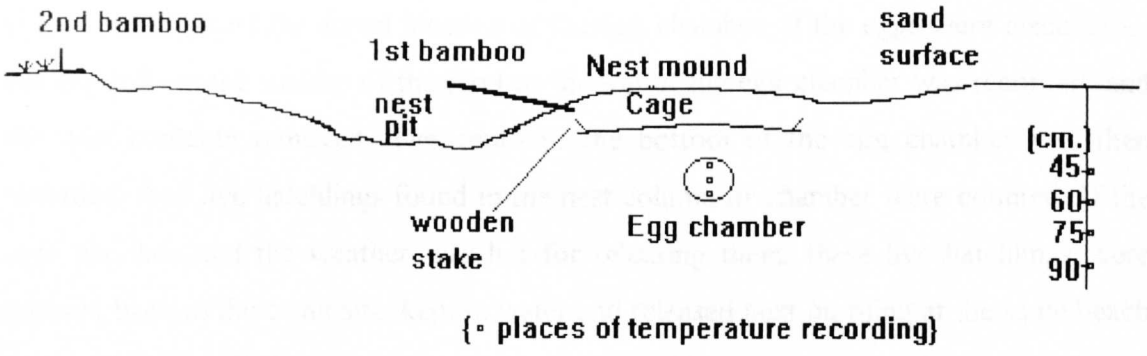


Figure 2.4. Profile of the nest area showing the placement of a cage over the nest, nest markings and depths where the temperature was recorded.(Two bamboo canes were placed, with the information written on them, one is just behind the nest and another near the vegetation. Cage is also fixed with 4 wooden stakes, one at each corner).

Later, sand temperatures on the beaches were investigated using tiny talks buried for 1-2 days or 1-2 weeks at turtle nest depths along the entire beach at 7, 14, 21, 28 meters distances from the sea in order to determine inter- and intra-beach thermal variation. The temperature of the sand was also measured at 2-3 different levels after excavating the nest, by means of temperature probes, and the time recorded. This will help to compare the temperature of that nest with other nests of which the temperatures were continuously recorded with the tiny-talks. Sand temperatures were also recorded just above the clutch (30, 20, 10 cm.) during the hatching period in order to understand emergence pattern of the hatchlings.

2.5. Hatching and stages of development:

If a nest was recorded as hatched, but only a few hatchling tracks were apparent, the uncaged nests' chamber was marked with a small bamboo cane and the nest was left for a few days to allow the majority of hatchlings to emerge in a natural way (some of the nests were caged during that period) and the nest was then excavated by hand roughly one

week after the first emergence. (This is a way of testing the development differences within the nest and between nests.). Care was needed during excavation, as a few live hatchlings could be found in the nest column. This final batch of hatchlings was also measured to compare with the first batch and other hatchlings. It was sometimes difficult to find the egg chamber, even when we saw the hatchling tracks. Thus nests were sometimes noted as having hatched, although excavation was not possible. Predation of nests also disguised the actual location of the egg chamber. If the eggs were discovered, the depth from the surface of the sand to the top of the egg chamber was recorded, and the nest contents removed. The depth to the bottom of the egg chamber was then recorded. Any live hatchlings found in the nest column or chamber were counted. If the time was late and the weather was hot for releasing them, these live hatchlings were brought back to the camp site, kept in water and released next morning at the same beach they were found. If the time was not late, these hatchlings were released immediately and allowed to crawl from the nest site to the sea unaided, whilst being carefully monitored, as predation from ghost crabs and birds was a threat. There was also a risk of overturning whilst manoeuvring around obstructions on the beach such as litter and wheel tracks.

A count of the eggs removed from the nest was made recording the number of hatched and unhatched eggs. Unhatched eggs were taken back home for detecting the level of development, opened in saline and the early stage embryos and samples for sex determination fixed in Bouin's solution, late stages and hatchlings in 10% formalin.

All the unhatched eggs or sampled eggs were classified into the following categories;

a) nonviable; these were non-yolked and often much smaller.

b) Yolked-unfertilised; these were yolked but there was no sign of development (There is no white spot on the eggshell or no reddish point on the yolk, since these are the early indications of development).

c) Spoilt eggs; These eggs were all in bad condition, eggshell may be black or different colour spots may be on it and the yolk was also dehydrated and different in colour (black or brownish) and occasionally had a bad smell.

d) Dead in shell embryos; Those have a visible embryo or have a white spot on the eggshell and/or reddish circle on the yolk and/or brownish spot on the inner side of the eggshell. These embryos were staged and some measurements were taken either at the camp site or back in Glasgow. The detailed methods will be explained in Chapter 6.

A few egg samples during oviposition and after predation were also taken in order the describe early developmental stages. These eggs were opened and the components of

eggs were weighed. In some of these occasions, the vitelline membrane ruptured and often tore through the embryonic area. After a few attempts, these eggs were preserved in formalin at least two days before being opened. This solidified the albumen and increased the viscosity of the yolk.

Before being opened, all data related to the egg were transcribed to a tag. Preserved and dead in shell eggs were opened in saline in a large bowl, the shell was cut into two nearly equal halves with scissors and the contents placed into the petrie dish. Large embryos were easily located and removed for storage in 10 % formalin. For small embryos, a dissection microscope was used when it was necessary and the embryos were preserved in Bouin's solution.

Those which had died post hatching, in the sand column or on the beach, were also recorded. A sand sample from the bottom of the egg chamber was also taken. All nest materials were removed from the beach to avoid confusion or an increase in predation and reburied under a heavy stone which we could find easily behind the vegetation zone.

2.6. Sand quality:

The volume of water in the sand and the porosity of the sand were also measured from the sand samples taken from the nests. For measuring the volume of water in the sand; sample was first weighed and then air dried and then weighed to a constant weight after 7-10 days. The percentage of water in the sand was then expressed as a volume of the original sample by dividing by the volume of the sand dried. A sub sample of the sand was also taken and dried to standardise the measurements and then poured into a cylinder. The sand volume in the cylinder was recorded as V1, and then we added the same amount of water (V2). We waited until there were no air bubbles rising from the sand and the total volume was recorded as V3. The porosity of the sample was calculated as

$$\frac{(V_1 + V_2 - V_3)}{V_1} \times 100$$

Sand samples were also taken from some of the nests back to Glasgow for sand composition and grain size analyses, to investigate whether there is a relationship between these parameters and hatching parameters.

2.6. Collection of samples for genetic analysis:

Heart samples from newly dead hatchlings and from the hatchlings sacrified for sex determination were collected and fixed in absolute alcohol for mt-DNA analysis. Dissection equipment was sterilised in 1% HCl between the samples.

2.7. Collection of tissue samples for heavy metal analyses:

Samples (eggshells, yolk and liver) were collected from four Turkish beaches (Dalyan, Fethiye, Patara and Kizilot). Just before hatching, some eggs were removed for sex determination, and the eggshells, yolks and livers of these embryos were used for metal analyses. About one week after the last hatching track was seen, nests were excavated, and samples were collected from dead-in-shell embryos. Heavy metal concentrations were measured in the above tissues to determine inter-clutch and intra-clutch variations, and also variation between organs of individuals.

2.8. Data analyses: All the temperature data were offloaded to a lap-top computer and the data analysed by using Minitab package program and graphics were plotted by Cricket Graph.

Chapter 3. Temperatures of nests and sex ratio:

This chapter gives the data on temperatures and sex ratio of loggerhead and green turtle nests in the Eastern Mediterranean.

3.1. Introduction:

Laboratory and field experiments have shown that sex in many turtle species is determined by egg incubation temperature, usually during the middle third of development (Yntema, 1979; Yntema and Mrosovsky, 1980; Janzen and Paukstis, 1991; Mrosovsky, 1994). Few studies have monitored incubation temperatures in the field, but experiments using artificial nests, or incubators with cyclic temperature fluctuations, suggest that sex is determined as though eggs were incubated constantly at the mean temperature. When eggs are incubated at constant temperatures, there is a narrow range of temperatures over which around 50% of each sex will be produced (pivotal temperature or threshold temperature), and wider ranges above this temperatures produce females and below this threshold produce males (Bull, 1980). Pivotal temperatures for all sea turtle species are reported to lie within a 1 °C range (28.6-29.7 °C), and the variety of relationship between pivotal and beach temperatures suggests that diversity of sex ratios in different populations should be expected (Mrosovsky, 1994). Pivotal temperatures for sea turtles (Mrosovsky, 1994) seem to vary slightly due to methods of measuring temperature, constancy of incubators or estimation made from field studies.

For sea turtles, population survival is dependent on the occurrence of a sufficient range of incubation temperatures to produce offspring of both sexes. If the temperature of a nest during the middle third of development is known, then the sex ratio of hatchlings from that nest can be predicted. If in turn this information is known for all parts of a beach throughout a nesting season, then the overall primary sex ratio can be predicted for all hatchlings produced from that beach (Standora and Spotila, 1985). Estimates of the sex ratio have also been obtained by combining the nesting distribution with the sexing of samples of hatchlings from different times during the season and termed Seasonal Sex Production Profiles (SSPPs) by Mrosovsky (1994). Estimates of sex ratios have also been obtained from pivotal incubation durations (Marcovaldi *et al.*, 1997).

Most of these data on temperature of nests come from either a correlation of sand temperatures and air temperatures with few nest temperatures taken or adapting the short term temperature records from the nests to the whole incubation period. In

previous studies methods of measuring temperature, constancy of incubators, and number of thermometers tested (i.e., resolution) varied among studies. Most studies used digital thermometers, copper-constant thermocouple thermometers, thelethermometers, or thermistor probes calibrated against a mercury thermometer, and data loggers (Bull, 1985; Mrosovsky and Provancha, 1989; Hays *et al.*, 1995; Mrosovsky *et al.*, 1995; Godfrey *et al.*, 1997; Marcovaldi *et al.*, 1997; Kaska *et al.*, 1998). From these studies, the temperature difference between the different zones on the beach and effects of these differences on the sex ratio can be found, but there are no data showing the temperature of the whole incubation period and the temperature differences within the clutch. In this Chapter, intra-clutch temperature differences of two species of turtles nesting in the Mediterranean, and the sex ratio of these nests by sexing a sample of hatchlings from each level where temperatures were recorded, and the pattern of emergence by loggerhead and green turtle hatchlings by examining the thermal environment of the clutch and sand adjacent to nests to identify any temperature differences within the clutch and to relate these to emergence times of the hatchlings and thermal cues used by the emerging hatchlings, is explained.

3.2. Materials and Methods

Methods used during the field work were explained in Chapter 2. Temperature data were offloaded to a computer and gonads of the sacrified hatchlings dissected and preserved in Bouin's solution for sex determination. The gonads were cut in half transversely and one half was embedded in paraffin wax, sectioned at 8-10 μm from the middle of the gonad, and stained with the Periodic Acid Schiff reaction (PAS) and Harris' hematoxylin. Sex designation was based on development of cortical and medullary regions and presence or absence of seminiferous tubules (Yntema and Mrosovsky 1980). The middle third of the incubation period was calculated from the total incubation period, from the night of laying to the day of first hatching. Data related to the temperatures of air (min., max.,) and sea water were obtained from Statistical Yearbook of Turkey (1997). These temperatures were taken 3 times (0700, 1400, and 2100 hours, local time) a day and the means of these data were taken as a monthly data. Sand temperatures at the 60 cm. depth from the middle (open) part of the beach (approximately 21 meters from sea) were also taken at different times during the nesting season via tiny talks or digital thermometers (Chapter 2). The monthly temperature readings at the beaches from May to October were pooled and averaged by months. The mean of the temperatures taken

during 4 -15 different days each month were taken as monthly sand temperatures to compare with air and sea water temperatures.

3.3. Results

3.3.1. Temperatures of nests and sex of hatchlings:

Temperatures of green turtle nests and loggerhead nests were recorded on six Eastern Mediterranean beaches (Figure 2.1) during the nesting season of 1995 and 1996. The information on these nests is shown in Table 3.1. The nesting season started at almost the end of May and continued until mid August in both years on Turkey and Cyprus. The majority of the nests were recorded during June and July, since the peak nesting season was during that time. The distances of nest from sea varied from 10 meters to 30 meters. The depths of top and bottom level of any green turtle nest was around 70-90 cm., and 30-50 cm. for loggerhead turtle nests. The clutch size varied from 65 to 95 for *C. caretta* and 78 to118 for *C. mydas*. The incubation period for green turtle nests was slightly longer than loggerhead turtle nests. Two of the loggerhead nests did not hatch. One of these was inundated twice during the middle third of incubation period and the other was under a vehicle track. The temperature data from these unhatched nests were excluded from the general data below. The hatching success of other nests varied between 48% and 94%.

The mean temperature of the whole incubation period for 8 loggerhead turtle nests ranged from 28.1 to 31.7 °C. Maximum temperature increase during the incubation period for loggerhead turtle nests was 9.6 °C (min. 24.5 °C , max. 34.1 °C). Mean temperature differences between the top and bottom of the loggerhead turtle nests was 0.9 °C (min. 0.3 °C , max. 1.4 °C). Temperature of the middle third of the incubation ranged from 27.4 to 32.5 °C . All the nests during the middle third of the incubation period experienced above the pivotal temperature, except for one nest (no:5). Mean temperature differences during the middle third of the incubation period between the top and bottom of loggerhead turtle nests ranged from 0.3 °C to 1.3 °C . The top level of a loggerhead turtle nest was warmer (min 0.3 °C , max. 1.4 °C) than the bottom level and the same or warmer (max. 0.7 °C) than the middle level of the nest. The top of the loggerhead nest was warmest, and the bottom was coolest, except for a few days after inundation caused by high tides (Figure 3.1A). The temperature at the center of nest was intermediate early in the nesting season. It rose to the same as the top of the nest later in the season. Marked diel cycles in loggerhead nests were detected with a range of up to 1.5 °C during the incubation period.

Table 3.1. Information on the recorded nests.

Nest no	Beach	Oviposition Date	Distance (m.) from Vegetation and Sea	Top and Bottom depths of the nest (cm.)	Clutch size	Incubation period (days)	Hatchling emergence success (%)
C.mydas 1	Akdeniz	8.6.1995	25-10	70-85	118	63	59
C.mydas 2	Akdeniz	21.6.1995	10-18	69-83	78	60	73
C.mydas 3	Akdeniz	24.6.1995	24-16	75-85	109	54	94
C.mydas 4	Akdeniz	10.7.1995	15-15	70-90	96	59	75
C.mydas 5	Karpaz	25.7.1996	10-15	75-90	87	55	96
C.caretta 1	Akdeniz	4.6.1995	10-10	30-50	83	-	0.0
C.caretta 2	Akdeniz	10.6.1995	26-16	35-47	95	54	48
C.caretta 3	Akdeniz	11.6.1997	18-16	30-50	73	53	75
C.caretta 4	Akdeniz	27.7.1995	11-18	30-50	77	50	68
C.caretta 5	Kizilot	27.6.1996	15-20	30-48	90	51	89
C.caretta 6	Kizilot	27.6.1996	5-25	30-54	65	50	68
C.caretta 7	Fethiye	2.7.1996	10-10	30-50	78	61	88
C.caretta 8	Fethiye	7.7.1996	11-15	30-48	75	-	0.0
C.caretta 9	Fethiye	7.7.1996	10-13	30-55	93	55	73
C.caretta 10	Patara	21.7.1996	25-30	30-55	62	52	79
C.caretta 11	Fethiye	22.7.1996	10-12	30-46	77	54	88
C.caretta 12	Dalyan	23.7.1997	30-25	30-48	72	50	83
C.caretta 13	Dalyan	23.7.1997	30-20	30-50	71	48	87
C.caretta 14	Dalyan	23.7.1997	30-15	30-50	75	52	88
C.caretta 15	Karpaz	24.7.1996	20-35	30-50	88	54	87
C.caretta 16	Dalyan	25.7.1997	30-30	30-45	70	54	90
C.caretta 17	Dalyan	2.8.1996	25-35	30-50	85	53	85
C.caretta 18	Dalyan	2.8.1996	20-27	30-55	77	52	81

Following an inundation caused by a high tide, the nest experienced a 2-4 °C drop in nest temperatures for a few days. If this inundation occurs early in the incubation period, development of the embryos in that nest were suspended. Details of this mortality will be explained in Chapter 5.

Between 1320 - 1710 readings were taken per nest. The daily mean (mean of 30 readings) temperatures of the nests were not constant, increasing during the incubation period for both species (Figure 3.1A, B).

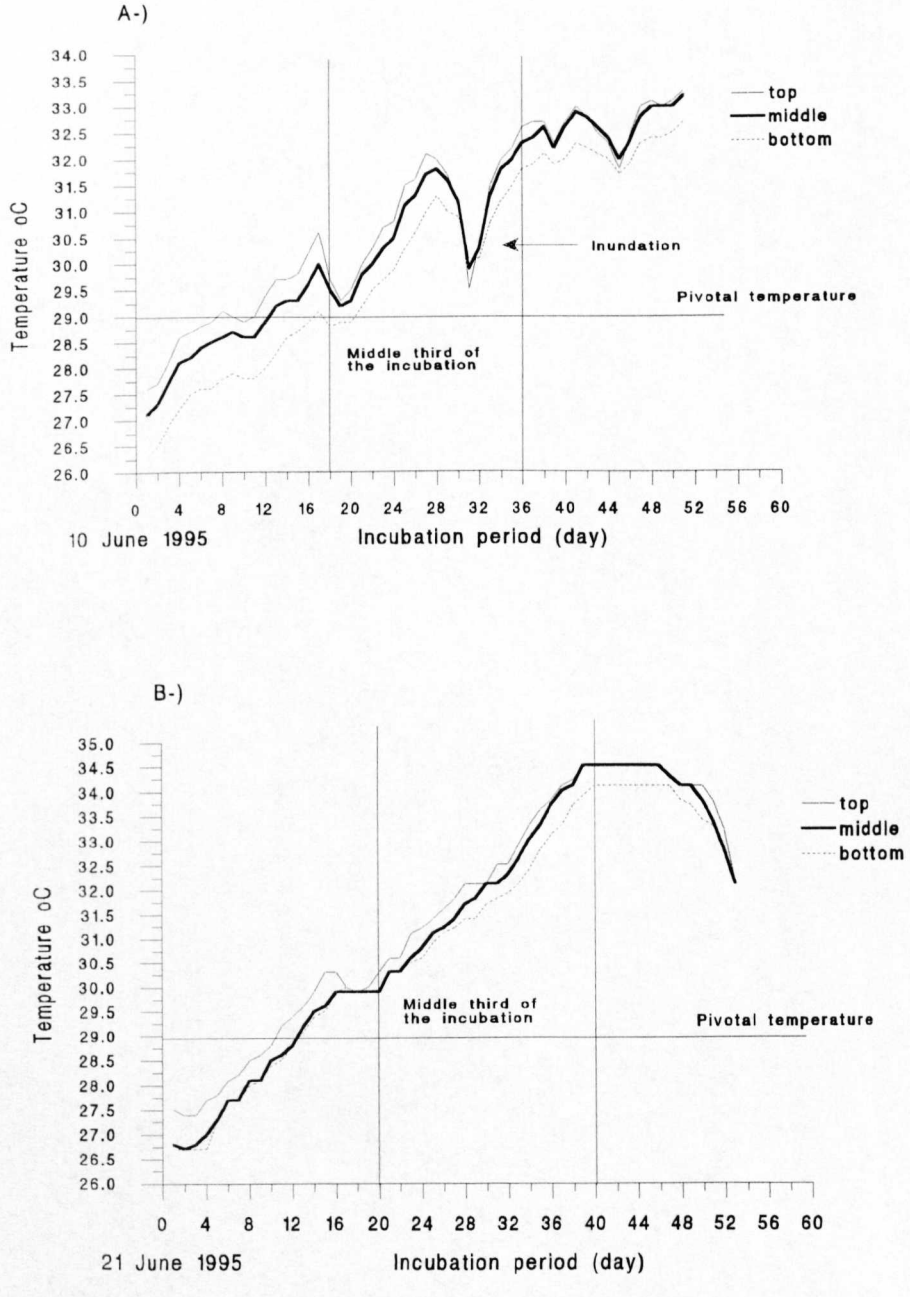


Figure 3.1. The incubation temperatures of sea turtle nests in the Mediterranean.

A: Loggerhead nest, B: Green turtle nest.

The mean temperature of the whole incubation period for 5 green turtle nests ranged from 29.5 to 31.3 °C. Maximum temperature increase during the incubation period for green turtle nests was 9.6 °C (min. 24.9 °C , max. 34.5 °C). Mean temperature differences between the top and bottom of the green turtle nests was 0.6 °C (min. 0.4 °C , max. 0.8 °C). Temperature of the middle third of the incubation ranged from 29.8 to 32.1 °C . All the nests during the middle third of the incubation period experienced above the pivotal temperature. Mean temperature differences during the middle third of the incubation period between the top and bottom of green turtle nests ranged from 0.4 °C to 0.8 °C . The top level of a green turtle nest was warmer (min 0.4 °C , max. 0.8 °C) than the bottom level and also warmer (min., 0.1 °C , max. 0.3 °C) than the middle level of the nest. For the first third of the incubation (about 18 days) the top of green turtle nest was the warmest, and the bottom was the coolest with the middle of the nest temperature being intermediate. During the next 18 days the temperature of the middle increased presumably due to metabolic heat and became the same as the temperature of the top of nest, and during the last third of the incubation period sometimes became warmer (Figure 3.1B). There were no diel cycles in green turtle nest temperatures.

Table 3.2 summarises the data on temperature and sex results of the green and loggerhead turtle hatchlings. The sex ratio of hatchlings for all nests was also calculated. 10-18 hatchlings per nest were sexed, and the results showed a female biased sex ratio (Table 3.2) for both species, except for one loggerhead nest which had experienced lower than the pivotal temperature during the middle third of the incubation period.

There is a positive correlation between the mean temperature of the middle third of the incubation period (r^2= 0.96) and sex ratio (percent female), but inverse relation between the mean temperature of a nest and incubation period (Figure 3.2). The mean incubation temperatures can be use for estimating the incubation period. In general, we can say that a 1 °C decrease in the mean incubation temperature means 4 days increase in incubation period (Table 3.1 and 3.2). Since the top of nests was warmer than the bottom during the middle third of incubation, we can expect the percentage of females to be higher among eggs at the top of the nest. This was the case. The percentage of females in eggs sampled from the top of nests was higher than in samples from the bottom of the same nest in 20 of 23 nests, was 100% at all levels in two and was 60% in all levels in one. For both species the overall difference in numbers of males and females between top and bottom of nests was statistically significant (green turtle nests $x^2_{1d.f.}$=6.86, loggerhead turtle nests $x^2_{1d.f.}$= 9.82 $p<0.01$ in both).

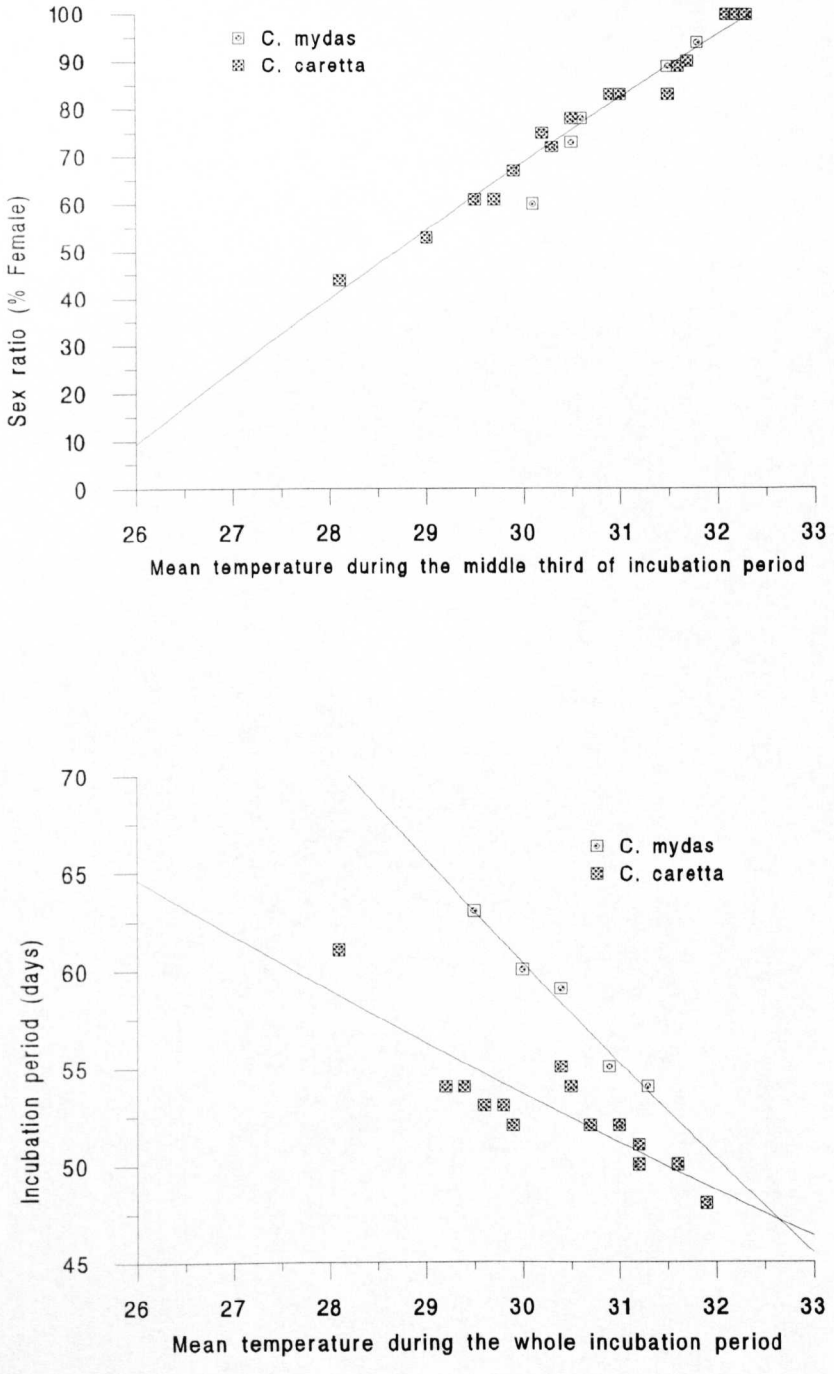


Figure 3.2. The relationships between the mean incubation temperature during the middle third of incubation period and sex ratio (top figure; pooled species since there was no difference in the relationship between species) and mean incubation temperature and incubation period for each species (bottom figure).

Table 3.2. The results of temperature and sex ratio of green and loggerhead turtle nests.

	TOP (T)		MIDDLE (M)		BOTTOM (B)		MEAN OF		Sex
Nest no	Mean temp.±SE	Middle third±SE	Mean temp.±SE	Middle third±SE	Mean temp.±SE	Middle third±SE	Mean temp.	Middle third	(%Female) Mean (T-M-B)
C.mydas 1	29.8±0.05	30.2±0.03	29.9±0.05	30.2±0.03	29.4±0.05	29.8±0.02	29.5	30.1	60(60-60-60)
C.mydas 2	30.3±0.03	30.9±0.03	30.0±0.04	30.6±0.03	29.5±0.04	30.1±0.02	30.0	30.5	73(100-82-40)
C.mydas 3	31.5±0.05	32.1±0.04	31.3±0.06	31.8±0.05	31.0±0.06	31.5±0.04	31.3	31.8	94(100-100-80)
C.mydas 4	30.7±0.03	31.0±0.03	30.4±0.04	30.5±0.03	30.0±0.04	30.2±0.02	30.4	30.6	78(100-83-50)
C.mydas 5	31.2±0.05	31.9±0.05	30.9±0.05	31.6±0.05	30.6±0.05	31.1±0.04	30.9	31.5	89(100-83-83)
C.caretta 1	29.4±0.03	29.8±0.07	28.6±0.03	29.1±0.05	28.2±0.03	28.8±0.03	28.7	29.2	---
C.caretta 2	30.9±0.04	31.3±0.05	30.6±0.04	31.1±0.04	30.0±0.04	30.6±0.04	30.5	31.0	83(100-83-67)
C.caretta 3	29.6±0.03	29.1±0.04	29.7±0.03	29.2±0.03	29.4±0.02	28.8±0.04	29.6	29.0	53(60-60-40)
C.caretta 4	31.7±0.03	32.2±0.03	31.7±0.02	32.2±0.01	31.4±0.02	32.0±0.01	31.6	32.1	100(100-100-100)
C.caretta 5	31.6±0.03	32.1±0.03	31.1±0.03	31.5±0.02	30.8±0.03	31.2±0.02	31.2	31.6	89(100-83-83)
C.caretta 6	32.1±0.03	32.5±0.02	31.8±0.03	32.1±0.02	31.1±0.04	32.1±0.01	31.2	32.2	100(100-100-100)
C.caretta 7	28.8±0.01	28.7±0.02	28.1±0.01	28.1±0.01	27.4±0.02	27.4±0.01	28.1	28.1	44(50-50-33)
C. caretta 8	27.7±0.01	27.6±0.01	27.3±0.01	27.2±0.01	27.0±0.01	27.0±0.01	27.3	27.3	---
C.caretta 9	30.9±0.02	30.2±0.01	30.4±0.02	30.2±0.01	29.8±0.02	30.1±0.01	30.4	30.2	75(83-80-60)
C.caretta 10	30.5±0.01	30.8±0.01	29.9±0.01	30.2±0.01	29.3±0.01	29.8±0.02	29.9	30.3	72(83-83-50)
C.caretta 11	*	*	*	*	29.2±0.01	29.5±0.01	29.2	29.5	61(67-67-50)
C.caretta 12	31.2±0.09	31.5±0.01	*	*	*	*	31.2	31.5	83(100-83-67)
C.caretta 13	31.9±0.01	32.3±0.01	*	*	*	*	31.9	32.3	100(100-100-100)
C.caretta 14	30.9±0.02	31.3±0.01	*	*	30.5±0.03	30.6±0.08	30.7	30.9	83(100-83-67)
C.caretta 15	*	*	29.6±0.01	29.9±0.01	29.2±0.01	29.5±0.01	29.4	29.7	61(67-67-50)
C.caretta 16	*	*	30.7±0.01	30.7±0.01	30.3±0.01	30.3±0.00	30.5	30.5	78(83-83-67)
C.caretta 17	30.0±0.01	30.1±0.01	*	*	29.6±0.00	29.7±0.00	29.8	29.9	67(83-67-50)
C.caretta 18	31.6±0.02	32.1±0.03	31.0±0.02	32.0±0.01	29.5±0.03	31.1±0.02	31.0	31.7	90(100-83-67)

3.3.2. Temperatures of sand:

Decreasing temperature with increasing depth (Figure 3.3A) was observed and there were also remarkable differences in temperature of the same depth on different beaches and different distances from the sea on the same beach (Figure 3.3B).

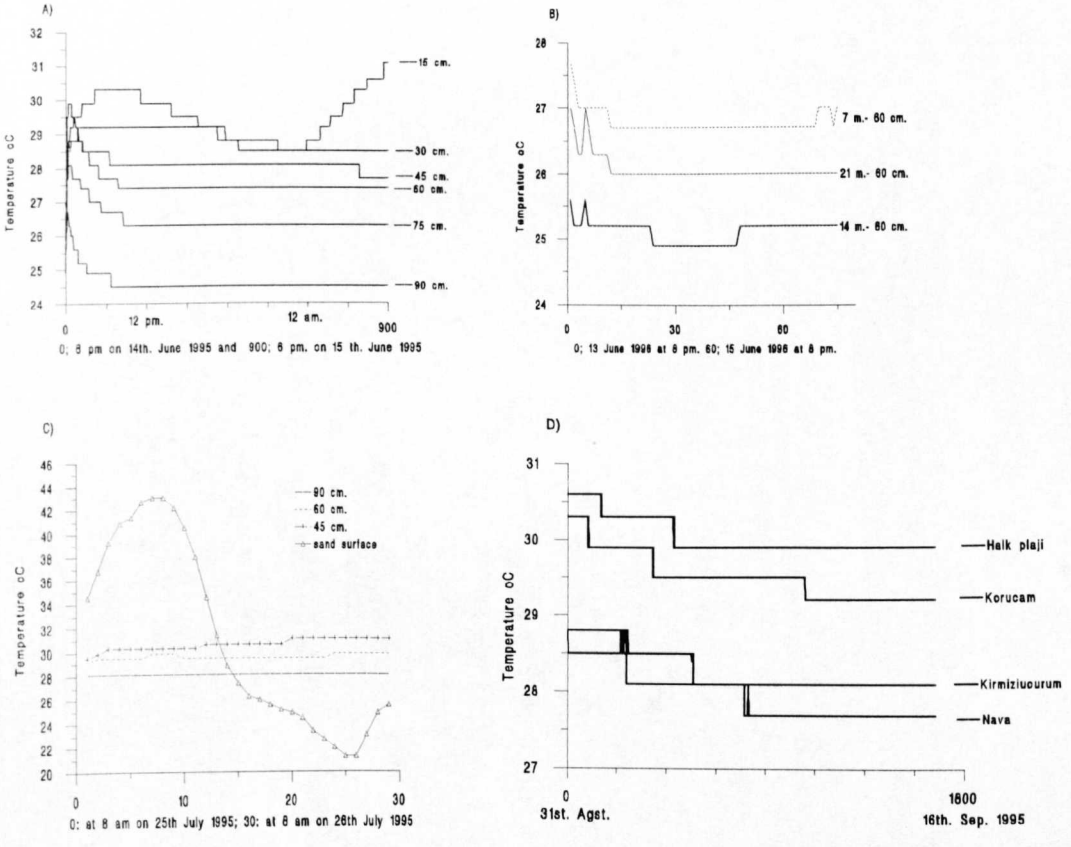


Figure 3.3. Sand temperatures changes on the beaches.

(A: Decreasing temperatures with increasing depth from Akdeniz beach, B: Temperature changes at the 60 cm. depth in relation to distance from sea, C: Sand surface temperatures and the temperatures at the depth of sea turtle nests (14 m. from sea), D: Inter-beach temperature differences at 70 cm. depth and 21 meters from the sea at thefour bays of Akdeniz beach).

The temperatures at 45, 60 and 80 cm. depth were warmer close to the sea but lower at 14 m. and warmer further inland (Figure 3.3). Maximum temperature differences were up to 1.0 °C at 45 cm., up to 1.7 °C at 60 cm., and up to 0.9 °C at 80 cm. depths. Variation within a 24 -h span was less, of course, at 80 cm. Sand surface temperature was not closely correlated to the temperatures at turtle nest depths (Figure 3.3C). There was up to 1.2 °C temperature difference at a depth of 50 cm and up to 1.8

°C difference at 70 cm depth between the bays of Akdeniz beach within a 15 km. long zone (Figure 3.3D). I was able to compare the sand temperatures with the temperature of a nest at the same depth. The mean sand temperature was lower than the temperature of a turtle nest at the same level. In general, the daily mean temperature of sand was 1.1-1.9 °C lower than the daily mean temperature of a loggerhead nest, and 0.4-1.0 °C lower than that of a green turtle nest, for the same period of time at the same depths.

The times of emergence of loggerhead hatchlings from 8 nests were monitored. The mean hatching success of these nests was 76.1%. Hatching times varied between 2100 and 0530 h. Sand temperatures just above the clutch during the time of hatching were cooling (Figure 3.4). Hatchlings from these nests and other nests, determined by counting the tracks of the hatchlings, always emerged on more than one night. The mean nightly number of hatchlings that emerged from green turtle nests was higher in the first two hatchings (77%) and then showed a decrease. The mean nightly number of hatchlings that emerged from loggerhead turtle nests was higher in the first three hatchings (75%) and then showed a decrease. The hatching intervals of green turtles were shorter (mean=3 nights, range 1-5, n= 45) than at loggerhead nests (mean=6.2 nights, range 2-8, n=75).

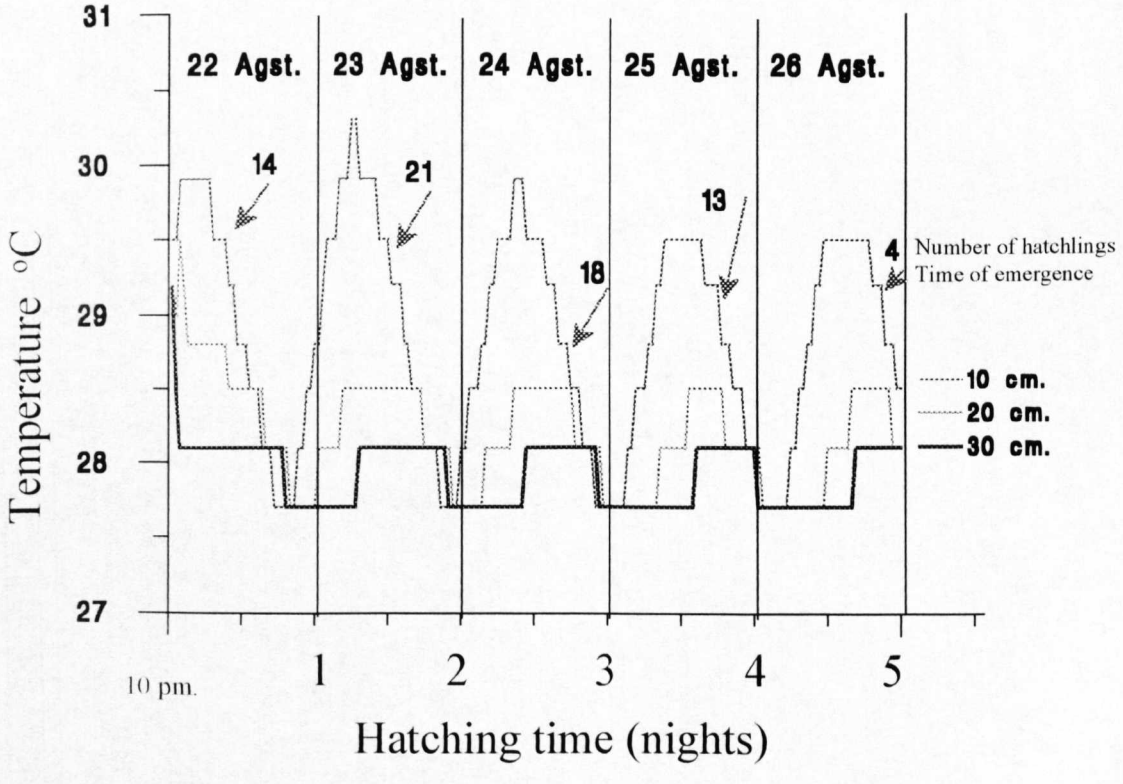


Figure 3.4. Sand temperatures during the hatching period.

Eggs that were taken with the temperature recorders also hatched at different times during the 24h period. Eggs of green turtles (n=78) hatched over 4 days, of which 50 % on the first day, 29.5 % on the second day and 19 % on the third day and only one egg hatched on the fourth day. Eggs (n=138) taken from loggerhead nests hatched over 6 days. Percentage of the hatching for each day were 23.9, 32.6, 18.1, 6.5, 9.4, 9.4 respectively.

The monthly temperature readings at the beaches taken from May to October 1996 (except for Akdeniz where temperatures were taken during 1995) were pooled and averaged by months as shown in Table 3.3. July and August were the warmest months and the mean sand temperatures were above 29 °C. Sand temperatures during May, June and September were cooler and below 29 °C. Three locations were chosen because they are the closest places to the beaches where nests were studied. These are Mugla, Antalya and Anamur (Figure 2.1).

Table 3.3. The temperatures of sand at 50 cm depth, air and sea water on the beaches along the Eastern Mediterranean.

Beaches	Mugla	Fethiye	Fethiye	Dalyan	Patara	Antalya	Antalya	Kizilot	Anamur	Alanya	Akdeniz
Nesting season	Air	Sea water		Sand		Air	Sea water	Sand	Air	Sea water	Sand
May	17.4	22.3	24.0	25.3	24.0	20.4	20.1	24.0	20.7	20.7	25.2
June	22.4	25.0	26.0	27.7	27.5	25.0	23.2	28.5	24.9	24.0	29.0
July	26.0	27.2	29.5	31.3	30.0	28.1	25.8	31.5	27.9	27.0	32.0
August	25.6	28.2	28.0	30.1	29.5	27.9	26.9	30.7	27.9	27.3	29.0
Septem.	21.6	26.7	26.4	28.2	27.0	24.7	26.0	29.5	25.1	26.8	28.5
October	15.8	23.9	25.1	26.7	26.0	19.9	23.7	27.0	20.9	24.2	27.5

3.4. Discussion

Bustard (1972) reported the optimal temperature range for sea turtle egg incubation as 27-32 °C. Carr and Hirth (1961) on Ascension Island recorded the temperatures of green turtle nests as 27.8- 28 °C, with an average gain of 2.3 °C during incubation. Hendrickson (1958) in Malaysia gives the range 28 °C to 30.4 °C but recorded approximately 6 °C rise in temperature during incubation for the same species. Reported temperature rises in natural nests are between 2 and 7 °C (Hendrickson, 1958; Carr and Hith, 1961; Bustard and Greenham, 1968). The data collected in this study generally indicate that the temperature of marine turtle nests in the Mediterranean is between 24 and 35 °C and rises by up to 9.6 °C during incubation.

Clutch temperatures are known to increase in nests of marine turtles (Hendrickson, 1958; Bustard and Greenham, 1968; Godfrey *et al.*, 1997) due to internal metabolic heating. Ackerman *et al.*(1985) noted that there is also an intimate association between the movement of water within the clutch and temperature variation between the centre and periphery of the clutch. Top eggs experience generally warmer conditions than bottom eggs within a nest (Thompson, 1988). My data also showed that top eggs were warmer and bottom eggs were cooler with the middle ones intermediate in the first third of incubation; but later in incubation middle temperatures become the same as the temperature of the top eggs or even sometimes warmer due to metabolic heat.

The results in this work did not show any evidence of a diel temperature cycle for green turtle nests, but up to 1.5 °C for loggerhead turtle nests. Presumably, since green turtle nests are very deep, diel temperature variations occurring near the surface are very much reduced at green turtle nests, but have some influence on loggerhead turtle nests. Hays *et al.* (1995) also did not detect any diel cycle in temperatures for green turtles on Ascension Island, but Standora and Spotila (1985) reported very small (0.5 °C) diel temperature variations for green turtle nests in Costa Rica.

Intra-beach thermal variation on turtle nesting beaches may be large. For example, at the green turtle rookery at Tortuguero, Costa Rica, nest temperatures may vary by up to 3 °C, depending on the extent of nest shading by supra-littoral vegetation (Morreale *et al.*, 1982). Hays *et al.* (1995) could not find intra-beach thermal variation because of the sparse supra-littoral vegetation on Ascension Island. Beach albedo is reported as a cause of intra- and inter-beach thermal differences (Limpus *et al.*, 1983; Hays *et al.*, 1995). For example, nesting beaches in Eastern Australia with visibly darker (brown) beaches 1-2 °C warmer than the visibly lighter (white) coloured beaches at Mon Repos than at Heron Island (Limpus *et al.*, 1983) and Hays *et al.* (1995) found the darkest beach was 4.2 °C warmer than the lightest coloured beach (between 12 beaches) at Ascension Island, South Atlantic. I did not attempt to measure the albedos of beaches, but the temperature of a same depth, observed on the beaches, was warmer close to the sea, became cooler with distance until 14 m., and became warmer again further up the beach. This would be quite important to know before a conservation step need to be taken, such as setting up a hatchery on a beach and relocation of nests on the beach or transfering to Styrofoam boxes.

Egg temperatures in the nest may rise above the temperature of the nesting beach, but this would probably not be by more than 2-4 °C (Hendrickson, 1958; Carr and

Hirth, 1961; Bustard, 1972; Godfrey *et al.*, 1997; Marcovaldi *et al.*, 1997). Greater temperature differences were recorded on loggerhead nests than green turtle nests, compared with the temperature of surrounding sand. This may be because the loggerhead nests are shallower than green turtle nests. Temperature drops due to inundation were also detected. This would indicate that weather can be major influence on nest temperature for loggerhead nests.

There was a female biased sex ratio from these results. Similar results were also reported elsewhere (cf. Mrosovsky, 1994). Inter-sexes were also reported for marine turtles, especially for *Dermochelys coriacea* (Dutton *et al.*, 1985), but identification of sexes from sections was quite easy as explained by Yntema and Mrosovsky (1980) for loggerhead turtles.

Mean incubation temperatures may be adequate to predict sex ratios only in sea turtles that have deep nests which experience little temperature fluctuation (Bull, 1980; Morreale *et al.*, 1982). The results of present work show that mean temperatures can be used for predicting the incubation period but provide a poor prediction of sex ratio.

The variety of relationship between pivotal and beach temperatures suggested that diversity of sex ratios in different populations should be expected (Mrosovsky, 1994). The combined pivotal temperature from North Carolina, Florida and Georgia of 29.0 °C (Mrosovsky, 1988) is below the 30.0 °C reported previously for loggerhead turtle eggs from Little Cumberland Island, Georgia (Yntema and Mrosovsky, 1982). The overall estimate of 29.0 °C for the pivotal temperature of North American loggerhead turtles (Mrosovsky, 1988) is close to the overall value of 28.6 °C given by Limpus *et al.* (1985) for Australian loggerhead turtles. The pivotal temperature of Surinam green turtles is about 28.75 °C (Mrosovsky *et al.*, 1984) and of Costa Rican green turtles, estimated from monitoring sand temperatures in the field, about 28.5 °C (Morreale *et al.*, 1982). From our results it can be said that the pivotal temperatures for sea turtles in the Mediterranean is just below 29 °C. As Standora and Spotila (1985) suggested, if the mean temperature of the nest during the middle third of the incubation period is known, then the sex ratio of hatchlings from that nest can be estimated.

Sea turtle hatchlings mostly emerge from their nests during the evening and thermal cues are believed to be important in controlling the emergence (Hendrickson, 1958; Bustard, 1967, 1972; Witherington *et al.*, 1990; Gyuris, 1993). It has also been suggested that hatchling sea turtles remain in the eggchamber until their siblings hatch, so that individuals emerge as part of a group and not singly (Carr and Hirth, 1961). I found

that hatchlings indeed hatched during the 24 h period, but emerged from the nests only during the night. I also found mean temperature variations within the clutch and therefore it might be that there was variation within the nest in the time to hatching since the rate of embryonic development and consequently the duration of the incubation period is dramatically affected by the temperature (Yntema and Mrosovsky, 1980; Miller, 1985).

Emergence asynchrony for sea turtles was reported previously (Hendrickson, 1958; Witherington *et al.*, 1990; Hays *et al.*, 1992; Gyuris, 1993; Kaska, 1993; Peters *et al.*, 1994). I observed that hatchling emergence from green turtle nests was less spread than from loggerhead nests. This may be because there was less variation in the mean incubation temperatures of green turtle nests than loggerhead nests. When turtle eggs are kept at constant temperature, incubation duration is longer at cooler temperatures, and a 1 °C decrease adds about 5 days in incubation (Mrosovsky and Yntema, 1980). In natural conditions, this study suggests that 1 °C temperature variation within the clutch causes about 4 days delay in both hatching and emergence of hatchlings. Therefore these data suggest that nests should not be excavated right after the first emergence since there may be some recently hatched eggs in the nest.

There was a considerable variation in sand temperature profiles at different sites at the same time, or at the same sites and same time of day but on different dates. Therfore hatching duration because of the temperature differences in sand, might be different for nests along the beach and there may be greater variation in the intra-nest time to hatching due to temperature differences within the clutch.

Cooling of sand temperatures at 15 cm. was suggested as a cue for the emergence of hatchlings (Hays *et al.*, 1992; Gyuris, 1993). I found that the time of emergence was not correlated with any fixed absolute temperature, and hatchlings emerged during the cooling period of the sand above the nest, suggesting that emergence is triggered by falling temperature rather than by a temperature threshold.

Sand, air or sea water temperatures were not closely correlated with nest temperatures. Therefore assumptions on sex ratio using these temperatures may not be reliable, because sex ratio can only be estimated if the middle third temperature of the incubation period is known.

Chapter 4. Predation, nest protection and sand characteristics:

This chapter considers the factors affecting the hatching success of sea turtles such as predation, inundation and sand characteristics, and also examines how hatching success can be increased by screening the nest against predation and relocating nests that are in unsuitable places.

4.1. Introduction:

Sea turtles lay their eggs in an egg chamber, where they are left under the sand for two months. During that period the nests are subjected to numerous biotic and abiotic threats, either natural or human induced: e.g., predation, tidal inundation, and beach erosion (Frazer, 1992; Pritchard, 1980). In the Mediterranean, the most common cause of mortality in developing eggs and hatchlings is nest predation and inundation (Baran and Kasparek, 1989; Canbolat, 1991; Kaska, 1993; Broderick and Godley, 1996). The list of reported nest predators is extensive and includes various canids, birds, rats, lizards, beetles and crabs (Geldiay *et al.*, 1982; Baran and Kasparek, 1989; Erk'akan, 1993; Kaska, 1993; Baran *et al.*, 1994; Brown and Macdonald, 1995; Baran and Turkozan, 1996, Broderick and Godley, 1996; Broderick and Hancock, 1997; Yerli *et al.*, 1997). The protection techniques focus primarily on artificial rearing of the eggs and/or hatchlings. Egg protection strategies include covering nests with cages to reduce predation and relocating eggs for incubation under natural conditions. Relocating and screening have proven useful for protecting sea turtle nests from predators and natural catastrophies.

The early stages of embryonic and hatchling life represent a crucial period in the life history of sea turtles when mortality levels are extremely high (Stancyk, 1982; Wyneken *et al.*, 1988). The achievement of a successful sea turtle nest protection operation depends upon the ability to obtain the maximum yield from eggs artificially incubated or transplanted. Sensitivity of turtle eggs is greatest early in the incubation period and does not totally abate after 20 days (Parmenter, 1980). Therefore, relocation should be done either very soon after laying or after 20 days in incubation.

Predation and screening was conducted on five closely separated beaches of Akdeniz on the west coast of Northern Cyprus where up to 94% predation was reported previously (Godley and Broderick, 1994) and relocation and screening studies were done both in Cyprus and southwest beaches (Fig. 2.1) of Turkey to examine predation patterns of foxes and crabs, to assess the success of caging and relocation to protect nests, and to examine embryonic mortalities. Sand grain size and water potential in the

sand were also investigated in relation to effect on hatching success and optimum conditions for incubation as an aid to hatchery activities.

4.2. Materials and Methods:

The general fieldwork procedure is explained in Chapter 2. The predation work was carried out in the summer of 1995 and both relocation and screening in the summers of 1995 and 1996.

The methods for studying nest predation by foxes were also explained in Chapter 2. Ghost crab (*Ocypode cursor*) predation was also investigated by looking at the crab burrow size and distribution around the nests at Kirmiziucurum beach (Figure 2.2). This was done in 19 different places. Before any quantitative work, notes were made on dispersal patterns of the crabs and it was found that crab burrows tended to be closer to sea. Therefore the beach was divided into three zones, each 5 m long, from high tide up to the beach. A transect was established 5 m in width, giving three 5mx5m quadrats. At the beginning of each week from June to September, the number of crab burrows in each quadrat was counted. The mean of the four counts for each count was converted to the number of burrows per meter square. The sizes of the burrows were also recorded by simply measuring with the finger thickness (one finger thickness is accepted as 1 cm in diameter and so on) early in the morning of counting. These sizes were classified as smaller or equal to 1 cm, 2 cm, 3 cm and 4 cm or bigger. The results were analysed as a spatial and seasonal distribution of the crab burrows.

When turtle tracks were seen during morning survey, they would be followed up the beach to establish whether or not a clutch had been laid. If the nest was in a "suitable" location then the nest's position was marked (Chapter 2). If the nest was in a poor location [i.e. in a busy part of the beach, below the high tide line (usually within 10 meters of the zone from the sea), or partly predated soon after laying etc.] then one out of every two clutches would be moved. Relocation was usually to a suitable site towards the back of the beach. Relocating a clutch is a delicate process, which should be done within a few hours of it being laid. Before relocation is carried out certain measurements of the egg chamber were made. The philosophy was to move the eggs to an environment as similar to that created by the mother as possible. Hence the top egg depth and the bottom egg depth of the nest would be noted. Then at the chosen site an egg chamber would be dug, to the size measured at the turtle nest, the eggs placed in it and the sand replaced as closely as possible, in the order that it was removed. Then the nest was screened (Chapter 2) and sand packed as by the turtles, by moving on the nest in a

sitting position. In this way we hoped to minimise the possible negative effects of relocation by copying the parents behaviour, working on the assumption that natural selection has dictated that the parent produces the optimum egg chamber.

The water potential of sand was investigated in the field (Chapter 2) and sand grain size and composition analyses were carried out in Glasgow. Dried sand samples were first divided into two halves and one was seived with the shaker for 10 minutes using the sive sizes 63 μm (4ϕ), 125 μm (3ϕ), 250 μm (2ϕ), 500 μm (1ϕ), 118 mm (0ϕ), and the amounts of the sand retained in each seive were weighed to an accuracy of 0.01 gram. The results were analysed using a computer programme, developed by Ms. M. Kirkham, in the Sedimentology Unit of Glasgow University. The kurtosis, skewness of the sand and mean grain sizes were calculated, to describe the differences in composition between the sands from different nests and beaches. Three samples from each beach were weighed (50 100 gms), then ashed at 480 °C overnight to remove any organic material and reweighed. Two one gram samples of each sample were then digested in excess 10 % HCl to remove any calcium carbonate. The sample was filtered using filter paper of a known weight, dried in an oven (60 °C), and then reweighed.

4.3. Results:

4.3.1. Predation at the west coast of Northern Cyprus:

The main laying season was June and July and during this period there were a total of 482 nesting activities recorded, 167 of these were successfully laid nests, of which 113 were loggerhead and 54 were green turtles. A total of 113 hatched nests were recorded (69 loggerhead and 44 green turtles) (Figure 4.1).

Predation occured at 133 (79.6%) nests. A total of 39 nests (29 loggerhead and 10 green) did not hatch because of predation. Main nest predators were red foxes, feral and domestic dogs, ghost crabs, scavenging birds, lizards and hedgehogs. 25 loggerhead nests were totally predated(**tp**). Although 32 (29 loggerhead and 3 green turtle nests) nests were predated to some extent (partly predation **pp**), 27 (14 loggerhead and 13 green turtle nests) were assessed to have hatched prior to predation(**hp**). Although loggerhead nests were vulnerable to predation during the whole incubation period, the majority of the predation occurred early or late in the incubation period. Green turtle nests suffered predation just before or during the hatching period. The majority of the loggerhead nests (86.5%) were predated during the months of July and August, but predation occured on green turtle nests mainly in August and September. Assuming a mean of 70 eggs per nest for both species (which is the mean of the eggs known from

the nests), it appears that, the eggs of totally predated nests and partly predated nests, 46.5 % of eggs were consumed in the vicinity of predated nests.

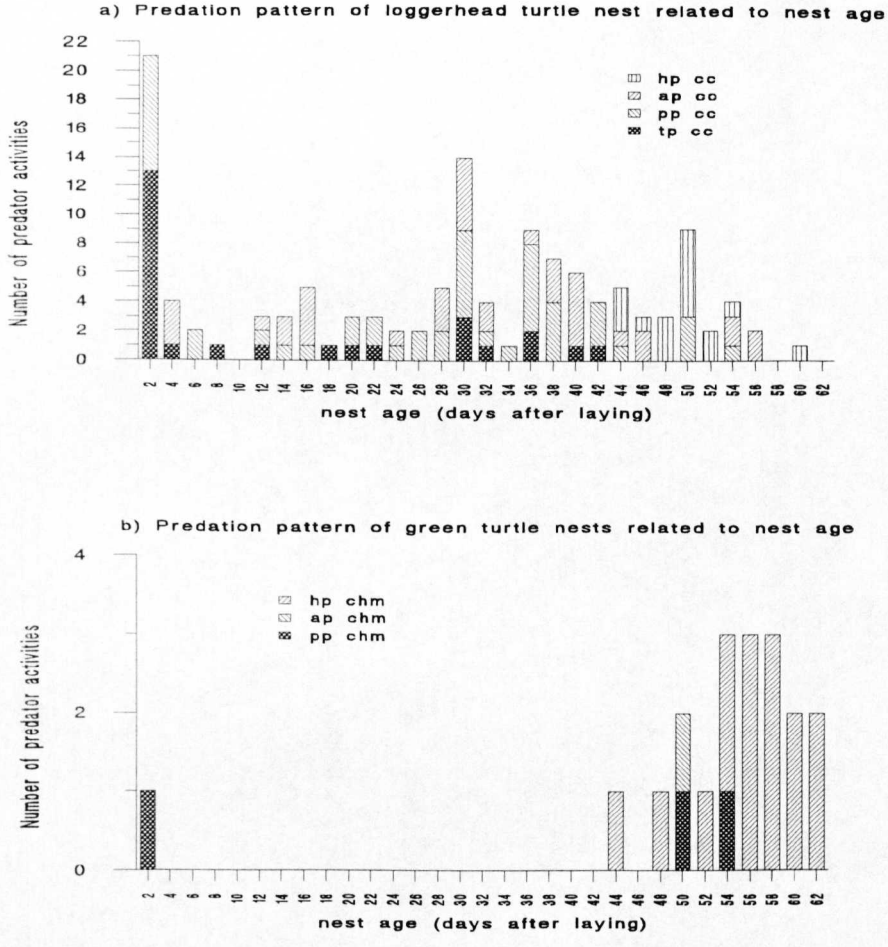


Figure 4.1. Predation pattern of sea turtle nests at the west coast of Northern Cyprus.
(**tp**; total predation, **pp**; part predation, **hp**; hatching and predation, **ap**; attempted predation).

4.3.2. Screening against predation:

A total of 80 (47.9%) nests (50 loggerhead nests and 30 green nests) were caged either next morning after laying, after predation or before hatching. Two types of cages were used, wire mesh and mesh grids (Chp 2, Fig.2.3.). Figure 4.2 shows the number of caged nests in relation to nest age. As a total, 21 loggerhead nests were caged next morning after laying and 29 at different time during the incubation. Six of the predated nests were covered by plenty of sand after predation but left uncaged. This was done mainly to test ideas on predator behaviour. After a few days, these nests were predated again so thereafter all the partly predated nests were caged. A total of 86 (51.4%) nests were used as controls of which 19 were totally predated and 29 of them as mentioned above screened after partly predation during the incubation period, therefore 48 (55.8%) of the

all control nests suffered due to predation. Hatching success at nests which were protected after predation was significantly greater than that at control nests which were attacked (Mann Whitney U=125, P=0.001). Only four (8% of the caged) nests caged after predation suffered part predation due to wrong placement of screen and because of the weak flexible cage material. All of these were wire meshes. None of the nests screened with mesh grids were predated again.

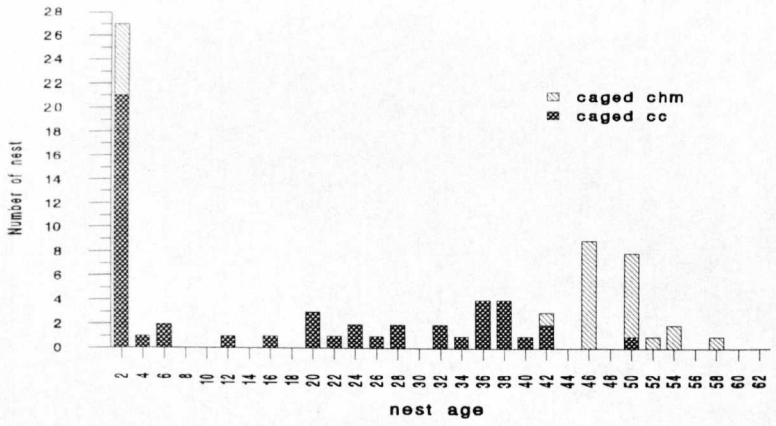


Figure 4.2. Number of cages put over nests at the west coast of Northern Cyprus in the summer of 1995 in relation to days of incubation. (Chm; *Chelonia mydas*, cc; *Caretta caretta*)

Only six green turtle nests were screened next morning after laying and 24 nests caged mainly during the hatching period and few earlier in the incubation period. As a total 24 nests were used as controls and 14 (58.3 %) of them suffered predation (only during the hatching period). Only two (6.6 %) of the screened nests suffered predation during hatching both due to misplacement of the screen and because of the weak flexible cage material. Figure 4.3 shows some of these examples.

In total, 80 nests were caged with wire mesh and 41 with mesh grids. Hatching success was greater for protected than unprotected nests (Mann Whitney U=540, P<0.001). Six of the nests screened with wire mesh were depredated and another 24 of these cages nearly removed from the nest by predators and these were rescreened the next morning after attempted predation. Only one nest screened with wire grid was predated and another 5 of them faced attempted predation but predators failed to reach the eggs. Thus wire grids provided significantly better protection against predation ($x^2_{1.d.f.}=6.7$, $P<0.01$).

Figure 4.3. Screening of turtle nests against predation.

A-) An example of nest predation by foxes.

B-) A nest screened but partly predated.

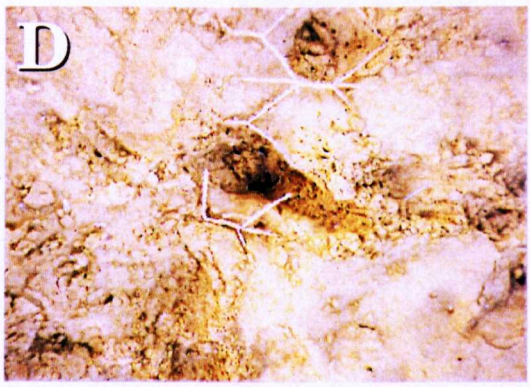
C, D, E-) Examples of screened nests facing attempted predation.

F-) A screened nest hatched successfully.


A


B


C


D


E


F

4.3.3. Crab burrow distribution on the beach:

The diameter of the *O. cursor* burrows was found were between 1-4 cm. The number of crab burrows is increased throughout the season until the end of August, and decreased in September, these changes were statistically significant (one-way ANOVA $F_{3,188}=52.06$, $P<0.01$) over the months. Data regarding the number of crab burrows are presented in Table 4.1.

Table 4.1. The mean daily numbers of crab burrows in relation to spatial and temporal distribution on Kirmiziucurum beach in 1995. (Four counts were made for each month).

Month	June				July				August				September			
Burrow size (cm)	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Zone 1 (sea-5m.)	9	5	3	2	6	17	5	2	5	20	15	0	0	5	10	5
Zone 2 (5-10m)	12	16	20	12	6	25	20	15	4	35	25	26	0	5	10	15
Zone 3 (10-15 m.)	0	17	6	4	1	8	12	18	0	40	40	30	0	5	10	15
Size total	21	38	29	18	13	50	37	35	9	95	80	56	0	15	30	35
Grand total	106				135				240				80			

The number of crab burrows per m^2 per day for each month were also different, being lower in June and September 1.4 and 1 per m^2 respectively; and slightly higher in July as 1.8 and the highest in August as 3.2 per m^2 per day. A significant difference (one-way ANOVA $F_{2,188}=61.29$, $P<0.01$) was found between the numbers of burrows which occured in each zone of the study area, being higher in the second zone covering 5-10 meters than the others. Around 19% of the burrows occurred in zone 1, 44% in zone 2 and 37% in zone 3. The majority (98%) of the smallest sizes of crab burrows were found in zone 1 and 2 and no crab burrows smaller than 1 cm were found in September. Within each zone, there were significant differences between the number of burrows belonging to each size category (one-way ANOVA $F_{3,188}= 25.8$, $P<0.01$). During the hatching season of 1995 at the west coast of Northern Cyprus, only one crab burrow was observed diving in to a nest, but 35 (30 loggerhead and 5 green turtle) hatchlings were found to be caught and taken by crabs to their burrows. These were observed by following the hatchling tracks and ending at the crab burrow. All these burrows were in the 4 cm diameter category.

4.3.4. Relocation:

All the relocated nests were screened. As a total 12 partly predated loggerhead nests were relocated to a fresh site and screened, and 17 left as a control. Although the differences of predation rate between the controls and relocated nests is statistically not significant ($x^2_{1.d.f.}=3.2$, $P>0.05$), four of the controls suffered predation and none of the relocated ones were predated.

18 loggerhead nests on Akdeniz beaches of Northern Cyprus and 26 loggerhead nests on three beaches of Turkey (Fethiye, Patara and Kizilot; 8, 10, 8 nests respectively) were relocated because of risk of inundation. These nests were chosen from the zone of 7 meters to Sea on each beach and relocated to a suitable site further up the beach and screened. 15 nests on Akdeniz, 7 nests on Fethiye, 9 nests on Patara and 7 nests on Kizilot beaches were left as controls. All of the control nests on Akdeniz and Patara beaches were inundated, six of the controls on Kizilot and two on Fethiye were also inundated and did not hatch at all. Hatching success of the relocated, controls and all loggerhead turtle nests are summarised in the following table. Hatching success for relocated nests was significantly greater than controls ($x^2_{1.d.f.}=24.26$, $P<0.001$). On Patara beach, which is the widest beach in Turkey (>100m) and which was especially liable to inundation by storms and beach erosion, there was a significant positive correlation between distance from the nest to high tide line and percent hatching success (Spearman rank; $r_s=0.48$; $P=0.05$; $n=17$). There was not any such correlation on other beaches.

Table 4.2. The hatching success of the loggerhead turtle nests.

Beaches	Relocated nests	Control nests	Overall Hatc. Suc.
Akdeniz	92.8	0.00	58.7
Fethiye	91.2	40.8	75.6
Patara	81.2	0.00	49.5
Kizilot	87.5	8.1	81.3

4.3.5. Sand characteristics:

The water in the sand was expressed as a percentage volume and this ranged from as low as 1.30% up to 13.8% on the beaches. All the samples from beaches were classified according to distances from sea for every 10 meters. The mean values of 15 samples from every 10 meters zone were calculated and these were 11.2%, 7.6% and 3.1% from

sea side to inland respectively. The moisture content of the sand was found to be greatest for those samples taken from nearest the sea. The values than decreased as the distance away from the sea increased. These changes were statistically significant (one-way ANOVA $F_{4,56}=52.67$, $P<0.01$).

The volume of air filled space of the sand (porosity) was measured and this ranged from 19% up to 41.9% on the beaches. The amount of organic material in the sand from the beaches were also calculated by ashing the sands as the weight loss in the sand is attributable to losses of organic carbon. These percentage lossess ranged from 1.09% to 2.95%. On acid digestion of 15 samples, the percentage losses ranged from 25.1% up to 79.5%. This weight loss is primarily due to removal of calcites. These data are summarised on a beach basis together with the mean sand grain size results in Table 4.3.

Table 4.3. The mean of the results of grain size, porosity and component analyses of the sands from the beaches in the Eastern Mediterranean. (M.G.S.; mean grain size, S.D.; Standard deviation, O.C.; organic carbon).

Beach	M.G.S. (ϕ)	S.D.	Skewness	Kurtosis	% air	% O. C.	% calcite
Dalyan	1.2547	0.8895	1.5067	-0.2820	37.02	1.78	50.0
Fethiye	1.0063	0.7156	1.9883	1.5426	32.12	1.18	30.9
Patara	1.7805	0.5325	-0.1235	-0.2171	34.25	0.77	28.9
Kizilot	2.2050	0.9345	0.1785	-0.3270	38.25	0.56	25.1
Korucam	0.4980	0.6668	0.2201	0.8288	40.45	2.01	69.8
Kirmiziucurum	1.6088	0.4754	0.0109	0.6006	34.21	2.95	79.5
Nava	1.7446	0.5220	-0.0816	-0.7540	32.20	2.14	55.2
Halk plaji	0.5090	0.8107	-0.5237	2.0201	36.10	1.09	30.1
North Karpaz	1.3130	0.4946	-0.0333	1.6326	30.40	1.85	48.2
Dipkarpaz	2.0960	0.5777	-1.0979	6.9366	34.35	1.80	48.0

Mean grain size, skewness of the distribution, standard deviation (or sorting of the sand) and kurtosis were all calculated for 59 samples from 10 beaches along the Mediterranean. The results of these samples were summarised on a beach basis. The mean grain size ranged from 0.49 to 2.20 phi (ϕ).

According to Folk (1974) classification (Table 4.4), the sand of the beaches can be classified as follows: Halk plaji and Dipkarpaz beaches have a fine sand; Dalyan, Fethiye, Patara, Kirmiziucurum, Nava and North Karpaz beaches have a medium sized sand; and only Kizilot and Korucam beaches have a coarse sand.

The small standard deviations obtained indicate a uniformity of sand grain size resulting from a well sorted beach. All samples can be classified as moderately sorted, having sorting values between 0.48 and 0.93. The value of skewness close to "0" means the sand particle distribution to be symmetrical, negative values show these have coarse skewed particle distribution, and positive values are described as being fine skewed.

Table 4.4. Particle size classification of sand (Folk, 1974).

Sand type	Range of particle size	
	Phi (ϕ)	metric (mm)
Very coarse sand	-1.0 to 0.0	2 .0 to 1.0
Coarse sand	0.0 to 1.0	1.0 to 0.5
Medium sand	1.0 to 2.0	0.5 to 0.25
Fine sand	2.0 to 3.0	0.250 to 0.125
Verry fine sand	3.0 to 4.0	0.125 to 0.0625

Therefore the sand from Nava, Halk plaji, Dipkarpaz, North Kapraz, and Patara beaches have a coarse skewed particle distribution; Korucam, Kirmiziucurum and Kizilot have a symmetrical particle distribution; Fethiye and Dalyan beaches have a fine skewed particle distribution. The relatively low kurtosis values imply that all the sand samples are platykurtic. That is, the sand particles represented in the "tails" of the frequency curve were better sorted than the sand of the central portion of the curve. Only sand samples from Dipkarpaz have a high value of kurtosis, and therefore are leptokurtic. When all ten beaches are considered, a Spearman rank test demonstrated no significant correlations between mean particle size and hatching success for 59 nests ($P > 0.05$).

4.4. Discussion:

Fox predation on sea turtle nests in Northern Cyprus and Turkey is high (Baran and Kasparek, 1989; Erk'akan, 1993; Brawn and Macdonald, 1995; Broderick and Godley, 1996; Yerli *et al.*, 1997). This present study was completely successful in preventing this by the placement of wire mesh grids. This method is now being used to protect nests (Erk'akan, 1993; Kaska, 1993; Yerli *et al.*, 1997). Foxes located the protected and unprotected nests during this study, but slightly less at protected ones. This may be because foxes are influenced by researcher's activities.

The significantly higher hatching success of nests protected after a first raid in comparison to those controls left unprotected indicates that the grids interrupt the

sequence of predation. Natural nest hatching success was significantly lower than that of relocated nests. While natural nests are subject to the whims of nature and the turtles' choice of sites, relocated nests were placed in the best sites available. Thus, reburied nests were not subject to even incidental wave washover, or burial by callapsing dune scarp or drift sand.

Within the study area at Kirmiziucurum beach, the distribution of the crabs up the shore was seen to vary with the size of the crab. Juvenile crabs were generally found closer to the sea with the larger adults being located at a greater distance from the sea. Similar results was found for the *O. cursor* population of north Atlit, northern Israel (Shuchman and Warburg, 1978). Only adults were found to be able to catch hatchlings. Quantitatively, it appears that loggerhead hatchlings were predominantly caught by crabs. This may be because loggerhead hatchlings are slightly smaller than green turtle hatchlings. In their younger stages, ghost crabs must remain nearer the water to keep their gills wet (Phillips, 1940). Therefore, because of this life cycle, the ghost crab appears to be more prevalent near nests later in the nesting season, that is mainly during the hatching season and probably this may help them to catch the hatchlings immediately after emergence. Ghost crabs have previously been reported to eat turtle eggs and hatchlings (Hendrickson, 1958; Hirth and Carr, 1970; Broderick and Godley, 1996).

Abiotic factors affecting the hatching success were generally saltwater inundation by high tides and severe beach erosion. Inundation was especially dominant on the west coast beaches of Northern Cyprus and Patara beach since a total 32 nests were inundated out of 210 nests studied on these beaches.

As biotic factors affecting the hatching success, nest predation of foxes and ghost crab predation were considered. Fox predation was dominant on the west coast beaches of Northern Cyprus, 39 nest completely predated and almost the same amount again of nests were predated to some extent. Transplanted nests after part predation were less heavily preyed upon by foxes than controls left in the original nest site, suggesting that foxes may detect their predated nests easily. This may be because of lack of clearance of nest material after predation. Little information is given in the literature regarding predation in relation to nest age, some studies noting that predation is greatest within 24 to 72 h after egg deposition, but in this study although some newly laid eggs were also heavily predated, predation was also considerable later in the incubation period dependent on the species. Loggerhead nests faced predation throughout almost the entire incubation period, whereas green turtle nests were mainly predated during the hatching.

This may also be related to predator behaviour, since different predation may be observed on other beaches in the Mediterranean (Brawn and Macdonald, 1995; Yerli *et al.*, 1997).

A total 80 nests were screened and only 6 of these suffered part predation due to wrong placement of the screen and the weak flexible screen material. Only one nest screened with wire grid was predated and another 5 of them faced attempted predation but predators failed to reach the eggs. Thus wire grids provided significantly better protection against predation, this may be because they were made up from a strong inflexible material. The hatching success of loggerhead nests (58.7%) was lower than green turtle nests (75.5%) due to heavier predation. Screening is one way of protecting these nests during the whole incubation period, or if the temperature profile of the beach is known, relocation of these nests to a fenced area would be an alternative.

Chapter 5. Embryological development of sea turtles

This chapter presents the staging of embryonic development measurements taken from embryos of sea turtles in the Mediterranean.

5.1. Introduction:

Few studies are available on embryonic development of sea turtles (Agassiz, 1857; Parker, 1880; Dereniyagala, 1939; Penyapol, 1958; Fujiwara, 1966; Domantay, 1968; Crastz, 1982). More recently the study of Miller (1985) gave a stage-by-stage description of sea turtle development using samples from Australia. Although marine turtles have been considered to a certain extent in most studies of turtle development, the emphasis has always been on more accessible non-marine turtles. In 1857, Agassiz compared the development of several species of turtles including terrestrial, freshwater and one marine species. This work has remained definitive because embryonic descriptions are complete for only two species, *Chelydra s. serpentina* (Yntema, 1968) and *Chrysemys picta belli* (Mahmoud *et al.*, 1973), both of which were studied by Agassiz. Crastz (1982) described 31 stages for *Lepidochelys olivacea*, and Miller (1985) described the embryonic development of six marine turtle species.

Embryonic staging alone contributes little to the understanding of a species or to its survival, but in the context of other studies on management problems and the effects of human intervention, basic embryological information can remove some of the guess work from decision making.

The success of a clutch of turtle eggs depends upon the interaction of a number of factors, such as salinity, humidity, temperature, gas flow, rainfall, tidal inundation, erosion and predation (Hendrickson, 1958; Bustard and Greenham, 1968; Prange and Ackerman, 1974; Ackerman, 1980; Mrosovsky, 1980). In conservation efforts, improper handling of eggs during movement to hatcheries may increase mortality (Limpus *et al.*, 1979; Blanck and Sawyer, 1981).

Knowledge of development under controlled and natal beach conditions helped the understanding of sexual differentiation. Therefore, detailed information on the temperature of the beach and developmental stages of sea turtles in the Eastern Mediterranean is important, especially for relocation and setting up hatcheries to protect these endangered species.

All marine turtle eggs normally have pliable, parchment-like shells and are typically spherical although not turgid at oviposition (Bustard, 1972; Ewert, 1979). Odd-shaped and yolkless eggs are occasionally reported (Bustard, 1972; Miller, 1985). When

laid, the eggshell is creamy-white. During the first one to two days, the heavy yolk settles downward, and the eggshell at the top starts "chalking" as the mucus dries and the egg absorbs water (Blanck and Sawyer, 1981; Miller, 1985). Simple candling of turtle eggs can be used to estimate the stage of development, date of laying, time of hatching, and the cycle of the female parent, as well as helping to avoid excessive mortality if eggs must be moved. Embryonic staging has traditionally been based upon morphological changes as well as chronological age and size of the embryo (Agassiz, 1857; Mahmoud *et al.*, 1973; Crastz, 1982), or by correlation between constant temperatures of incubation and time together with morphological changes (Yntema, 1968) or both (Miller, 1985). Although the description of the stages of development by Miller (1985) is quite detailed, it is difficult to identify especially the early stages in the field.

In this work, I used eggs from partly predated nests, of which the date of laying was known, to describe the stages of development in field conditions and identified the stages of dead-in-shell embryos i.e. those found to have died at some time after laying.

5.2. Materials and Methods:

Beaches were patrolled daily early in the morning to monitor nesting, predation and hatching (Chapter 2). Each nest was marked and noted with the date of laying and the species on a bamboo cane or a small stone and placed one 1 meter behind the nest and another near vegetation (Chapter 2). Therefore when a nest had been predated, any damaged eggs could be taken or other sampled eggs along with the data written on the nest mark to the camp site to describe the stages of development. In addition to damaged eggs from predated nests, small numbers of intact eggs (35 green turtle eggs; 75 loggerhead eggs) were sampled from nests predated in the first 10 days of incubation, in order to ensure that a complete series of stages could be described. These eggs were weighed and measured and used to access the components of the eggs, as well as embryological stages. The post-ovipositional development of loggerhead and green turtle embryos were described from a total 1882 (75 eggs sampled, 270 partly predated eggs and 1537 dead in shell embryos) loggerhead turtle and 1169 (35 eggs sampled, 105 partly predated eggs and 1029 dead in shell) green turtle embryos, collected in three consecutive seasons (1994 to 1996). Initially, all the measurements and morphological changes in the embryos of which the age was known were recorded and a simple staging guide for Mediterranean sea turtles was developed and later these results compared with Miller (1985) and it was found that the appearances of the morphological characters of the embryos at the different stages matched very closely with Miller's description. The

description of days of development were compared with the published work on development of sea turtles (Miller, 1985). After staging, morphometric measurements of the embryos were taken. In addition to these measurements, head, tail and limb development during the early stages and any abnormal developments noticed were examined by either light or Scanning Electron Microscopy. The percentage of abnormalities was calculated as a proportion of the total number of eggs observed.

Morphometric measurements of the embryos were taken. These were either measured using standard calipers to an accuracy of 0.1 mm or under microscope to an accuracy of 0.01 mm as follows. One way analysis of variance tests among the measurements for each characteristic by stages for both species were done. The results showed no significant differences between two species for each character.

Crown-Rump Length (CRL): This is measured as the straight-line length of the blastodisc in the early embryos and straight-line length from the anterior end of the brain to the rump in later stage embryos. This measurement was taken for embryos less than two weeks old (stage 20) under a dissection microscope; the number of somites were also counted when it was possible.

Straight Carapace Length (SCL): The greatest straight-line distance between the posterior border of the supra-caudal scales and the anterior border of the nuchal scales.

Straight Carapace Width (SCW): The greatest straight-line distance across the middle part of the carapace.

Head Width (HW): The greatest width of the head.

Head Length (HL): The distance from the rostal extension to the posterior of the head in early embryos or to the posterior extension of the supra-occipital in older embryos.

Bridge Length (BL): The shortest distance from the anterior border to the posterior border of inframarginal scales.

Forelimb Length (FL): The greatest straight-line length of the forelimb when held in the embryonic position as a total length of the limb in early embryos. In older embryos, it is measured from the wrist to the distal tip of the flipper.

Hindlimb Length (HlL): The greatest straight-line length of the hindlimb when held in the embryonic position as a total length of the limb in early embryos. In older embryos, it is measured from the knee to the distal tip of the flipper.

Interclaw Distance (ID): The straight distance from the proximal edge of the proximal claw to the proximal edge of the distal claw or to the presumptive future location of the distal claw.

Tail Length (TL): A total length of the tail as in early and late embryos and greatest straight-line length of the tail when curled anteroventrally in middle stages.

Yolk Diameter (YD): The greatest diameter of the yolk. This was only measured for late-stage (27-31) embryos.

(Embryo weight was also measured from embryos dried of all surface fluids, but not all the embryos were weighed before fixation, therefore the results of these measurements are not presented).

Early stage embryos were examined by scanning electron microscopy (SEM). For this, Bouin-fixed embryos were washed in several changes of distilled water to remove the fixative. The following procedure has been followed.

- 2.5% Glutaraldehyde in 0.1 M Phosphate (PO_4) Buffer fix (1 hr)
- Three times 0.1 M Phosphate (PO_4) Buffer rinse (5 mins)
- 1 % Osmium tetroxide (OSO_4) (1 hr)
- Three times distilled water rinse (10 mins)
- 0.5 % aqueous Uranil acetate (1 hr, in the dark)
- Two quick changes of distilled water
- Acetone dehydration; 30%, 50%, 70%, 90%, Absolute Acetone (Twice), Dried Absolute Acetone (15 mins each)
- Critical Point Drier (1 hr 40 min)
    -period in CO_2 (1 hr 40 mins)
    -Compression time (15 mins) and decompression time (15 mins)
- Mounting the samples on Stubsand Gold coat in sputter coater.
-Viewing on SEM and saving the pictures into a computer.

5.3. Results:

5.3.1. Eggs:

The mean ovipositional diameters were 4.37±0.38 cm.(range 4.01-4.75) for green turtle eggs (N=35) and 3.89±0.18 cm (range 3.59-4.28) for loggerhead turtle eggs (N=75). Eggs sampled for the description of early embryonic development were also used for measurements of egg components. The mean total weight of 20 ovipositional loggerhead turtle eggs was 33.54 ±0.33 grams. The components contributed different amounts to the total as 4.6% shell, 44.4% albumen and 51% yolk. The mean total weight of 12 ovipositional green turtle eggs was 34.79 ±0.80 grams. The components contributed

different amounts to the total for green turtle eggs being 4.7% shell, 43.1% albumen and 52.2% yolk.

5.3.2. Descriptions of stages and ages of the embryos:

The following explanations apply to both *C. caretta* and *C. mydas*. The stages referred to Miller (1985) are given in parenthesis.

Day 0; Time of laying; there is no white chalked area on the shell (Stage 6).

Day 1; Externally, the white-chalked area is initiated at the apical region (top) of the egg and covers less than half of the egg (usually the diameter of the white area is smaller than 15 mm.). Internally, the blastopore is shaped as a posteriorly facing opening and U shaped crescent (Fig. 5.1A): the blastopore opening is getting narrower and slightly arched (Stages7,8).

Day 2; Externally, the white area covers less than half of the egg (Fig. 5.1B). Internally, the neural folds are visible; there are no somites (Stage 9).

Day 3; The neural folds are touching or fusing along the midline of the head and there are up to 6 pairs of somites (Stages 10,11). The neural folds have risen along the length of the embryo and they surround the blastopore posteriorly; the head process is slightly raised (Fig. 5.1C).

Day 4; the amnion covers approximately one-half of the total body length. Eight to14 pairs of somites are present (Stages 12, 13). The posterior region of embryo is wide and has become flat, the heart is present as initial tubes, and anterior neuropore is open (Fig. 5.1D).

Day 5; The tail bud is formed and the amnion covers the neurocentric canal. The heart is visible and S-shaped. Fifteen to 17 pairs of somites are present (Stage 14). The anterior neuropore is closed. The neural folds are fused anteriorly (Fig. 5. 1E).

Day 6; The whole embryo is covered by the amnion, and the mouth of the embryo is open. The first pharyngeal cleft is open. Nineteen to 21 pairs of somites are present (Stage 15). The heart is clearly visible (Fig.5. 1F).

Days 7 and 8; The first two pharyngeal clefts are distinct and open. Small limb buds are present on the lateral body folds. The lens is differentiated in the eye. Vitelline vessels are anastomosing and blood islands are visible in the area vasculosa. Somites number 23 to 27 pairs (Stage 16). Morphometric measurements taken from these embryos are summarised in Table 5.2.

Figure 5.1. Early post-ovipositional development of sea turtles.

A-) One day old *C. mydas* embryo. SEM view of blastopore opening. (x 216)

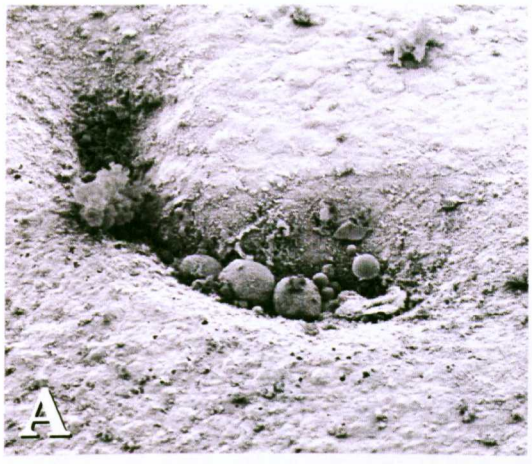
B-) Two days old *C. caretta* eggs. Chalking is visible on the apical pole of the eggs. Bar equals 2 cm.

C-) Three days old (Stage 10) *C. mydas* embryo. Bar equals 20 μm.

D-) Four days old (Stage 12) *C.caretta* embryo. Bar equals 30 μm.

E-) Five days old (Stage 14) *C.mydas* embryo. Bar equals 100 μm.

F-) Six days old (Stage 15) *C. caretta* embryo. Bar equals 100 μm.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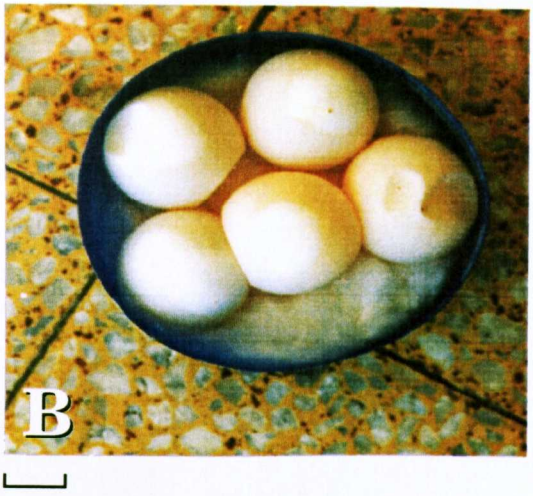
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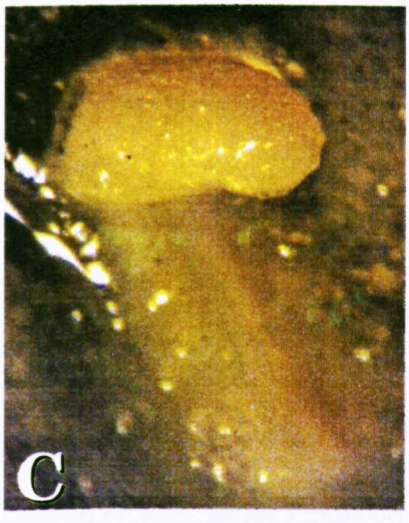
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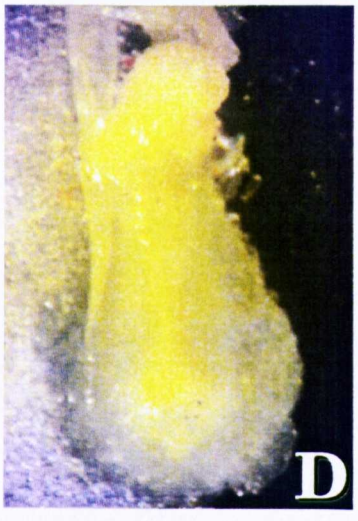
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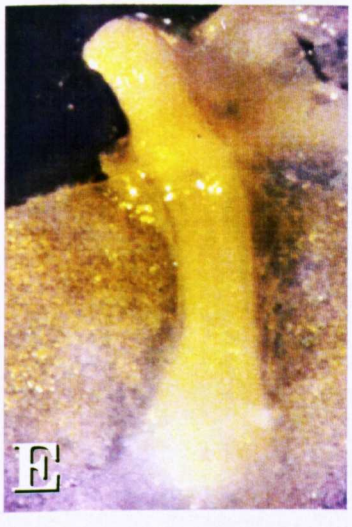
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The only reliable measurement that can be taken until the embryo is one week old is the crown-rump length. Table 5.1 summarises the crown-rump lengths of the embryos up until stage 15, and gives the numbers of embryos examined at each stage.

Table 5.1. The crown-rump lengths (measurements in cm) of the embryos until one week old.

Stage	*Caretta caretta* (mean ± S.E (N))	*Chelonia mydas* (mean ± S.E (N))
6	0.168 ± 0.015 (6)	0.177 ± 0.011 (4)
7	0.218 ± 0.013 (5)	0.205 ± 0.015 (4)
8	0.233 ± 0.011 (10)	0.236 ± 0.007 (5)
9	0.249 ± 0.006 (12)	0.241 ± 0.009 (6)
10	0.252 ± 0.006 (11)	0.255 ± 0.005 (12)
11	0.253 ± 0.005 (12)	0.255 ± 0.005 (6)
12	0.338 ± 0.011 (7)	0.371 ± 0.008 (6)
13	0.421 ± 0.008 (7)	0.430 ± 0.008 (5)
14	0.477 ± 0.007 (8)	0.466 ± 0.011 (6)
15	0.546 ± 0.007 (14)	0.520 ± 0.006 (9)

Days 8 and 9; All the pharingeal clefts (1-5) are open. Limb buds bulged laterioposteriorly. The lens is clearly visible and the retina is slightly pigmented. The tail is long and straight. The allantois is about the size of the eye. Blood islands at the periphery of the area vasculosa are anastomosing to form the sinus terminalis. This can be seen lateral to the embryo. Twentynine to 34 pairs of somites are present (Stage 17). Morphometric measurements taken from these embryos are summarised in Table 5.2.

Table 5.2. Morphometric measurements (in cm.) of embryos at stages 16 and 17.

	16		17	
	Caretta caretta (mean ± S.E (N))	*Chelonia mydas* (mean ± S.E (N))	*Caretta caretta* (mean ± S.E (N))	*Chelonia mydas* (mean ± S.E (N))
FL	0.107 ± 0.0009 (20)	0.103 ± 0.0014 (15)	0.117 ± 0.0060 (20)	0.152 ± 0.0139 (15)
HIL	0.085 ± 0.0010 (15)	0.050 ± 0.0002 (10)	0.110 ± 0.0040 (12)	0.082 ± 0.0075 (11)
CRL	0.616 ± 0.0464 (20)	0.525 ± 0.0185 (15)	0.626 ± 0.0441 (20)	0.676 ± 0.0431 (15)
HW	0.130 ± 0.0137 (16)	0.086 ± 0.0061 (12)	0.150 ± 0.0062 (15)	0.117 ± 0.0132 (11)
HL	0.150 ± 0.0117 (20)	0.121 ± 0.0095 (14)	0.161 ± 0.0103 (20)	0.505 ± 0.0108 (12)

Day 10; A flap of skin has developed on the anterior border of each pharyngeal cleft. The retina is completely pigmented. The limb buds are forming digital plates. The allantois is about the same size as the head. Thirtyfive to 40 somites are present (Stage 18). Morphometric measurements taken from these embryos are summarised in Table 5.3.

Table 5.3. Morphometric measurements (in cm.) of embryos at stage 18 and 19.

	18		19	
	Caretta caretta (mean ± S.E (N))	*Chelonia mydas* (mean ± S.E (N))	*Caretta caretta* (mean ± S.E (N))	*Chelonia mydas* (mean ± S.E (N))
FL	0.117 ± 0.0077 (20)	0.119 ± 0.0091 (10)	0.131 ± 0.0074 (25)	0.140 ± 0.0132 (11)
HIL	0.098 ± 0.0065 (15)	0.083 ± 0.0060 (9)	0.088 ± 0.0049 (15)	0.104 ± 0.0028 (19)
CRL	0.777 ± 0.0354 (21)	0.760 ± 0.0514 (15)	0.780 ± 0.0314 (21)	0.774 ± 0.0186 (15)
SCW	0.089 ± 0.0036 (7)	0.124 ± 0.0094 (5)	0.135 ± 0.0056 (11)	0.169 ± 0.0001 (9)
HW	0.215 ± 0.0154 (16)	0.198 ± 0.0127 (12)	0.228 ± 0.0113 (18)	0.263 ± 0.0063 (13)
HL	0.249 ± 0.0131 (17)	0.223 ± 0.0096 (14)	0.255 ± 0.0128 (21)	0.296 ± 0.0056 (17)

Days 11 and 12; The limb buds have definite paddles, and project laterally free from the body wall. The allantois is slightly larger than the head. The tail curls anteroventrally (Stage 19). Morphometric measurements taken from these embryos are summarised in Table 5.3. A green turtle embryo can be seen in Figure 5. 2A.

Days 13 and 14; The digital plates of the forelimbs and hindlimbs are partially or completely twisted flat against the body wall. Total body length is less than 10 mm. The iris is not pigmented. Pharyngeal clefts are nearly closed (Stage 20). Morphometric measurements taken from these embryos are summarised in Table 5.4.

Days 15-17; The carapace rudiment is visible laterally. The iris has pigmented along its posterior border. All pharyngeal clefts are covered by flaps of skin (Stage 21). Morphometric measurements taken from these embryos are summarised in Table 5.4.

Days 18-20; A distal ridge defines the limit of the limb from the digital plate, and proximal ridge delimits the limb. The marginal ridge of the carapace is marked by small, low serrations. The lateral edge of the plastron is evident. The tail is longer than the hindlimb (Stage 22). Morphometric measurements taken from these embryos are summarised in Table 5.5. A loggerhead turtle embryo can be seen in Figure 5.2B.

Figure 5.2. The mid and late stage development of sea turtles.

A-) An 11 days old (Stage 19) *C. mydas* embryo. Bar equals 1 mm.

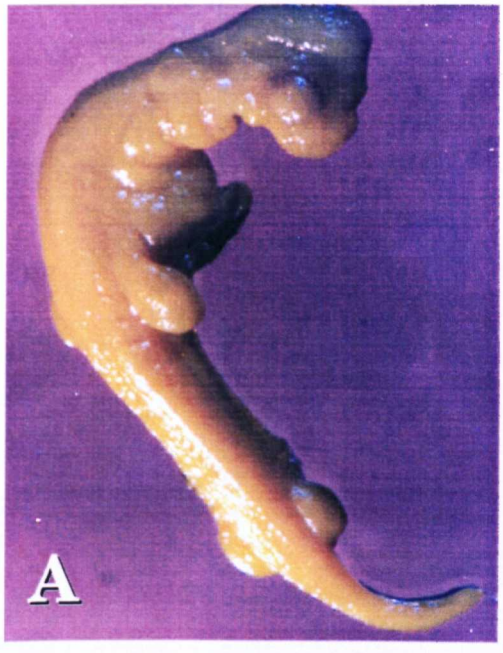
B-) A 19 days old (Stage 21) *C. caretta* embryo. Bar equals 2 mm.

C-) A 25 days old (Stage 24) *C. caretta* embryo. Bar equals 2 mm.

D-) A 30 days old (Stage 25) *C. mydas* embryo. Bar equals 0.5 cm.

E-) A 45 days old (Stage 29) *C. mydas* embryo. Bar equals 1 cm.

F-) A 51 days old (Stage 31) *C. caretta* embryo. Bar equals 1 cm.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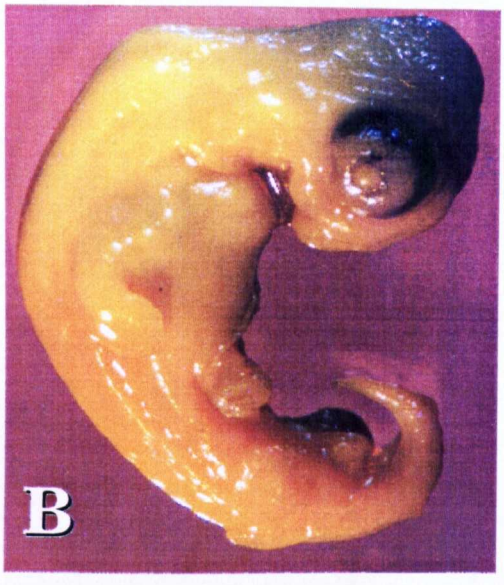
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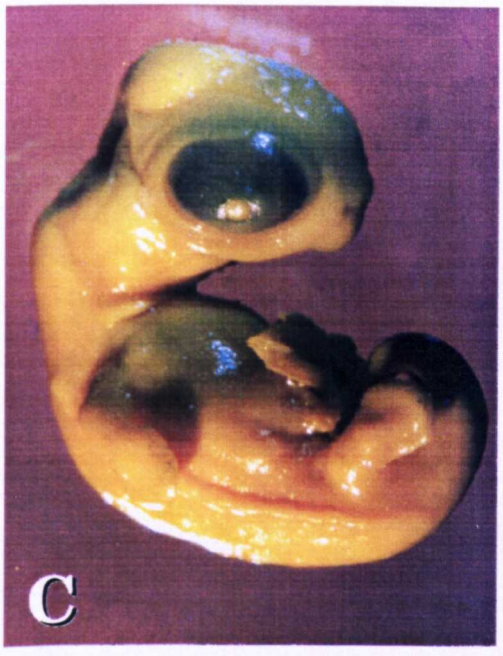
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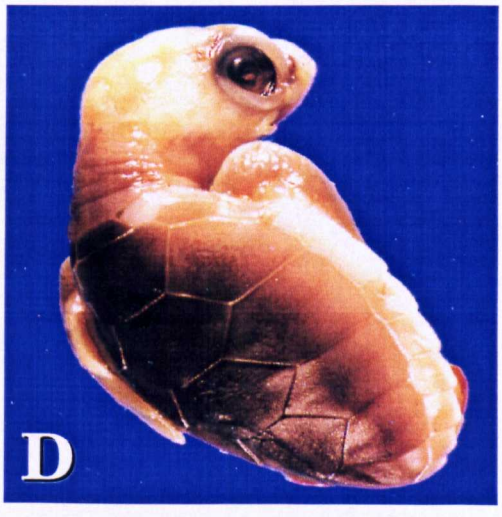
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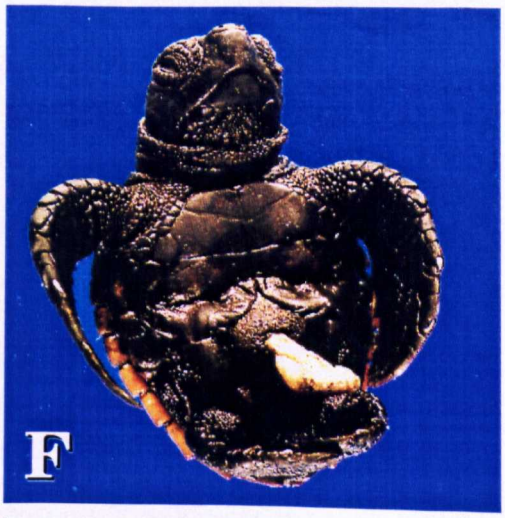
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Table 5.4. Morphometric measurements (in cm.) of embryos at stage 20 and 21.

	20		21	
	Caretta caretta (mean ± S.E (N))	*Chelonia mydas* (mean ± S.E (N))	*Caretta caretta* (mean ± S.E (N))	*Chelonia mydas* (mean ± S.E (N))
FL	0.162 ± 0.0069 (40)	0.194 ± 0.0062 (25)	0.254 ± 0.0061 (55)	0.265 ± 0.0041 (37)
HIL	0.112 ± 0.0046 (35)	0.137 ± 0.0073 (29)	0.166 ± 0.0052 (55)	0.208 ± 0.0039 (29)
CRL	0.953 ± 0.0181 (40)	0.877 ± 0.0359 (25)	1.181 ± 0.0280 (31)	1.103 ± 0.0251 (25)
SCL			0.840 ± 0.0112 (21)	0.875 ± 0.0065 (17)
SCW	0.158 ± 0.0048 (27)	0.212 ± 0.0113 (18)	0.250 ± 0.0119 (47)	0.310 ± 0.0074 (35)
HW	0.281 ± 0.0089 (36)	0.348 ± 0.0130 (25)	0.407 ± 0.0133 (46)	0.471 ± 0.0106 (32)
HL	0.319 ± 0.0098 (37)	0.379 ± 0.0119 (24)	0.470 ± 0.0131 (17)	0.520 ± 0.0101 (34)

Table 5.5. Morphometric measurements (in cm.) of embryos at stage 22 and 23.

	22		23	
	Caretta caretta (mean ± S.E (N))	*Chelonia mydas* (mean ± S.E (N))	*Caretta caretta* (mean ± S.E (N))	*Chelonia mydas* (mean ± S.E (N))
ID			0.107 ± 0.0027 (35)	0.108 ± 0.0026 (21)
FL	0.313 ± 0.0079 (48)	0.373 ± 0.0143 (37)	0.422 ± 0.0094 (53)	0.475 ± 0.0138 (45)
HIL	0.236 ± 0.0077 (45)	0.280 ± 0.0074 (26)	0.340 ± 0.0088 (53)	0.367 ± 0.0098 (36)
CRL	1.271 ± 0.0259 (41)	1.314 ± 0.0459 (35)	1.531 ± 0.0336 (51)	1.544 ± 0.0148 (38)
SCL	0.943 ± 0.0179 (49)	0.997 ± 0.0248 (27)	1.361 ± 0.0280 (49)	1.126 ± 0.0254 (37)
SCW	0.412 ± 0.0227 (47)	0.453 ± 0.0181 (35)	0.681 ± 0.0222 (53)	0.665 ± 0.0191 (36)
BL			0.320 ± 0.0049 (23)	0.473 ± 0.0082 (21)
HW	0.539 ± 0.0171 (46)	0.614 ± 0.0169 (37)	0.726 ± 0.0133 (46)	0.733 ± 0.0104 (37)
HL	0.565 ± 0.0148 (47)	0.724 ± 0.0263 (37)	0.843 ± 0.0135 (53)	0.770 ± 0.0188 (37)

Days 21-23; The foreflipper has elongated. Digital ridges are visible on the digital plates. The posterior part of the carapace has been completed. Scutes are not differentiated. Ribs are visible through the carapace. The tail is kinked inwards (Stage 23). Morphometric measurements taken from these embryos are summarised in Table 5.5.

Days 24-27; The digital ridges are well developed. The anterior part of the carapace has been completed and the scutes of the carapace are visible, but scales are not visible on the skin. The tail is slightly longer than the hindflipper. The border of the plastron is complete. The caruncle (enables hatchling to break the shell during hatching) appears as a white spot (Stage 24). Morphometric measurements taken from these embryos are summarised in Table 5.6. A loggerhead turtle embryo can be seen in Figure 5. 2C.

Days 28-32; The carapace has been completed and all the scutes have differentiated. The head is unscaled. The tail and hindflippers are equal in size. The phalanges are well defined and claws are present on the first digit (Stage 25). Morphometric measurements taken from these embryos are summarised in Table 5.6. A green turtle embryo can be seen in Figure 5. 2D.

Table 5.6. Morphometric measurements (in cm.) of embryos at stage 24 and 25.

	24		25	
	Caretta caretta (mean ± S.E. N:100)	*Chelonia mydas* (mean ± S.E N:75)	*Caretta caretta* (mean ± S.E N:100)	*Chelonia mydas* (mean ± S.E N:75)
ID	0.142 ± 0.0033	0.178 ± 0.0052	0.228 ± 0.0069	0.275 ± 0.0069
FL	0.596 ± 0.0168	0.787 ± 0.0170	0.933 ± 0.0204	1.119 ± 0.0236
HIL	0.427 ± 0.0090	0.539 ± 0.0103	0.689 ± 0.0124	0.705 ± 0.0163
CRL	1.783 ± 0.0253	1.968 ± 0.0238	2.477 ± 0.0401	2.542 ± 0.0541
SCL	1.381 ± 0.0238	1.609 ± 0.0235	1.890 ± 0.0288	2.001 ± 0.0376
CCL			2.383 ± 0.0311 (70)	2.619 ± 0.0383
SCW	0.984 ± 0.0237	1.134 ± 0.0288	1.537 ± 0.0314	1.610 ± 0.0344
CCW			2.086 ± 0.0300 (75)	2.108 ± 0.0403
BL	0.458 ± 0.0096	0.584 ± 0.0071	0.603 ± 0.0010	0.725 ± 0.0156
HW	0.825 ± 0.0087	0.847 ± 0.0089	0.894 ± 0.0050	0.872 ± 0.0196
HL	0.973 ± 0.0088	0.981 ± 0.0163	1.235 ± 0.0141	1.252 ± 0.0201

Days 33-36; The scutes of the carapace were becaming pigmented. All flipper scales are present and head scales are visible. The plastron exhibits definite coloration (Stage 26). Morphometric measurements taken from these embryos are summarised in Table 5.7.

Days 37-39; The yolk is bigger than the embryo (The diameter of the yolk is usually larger than 20 mm). The wrinkles of skin on the neck are well defined (Stage 27). Morphometric measurements taken from these embryos are summarised in Table 5.7.

Days 40-43; The yolk and the embryo are almost equal in volume (The diameter of the yolk is just around 20 mm) (Stage 28). Morphometric measurements taken from these embryos are summarised in Table 5.8.

Days 44-47; All hatchling pigmentation and morphology are present. The diameter of the yolk is >10 mm <20 mm (Stage 29). Morphometric measurements taken from these embryos are summarised in Table 5.8. A photograph of a stage 29 embryo is shown in Figure 5. 2E.

Table 5.7. Morphometric measurements (in cm.) of embryos at stage 26 and 27.

	26		27	
	Caretta caretta (mean ± S.E. N:125)	*Chelonia mydas* (mean ± S.E N:100)	*Caretta caretta* (mean ± S.E N:200)	*Chelonia mydas* (mean ± S.E N:175)
ID	0.357 ± 0.0096	0.417 ± 0.0034	0.615 ± 0.0052	0.964 ± 0.0066
FL	1.294 ± 0.0300	1.484 ± 0.0100	2.302 ± 0.0127	2.712 ± 0.0228
HIL	0.873 ± 0.0189	0.994 ± 0.0069	1.472 ± 0.0100	1.781 ± 0.0120
SCL	2.345 ± 0.0364	2.432 ± 0.0799	3.223 ± 0.0086	3.533 ± 0.0133
CCL	2.930 ± 0.0475	3.212 ± 0.0185	4.102 ± 0.0192	4.621 ± 0.0176
SCW	1.784 ± 0.0259	2.164 ± 0.0711	2.519 ± 0.0082	2.757 ± 0.0100
CCW	2.459 ± 0.0329	2.563 ± 0.0094	3.345 ± 0.0143	3.667 ± 0.0149
BL	0.718 ± 0.0119	0.929 ± 0.0063	0.942 ± 0.0075	1.254 ± 0.0058
HW	0.964 ± 0.0066	0.945 ± 0.0041	1.174 ± 0.0062	1.220 ± 0.0064
HL	1.504 ± 0.0123	1.451 ± 0.0033	1.759 ± 0.0020	1.918 ± 0.0036

Days 48-50; The remaining yolk mass is covered with pigmented membrane and the diameter of the yolk is smaller than 10 mm. The embryo breaks the eggshell with its caruncle at this stage (Stage 30). Morphometric measurements taken from these embryos are summarised in Table 5.8. A green turtle embryo at this stage can be seen in Figure 5. 2F.

Days 51-62; The yolk mass is mostly withdrawn into abdomen. The hatchling is outside the eggshell and ready to emerge or emerged (Stage 31). Morphometric measurements of either live hatchlings or dead in nest hatchlings were also taken from the beaches.

Table 5.8. Morphometric measurements of embryos at stage 28, 29 and 30.

	28		29		30	
	Caretta caretta (mean ± S.E. N:170)	*Chelonia mydas* (mean ± S.E N:150)	*Caretta caretta* (mean ± S.E N:160)	*Chelonia mydas* (mean ± S.E N:145)	*Caretta caretta* (mean ± S.E N:144)	*Chelonia mydas* (mean ± S.E N:125)
ID	0.766 ± 0.0059	0.921 ± 0.0056	0.802 ± 0.0048	1.050 ± 0.0067	0.872 ± 0.0074	1.121 ± 0.0035
FL	2.789 ± 0.0179	3.082 ± 0.0295	3.051 ± 0.0102	3.810 ± 0.0148	3.233 ± 0.0169	3.981 ± 0.0074
HIL	1.768 ± 0.0119	2.164 ± 0.0187	1.947 ± 0.0072	2.468 ± 0.0130	2.034 ± 0.0073	2.585 ± 0.0096
SCL	3.518 ± 0.0131	3.784 ± 0.0139	3.546 ± 0.0312	3.999 ± 0.0129	3.846 ± 0.0205	4.251 ± 0.0179
CCL	4.621 ± 0.0198	5.052 ± 0.0276	4.927 ± 0.0198	5.303 ± 0.0261	5.227 ± 0.0185	5.534 ± 0.0122
SCW	2.877 ± 0.0084	3.155 ± 0.0193	3.159 ± 0.0172	3.417 ± 0.0117	3.352 ± 0.0152	3.657 ± 0.0055
CCW	3.792 ± 0.0193	4.045 ± 0.0195	3.931 ± 0.0097	4.264 ± 0.0121	4.306 ± 0.0224	4.525 ± 0.0138
BL	1.022 ± 0.0078	1.326 ± 0.0074	1.119 ± 0.0171	1.349 ± 0.0060	1.082 ± 0.0090	1.367 ± 0.0076
HW	1.354 ± 0.0061	1.421 ± 0.0097	1.445 ± 0.0042	1.512 ± 0.0042	1.507 ± 0.0077	1.544 ± 0.0025
HL	1.898 ± 0.0022	2.094 ± 0.0123	1.971 ± 0.0033	2.088 ± 0.0064	1.996 ± 0.0081	2.179 ± 0.0020

From these observations, the only reliable measurement that can be taken was found to be the crown-rump length for embryos staged 6-20 and straight carapace length for embryos staged 21-31. There was also some most significant changes in the characteristics in embryos for each stage. I summarise, in the table below, a simple stageing guide for Mediterranean sea turtles.

Table 5.9. A simple stageing guide for sea turtles in the Mediterranean, (DD: days of development; CRL: Crown-rump length, SCL: Straight carapace length, measurements in cm.).

		C. caretta	*C. mydas*	Characteristics
Stage	DD	CRL	CRL	
6	0-1	0.16	0.17	anteriorly opening or transverse blastopore, no white chalking on the shell
7	1	0.21	0.21	posteriorly opening blastopore, white chalking starts on the shell
8	1.5	0.23	0.24	'n' shaped blastopore, white area enlarges <1.5 cm
9	2	0.24	0.24	head fold indicated, white area covers less than half of the egg
10	3	0.25	0.25	neural fold present, few somites
11	3.5	0.25	0.25	5-6 somites
12	4	0.33	0.37	8-10 somites, amnion covers 1/2 embryo
13	4.5	0.42	0.43	12-14 somites, heart is present
14	5	0.48	0.47	15-17 somites, tail bud is formed
15	6	0.55	0.52	19-21 somites, 1. pharyngeal clefts open, amnion complete
16	7-8	0.63	0.53	23-27 somites, 2. pharyngeal clefts open, limb buds are initiated, blood islands visible
17	8-9	0.63	0.68	29-34 somites, all pharyngeal clefts open, tail is elongates, lens visible in the eye
18	10	0.78	0.76	35-40 somites, closing of pharyngeal clefts starts, limb buds form digital plates.
19	11-12	0.78	0.77	>40 somites, tail is elongates, limb buds have paddles.
20	13-14	0.95	0.88	digital plates twisted flat against body, iris is not pigmented.
		SCL	SCL	
21	15-17	0.84	0.88	carapace rudiment indicated laterally, all pharyngeal clefts closed.
22	18-20	0.94	0.99	limb defined completely, marginal carapace have marked, tail is longer than hindlimb.
23	21-23	1.36	1.12	posterior of carapace and digital serrations indicated, ribs are visible.
24	24-27	1.38	1.60	anterior of carapace and digital serrations indicated but no scales present.
25	28-32	1.89	2.00	carapace completed with scutes, tail=hindlimb in length.
26	33-36	2.34	2.43	head and flipper scales present.
27	37-39	3.22	3.53	Diameter of yolk >20mm
28	40-43	3.51	3.78	Diameter of yolk ≅20mm
29	44-47	3.54	4.00	Diameter of yolk >10mm<20mm, hatchling pigmentation is present.
30	48-50	3.84	4.25	Pipping stage, diameter of yolk <10mm
31	51-62	3.97	4.51	Hatching stage, hatchling is outside of eggshell.

Table 5.10 gives some of the data recorded on hatchlings. As can be seen from this table, hatchling size may also vary from beach to beach. The mean of the measurements of the first emerged group of loggerhead hatchlings and last emerged hatchlings and dead in nest hatchlings from all the nests showed there is a statistically significant ($t=3.00$, $p<0.01$) difference between them, with the last emerged hatchlings being smaller.

Table 5.10. Some of the results of morphometric measurements of hatchlings on different beaches.

Species	Beach	Sample size	SCL± S.E.	SCW ± S.E.
C. caretta	Akdeniz	169	4.105 ± 0.0321	3.174 ± 0.0260
C. caretta	Fethiye	223	3.881 ± 0.1880	2.998 ± 0.1420
C. caretta	Patara	217	3.943 ± 0.0185	3.089 ± 0.0197
C. mydas	Akdeniz	156	4.513 ± 0.0388	3.477 ± 0.0243

5.3.3. Scanning electron microscopy:

Morphological changes on limbs, tail and head development observed externally on the embryos were examined under SEM for early stage embryos. Very few early stage embryos were examined with SEM, and the descriptions of the embryos were also obtained from other embryos examined with the light microscope. Some photographs of these embryos can be seen in Figure 5.3.

5.3.3.1. Head development;

The head fold is first visible as a semicircular area at the apex of the neural groove which appears as a shallow channel at the end of first day, and in day two, it forms a crescent anterior of the neural groove. The neural folds are fused behind the head and optic vesicles are present lateral of the head on the third day of development. By days four and five, the otic vesicles are just visible at the posterior site of the head and right ventral of this; the first pharyngeal groove is also evident. The mouth of the embryo shows a very small opening by day six and the lens is also formed in the eye. The second and third pharyngeal clefts are also indicated as grooves. In days seven and eight, the first three pharyngeal clefts are open. The first blood islands are also visible. By day nine, all the (1-5) pharyngeal clefts are open, and the retina of the eye is slightly pigmented. A flap of skin has developed on anterior border of each pharyngeal cleft by day ten.

Figure 5.3. The early development of sea turtles and development of head, limb and tail observed using scanning electron microscopy. (x magnification)

A-) Overview of a 3 days *C. mydas* embryo. (x13).

B-) Overview of a 4 days *C. caretta* embryo. (x 16).

C-) Overview of a 5 days *C. mydas* embryo. (x 16).

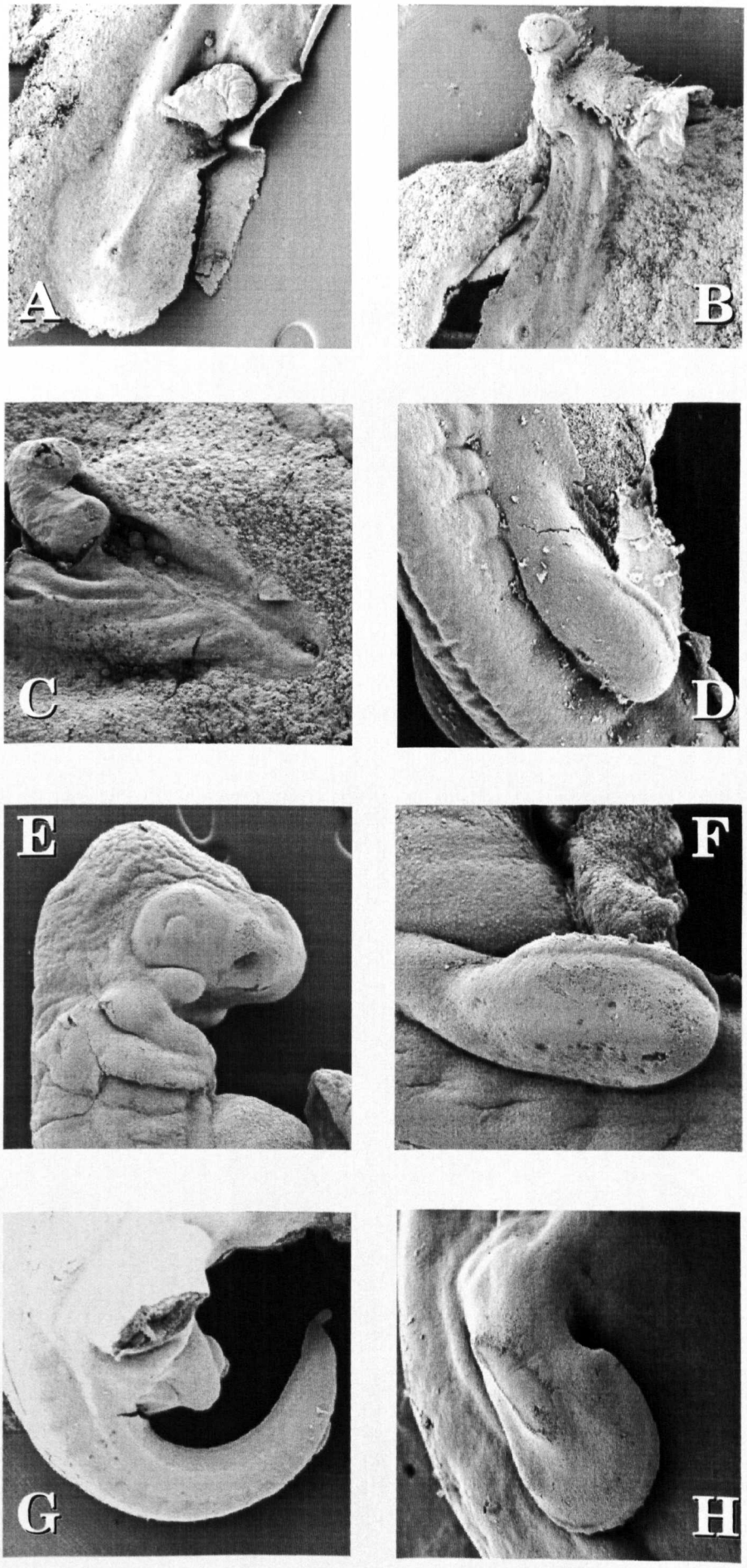
D-) Hindlimb development at 10 days *C. caretta* embryo. (x 32).

E-) The development of head, eye, nose and pharyngeal clefts at 12 days, *C. mydas* embryo. (x 16).

F-) Forelimb development at 12 days, *C. mydas* embryo. (x 29).

G-) Tail development of *C.caretta* at 18 days. (x 16).

H-) Forelimb development of *C.caretta* at 18 days. (x 16).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The snout, eye and differentiation of brain is very clear at the age of 12 days (Figure 5.3E). The iris is fully pigmented at 25 days old embryos (Figure 5.2B). The caruncle is slightly visible at this stage. The scales on the head are indicated and show the hatchling characters at the age of 35 days (Figure 5.2D).

5.3.3.2. Limb development:

The initiation of limb bud development has started at one week old as a small swelling of the body wall; the bud lengthen along an axis that is first perpendicular then becomes oblique to point caudally along the surface of the body at later stages. The base of the anterior limb bud elongates first and projects latero-posteriorly from the lateral body wall at 9 days old. The distal part of the limb shows a form of digital plates and apical ridge by 10 days (Figure 5.3D), and has definite paddles and extends laterally from the body wall at 12 days old (Figure 5.3F); each digital plate is bounded by a distinct apical ridge. A distal ridge bounds the digital plate and a proximal ridge delimits the limb at 18 days old (Figure 5.3F,H). Claws are starting to develop on the first digits at day 28 (Figure 5.2C).

5.3.3.3. Tail development:

A small tail bud starts developing at five days (embryo is 4-5 mm in length) and in the following days the tail process extends beyond the base of the hindlimb buds. At nine days, the tail is long, straight and blunt. The tail curls anterio-ventrally and typically has a terminal outward kink at 11 and 12 days. The tail is longer than the hindlimb at 18 days and kinked inwards (Figure 5.3F,G). The tail becomes equal in length to the hindflippers at the age of 28 days (Figure 5.2C). The measurement of tail is difficult since it curls and become a 'C' shape during the early stages of development. Because there were few data on tail length in relation to total body length and hindlimb lengths for both species, comparision of these characters during the embryonic development was not possible. This is mainly because of the difficulty of measuring the tail which shows "C" shaped in early stages. A straight-line length measurement of tail was also meaasured when it is possible and the following graph were plotted by combining the data from both species for 31 early stage samples (Figure 5.4).

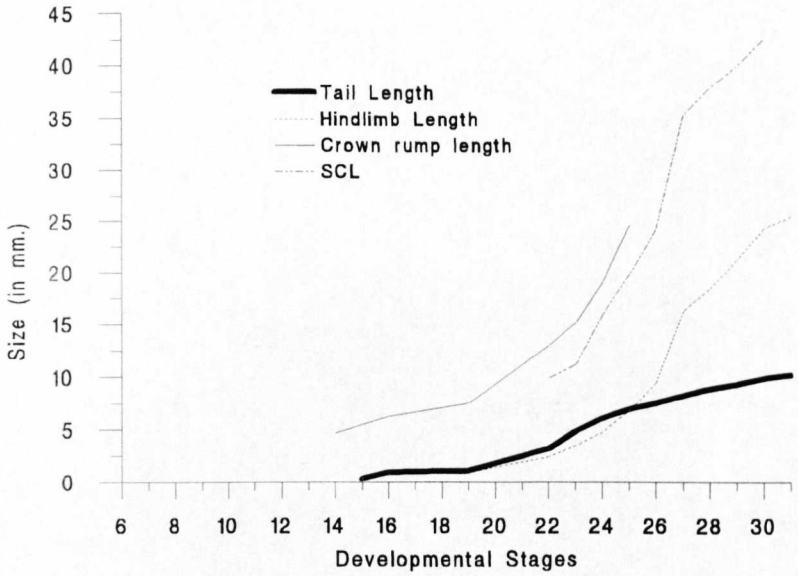


Figure 5.4. The measurement of tail, hindlimb, crown-rump and straight carapace lengths of the embryos (Data combined from both species).

To illustrate growth, measurements were plotted in the Figure 5.5. Only the measurements of crown-rump length, straight carapace length, forelimb length and head width were plotted. Typically, the rate of change is slow at first but accelerates later in the incubation period and slows again during the days before hatching (Figure 5.5). From these observations, the only reliable measurement that can be taken was found to be the crown-rump length for embryos staged 6-20 and straight carapace length for embryos staged 21-31 (Table 5.6).

5.3.4. Abnormal development and twinning:

Table 5.11 presents the summary data on the number and frequency of occurrence of different abnormalities observed. Typically an embryo exhibited more than one malformation.

The most common form of abnormality was supernumerary and /or subnumerary scutes (8 % of all embryos) on the carapace among normally pigmented embryos as well as among the albino forms. Albino forms were 1% of the total embryos. Malocclusion of jaw, lack of nostril, caruncle, eye (Figure 5.5F) and limb and head deformities (2 % of the embryos) were among other common forms.

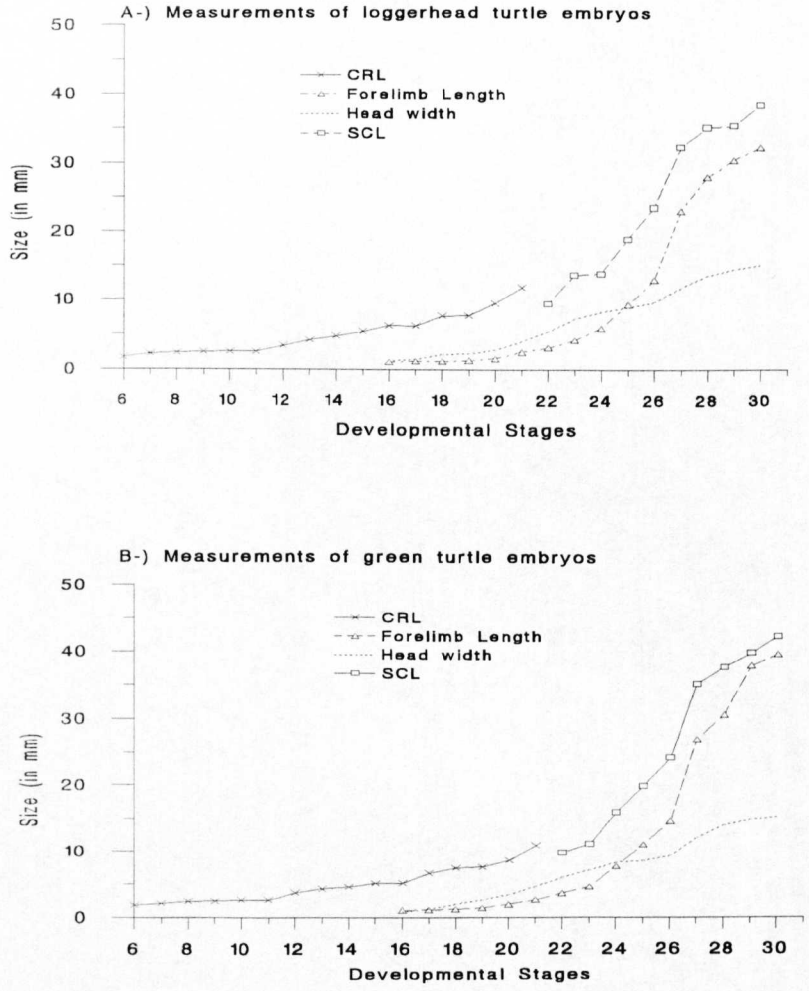


Figure 5.5. The plot of the measurements of turtle embryos.
(CRL: Crown-rump length and SCL: Straight carapace length).

Table 5.11. The number and frequency of the abnormalities observed on sea turtle embryos.

		Caretta caretta			*Chelonia mydas*		
Abnormality observed	Σ %	Sampled N:75	Predated N:270	Dead-in-shell N: 1537	Sampled N:35	Predated N:105	Dead-in-shell N:1029
supernumerary	4.0		12	30		35	45
subnumerary	4.0		5	42		29	49
Head abnorm.	2.1		5	20		10	28
Jaw,nostril,eye	1.6			17			33
Albino	1.0			9			22
Twinning	1.0			13	1		16

Twins (0.1 % of the embryos) of equal size and twins of unequal size were also detected. Twins of equal size were of normal pigmentation and of late stage (26-29), but each was smaller in carapace length than a normal single embryo of the same stage. Unequal sized twins had one member which was much smaller than the other. Both were fully pigmented. Early stage twins were also detected (Figure 5.6 A,B,C,D). 'Siamese' embryos were also detected. These are the embryos show a single body with joint two heads (Figure 5.6E). Head and scute abnormalities were much more common in albino embryos in comparison with non-albinos, because all the albino embryos showed abnormalities in their head, jaw, eye and nostril, whereas only 9.6% of the non-albinos showed head and scute abnormalities (Table 5.11 and Figure 5.7).

Figure 5.6. Twinnings and abnormal development of sea turtles.

A-) Early stage (14) *C. mydas* twinnings (Opposite orientation). Bar equals 50μm.

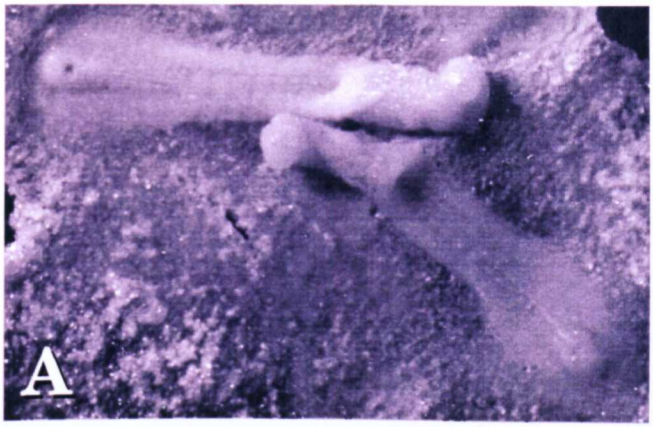
B-) Unequal sized twinning- late stage *C. caretta*. Bar equals 2 cm.

C-) Unequal sized twinning- late stage *C. mydas*. Bar equals 0.5 cm.

D-) Equal sized twinning- late stage *C. mydas*. Bar equals 0.5 cm.

E-) 'Siamese' twinning in a *C.caretta* embryo. Bar equals 1 cm.

F-) Abnormal *C. mydas* embryo with one eye and abnormal nose and mouth development. Bar equals 1 cm.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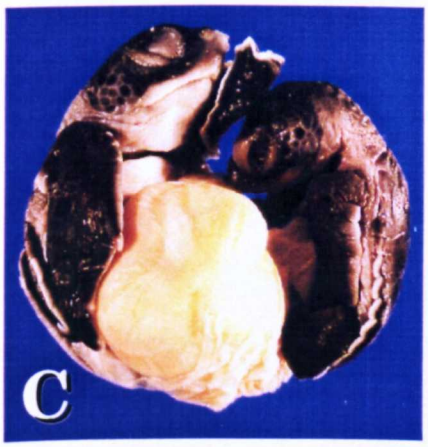
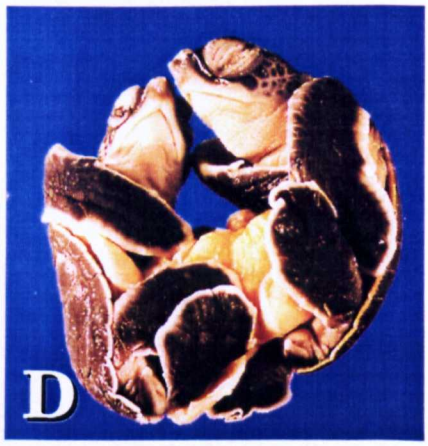
B


C


D


E


F

Figure 5.7. Albino embryos and some examples of abnormal development. Bars are equal to 1 cm.

A-) Head and face abnormalities.

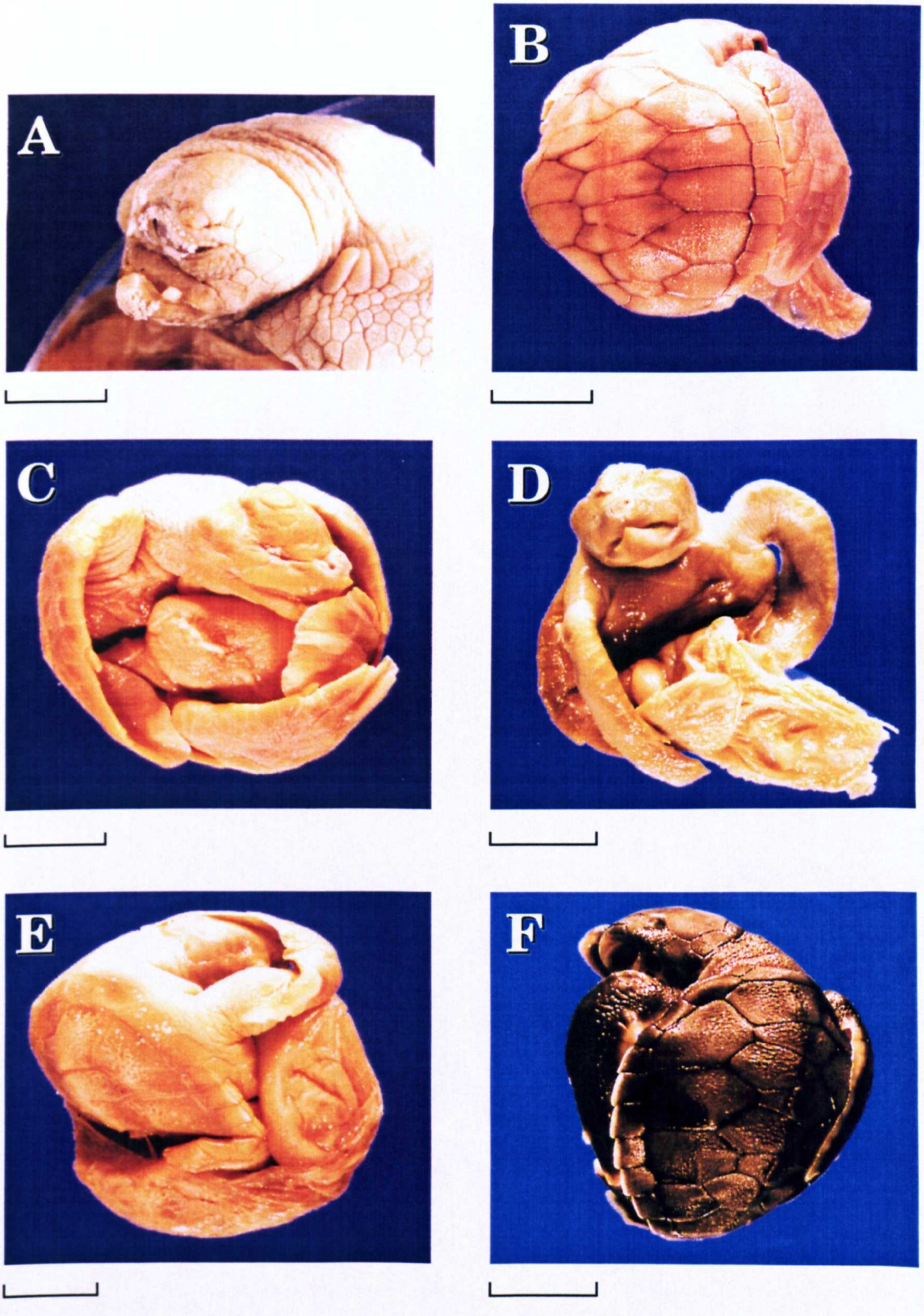
B-) Supernumerary of neural scutes and/or subnumerary costal scutes.

C-) Face and head abnormalities and unusual long limbs.

D-) Face and jaw abnormalities.

E-) A "Saddleback" abnormality of carapace.

F-) Supernumerary of neural scutes on a non-albino embryo.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5.4. Discussion:

The reproductive biology of sea turtles is quite similar across species, and therefore similar developmental characters occur in both loggerhead and green turtle eggs and embryos (Miller, 1985). The size of normal eggs varies among the turtle species and between populations (see Ewert, 1979, 1985; Miller, 1985). Larger eggs are heavier and produce larger hatchlings yet differences are reported in the proportion of shell, yolk and albumen in ovipositional eggs of different species. For example, it has been reported that the shell contributes about 4.3%, the yolk about 67.3%, and the albumen about 28.4% to the total weight of green turtle eggs and 5.8, 55.0, and 39.2 % respectively for loggerhead turtle eggs (Tomita, 1929 in Miller, 1985). The components of eggs were slightly different between the two species in my work. I found 4.7% shell, 43.1% albumen and 52.2% yolk in green turtle eggs and 4.6% shell, 44.4% albumen and 51% yolk in loggerhead turtle eggs. Thus for both species the percentage yolk was higher in eastern Mediterranean eggs than in those reported by Miller (1985), samples from elsewhere and see also Ewert (1979; Table 7) for a review on turtles.

The descriptions of Agassiz (1857) of sea turtle development and Crastz (1982) and Miller (1985) show slight differences between them, compared with the fresh-water turtles (Yntema, 1968; Mahmoud *et al.*, 1973). The first difference between them was the number of stages used by each researcher. The other differences among the descriptions result from the differences in the degree of development of the characteristics used, the rate of development and the temperature of incubation. The post-ovipositional developmental characteristics described in present study correlated very closely with Miller and Crastz. The work of Yntema (1968) on *Chelydra serpentina* is similar apart from the very early stages he used.

Agassiz (1857) examined an incomplete series of 7 *C. caretta* embryos in the context of a comparison with other species. Parker (1880) defined five stages in the development of *C. mydas*; these ranged from 27 somites to near the hatching stage. Deraniyagala (1939) published the details of four stages of *C.mydas* and *C. caretta*; these ranged from 16 to 37 days of incubation. Penyapol (1958) provided the most complete series on *C.mydas* which included 7 stages ranging from approximately 15 days to approximately 37 days. The early development of *C.caretta* was first described by Fujiwara (1966). Domontay (1968) described changes in the appearance of the egg during incubation and presented notes on development of 20 *C.mydas* samples. Deraniyagala (1939) worked on five, Crastz (1982) on one and Miller (1985) on six sea

turtle species. Miller (1985) found some developmental differences between the six sea turtle species, but not between the two species that I worked on. My results were not exactly comparable with the earlier works but quite similar with Miller's work. The slight differences were probably the result of comparing the eggs sampled from the field with ones incubated artificially under constant temperature. Another difference would be the early stages. For example a one day embryo may show the characteristics of stages 7 and 8 of Miller (1995). Similarly, a three day old embryo may show the characteristics of stages 10 or 11, a four days old embryo to show the characteristics of stages 12 or 13, and an eight day old embryo may show either stage 16 or 17. These differences may be because the days of development for early stages are very close. There is a temperature difference in the nest and in surrounding sand (Chapter 3), and also differences in the total incubation period, since the nesting grounds of both populations are quite isolated and far from each other. Temperature influences the duration of incubation and the rate of development (Miller, 1985). Low temperatures increase the duration of incubation and slow the rate of development, wheres high temperatures decrease the duration and increase the rate (Miller, 1982). For example in *Chelonia mydas*, incubation lasted 94 days at 23-25°C (Ackerman and Prange, 1972) and 47-49 days at 32°C (Bustard and Greenham, 1968). Heat increases the rate of development and cold retards it in most turtle embryos (Yntema, 1978; Kam and Lillywhite, 1994; Miller, 1985; Packard *et al.*, 1987). Temperature, hydric environment, and gas exchange act synergistically to embryo metabolism in fresh turtle embryos (Gettinger *et al.*, 1984; Packard *et al.*, 1987), and this may be similar for sea turtles. Sea turtles have a tolerance of development over a range of about 9 °C (Miller, 1985). Temperature two or three degrees higher or lower than normal range may cause malformation or death (Yntema, 1960; Bustard, 1971; Miller, 1985; Rosenburg and Kelly, 1996; Ciofi and Swingland, 1997).

Plots of data obtained from the measurement of most morphological characters follow a more or less sigmoidal shape (Figure 5.4 and 5.5). Typically, the rate of change is slow at first but accelerates during some portion of the incubation period, and slows again during the days before hatching.

Abnormalities of the scutes of the carapace and cephalic deformations are very common in my work and have been reported elsewhere previously (Bustard, 1972; Ewert, 1979; Miller, 1985; Kaska, 1993). Twinning among turtle embryos has also been reported (Yntema, 1970, 1971; Ewert, 1979; Fowler, 1979; Blanck and Sawyer, 1981; Miller, 1985; Whitmore and Dutton, 1985; Eckert, 1990; Peters *et al.*, 1994; Tucker and

Janzen, 1997). The percentage of twinning reported in these studies is between 0.01 % and 0.05 %. My result at 0.1 % is well above these, although there is no obvious reason for this difference. Albino embryos have also been reported previously (Bustard, 1972; Miller, 1985). In this study, head and scute abnormalities were more common in albino embryos than in non-albino ones. The abnormalities observed among sea turtle embryos may be because of incubation conditions as well as genetic factors, but this is still unknown. Some malformations, such as albinism, cleft plate, and even absence or reduction of one flipper are not necessary lethal. Many aberrant embryos die in early stages in development. Of the aberrant turtle embryos which do develop, most are unable to break out of their eggs as a result of their deformities. Probably, because of this, many abnormal embryos were found dead in shell.

Chapter 6. Genetic structure of Mediterranean sea turtle populations.

This chapter examines the genetic diversity of Mediterranean sea turtles, using samples from the west coast of Northern Cyprus, by determining mtDNA control region sequences. These sequences were also compared with the reported results in Genbank, and polymorphic sites were determined.

6.1. Introduction:

The natal homing hypothesis for the reproductive migration of sea turtles suggests that females return to breed and nest at the same beach from which they had hatched (Carr, 1967). Hatchlings and juveniles move among several habitats during development. Adults migrate between feeding and nesting grounds that are hundreds or thousands of kilometers apart, and both movements are difficult to track in the marine environment (Carr, 1980). Much of what is known about the life history of sea turtles has come from tagging experiments on nesting females.

The herbivorous green turtle is distributed circumglobally in tropical and subtropical oceans (Carr, 1967). The carnivorous loggerhead turtle occurs in the Mediterranean, Atlantic, Indian, and Pacific Oceans (Dodd, 1988). Nesting habitat for loggerhead turtles and green turtles overlap in some areas, for example the eastern Mediterranean. Only small populations of green turtles and loggerhead turtles nest in the Mediterranean, approximately 400 green turtle and 2000 loggerhead turtle females per season (Groombridge, 1990). One prediction of the natal homing hypothesis is that each nesting colony should comprise a group of isolated maternal lineages, as females assort themselves according to their natal beach (Carr, 1967). Hendrickson (1958) proposed an alternative explanation for female nest site fidelity, called the social facilitation hypothesis. Under this hypothesis, first-time nesting females follow experienced breeders from the feeding grounds to a nesting beach, and use this site for all subsequent nestings. Both of these hypotheses have proven difficult to test directly, as no tag applied to a hatchling has been recovered from an adult. Natal site philopatry, however, generates a testable prediction about the genetic structure of populations. If females return faithfully to their natal beach, then each nesting population should be effectively isolated in terms of female transmitted traits (such as mtDNA). In contrast, social facilitation would result in high rates of female-mediated gene flow between the beaches that share feeding grounds.

In recent reports, analyses of maternally transmitted mitochondrial DNA (mtDNA) have proven useful for resolving questions about nesting behaviour and population demography in sea turtles. mtDNA has the virtue of a maternal, nonrecombining mode of

inheritence, rapid pace of evolution, and extensive intraspecific polymorphism. It is tightly packed with genes for 13 messenger RNAs, 2 ribosomal RNAs, and 22 transfer RNAs. In addition to these 37 genes, an area known as the "D-loop", roughly 0.8 kilobases long, appears to exercise control over mtDNA replication and RNA transcription (Avise *et al.*, 1987). Patterns of variation of mtDNA have been used extensively for the study of population genetic structure, phylogeographic relationships, and other aspects of molecular ecology of various organisms (for reviews see Avise 1994; Moritz *et al.*, 1987). More recently, much attention has also been given to the application of mtDNA markers in the management of endangered or threatened species. In many cases, mtDNA studies have delineated the structure of populations, and thus have provided guidance into the level at which management priorities should be set for the protection of a particular species. In the case of sea turtles, mtDNA surveys of breeding colonies have focused primarily on the delineation of demographically independent population units with significance for conservation.

Bowen *et al.* (1992) have tested the natal homing hypothesis for 15 colonies of green turtles in the Pacific and Atlantic regions using restriction-fragment-length-polymorphisms (RFLP) of mtDNA. Their study identified significant differences among most colonies, thereby extending the earlier conclusions based on mtDNA analyses in favour of the natal homing hypothesis (Meylan *et al.*, 1990). Allard *et al.* (1994) and Lahanas *et al.* (1994) applied analysis of mtDNA control region sequences to problems in green turtle biology and more recently Encalada *et al.* (1996) employed mtDNA control region sequences to assess the population genetic structure and phylogeography of green turtles in the Atlantic Ocean and Mediterranean Sea. Schroth *et al.* (1996) also looked at the genetic diversity of the loggerhead turtle population in the Mediterranean. Their study showed that colonies of turtles separated by only 10s of km are genetically distinct and also female natal homing is precise with limited gene flow between turtle colonies is male-mediated.

The purpose of this study was to determine whether the Mediterranean populations of green turtles and loggerhead turtles are genetically distinct from their Atlantic relatives. Following published work (Encalada *et al.*, 1996 and Schroth *et al.*, 1996) with the same aim, and other mtDNA control region sequences presented at Genbank, my goal became a further exploration of the diversity present in the Mediterranean which I hoped would help refine estimates of the rate of genetic exchange with the Atlantic populations.

6.2. Materials and Methods:

6.2.1. Collection of tissues and preparation of DNA:

Tissues were dissected from 17 green turtle and 10 loggerhead turtle hatchlings from west coast beaches of Northern Cyprus (Figure 1) during the hatching season of 1995, either from the hatchlings sacrified for sex determination purposes or from hatchlings which had been found dead in the nest column during nest excavation after hatching. One sample per nest was taken. Heart, liver and brain samples from the hatchlings were dissected and preserved in absolute alcohol and stored at room temperature. Total DNA isolations from heart samples were conducted by digesting with proteinase K at 50 °C for 4 h. Contaminating proteins were removed by sequential extraction with equal volumes of phenol-chloroform and the DNA recovered from solution by ethanol precipitation in the presence of 1.25 M ammonium acetate and resuspended in TE buffer (10 mM Tris.Cl (pH: 8.0), 1 mM Ethylene Diamine Tetra Acetic acid [EDTA]) (Sambrook *et al.*, 1989).

6.2.2. Polymerase Chain Reaction:

The Polymerase chain reaction(PCR) is an in vitro technique which allows the amplification of a specific DNA region that lies between two regions of known DNA sequence. PCR amplification of DNA can be achieved by using oligonucleotide primers (Newton and Graham, 1994). These are short, single-stranded DNA molecules which are complementary to the ends of a defined sequence of DNA template. The primers are extended on single-stranded denatured DNA (template) by a DNA polymerase, in the presence of deoxynucleoside triphosphates (dNTPs) under suitable reaction conditions. This results in the synthesis of new DNA strands complementary to the template strands. These strands exist at this stage as double-stranded DNA molecules. Strand synthesis can be repeated by heat denaturation of the double-stranded DNA, annealing of primers by cooling the mixture and primer extension by DNA polymerase at a temperature suitable for the enzyme reaction. Each repeat of strand synthesis comprises a cycle of amplification. The target sequence product (amplicon) which is obtained contains the oligonucleotide primer sequences at its ends. Basic components of a PCR amplification are template DNA (1 µl), reaction buffer (2.5 µl), magnesium, dNTPs (2 µl each), 2 oligonucleotide primers (2 µl each) and DNA polymerase (0.2 µl) and optional additives such as Bovine Serum Albumin (BSA) (0.3 µl) and 15 µl distilled water make up 25 µl reaction mixture. The reaction components are overlaid with mineral oil and the tube placed in a thermal cycler. This is basically to prevent evaporation in the majority of the

thermal cyclers which do not heat the lids of the reaction tubes. The oil also helps prevent sample to sample contamination. The thermal cycler is an automatic instrument that takes the reaction through a series of different temperatures for varying amounts of time. This series of temperatures and time adjustments is referred to as one cycle of amplification. Each PCR cycle theoretically doubles the amount of targeted template sequence (amplicon) in the reaction. A typical temperature cycling profile has a three-step protocol. The first step is denaturation of template DNA at 95-100 °C for 15 seconds to 2 minutes. In the denaturation process, the two interwined strands of DNA separate from one another, producing the necessary single stranded DNA template for the thermostable polymerase. The next step is the annealing of primers. It is possible to anneal the oligonucleotide primers to the denatured template by lowering the temperature to 37-65 °C depending on the Tm (melting temperature) of the oligonucleotide primers. At this temperature, the oligonucleotide primers can form stable associations (anneal) with the separated target DNA strands and serve as primers for DNA synthesis by a thermostable DNA polymerase. This step lasts approximately 30-60 seconds. The last step is extension of the primers. This is usually performed at 72 °C, which is the optimum temperature for *Taq*/Amplitaq DNA polymerases. Thermostable DNA polymerases are enzymes (The thermostable DNA polymerase from *Thermus aquaticus* **(Taq)** has been the most extensively used enzyme in PCR) which catalyze the synthesis of long polynucleotide chains from monomer deoxynucleoside triphosphates synthesis of a new complementary strand. DNA synthesis always proceeds in the 5' to 3' direction since the polymerization is always from the 5' α-phosphate of the deoxynucleoside triphosphate to the 3' terminal hydroxyl group of the growing DNA strand. Deoxynucleoside triphosphates (dNTPs) used in natural DNA synthesis normally comprise deoxyadenosine triphosphates (dATP), deoxycytidine triphosphates (dCTP), deoxyguanosine triphosphates (dGTP) and deoxythymidine triphosphates (dTTP). These dNTPs attach to the free 3'-hydroxyl group of the primer and from a strand complementary to the template strand.The synthesis of new DNA begins when the reaction temperature is raised to 72 °C. Extension of the primer by the thermostable polymerase lasts approximately 1-2 minutes. These three steps constitute one cycle of PCR. The number of cycles is usually between 25 and 35. With increasing cycle numbers it is common to observe an increase in the amount of unwanted artificial products and no increase in the desired product.

6.2.2.1. Primers:

Four primers were used in this work. LTCM1 (5'-CCC AAA ACC GGA ATC CTAT-3'), LDCM1 (5'-AGT GAA ATG ACA TAG GAC ATA-3'), and HDCM1 (5'-ACT ACC GTA TGC CAG GTTA-3') developed by Allard *et al.* (1994) and LTCM2 (5'-CGG TCC CCA AAA CCG GAA TCC TAT-3') and HDCM2 (5'-GCA AGT AAA ACT ACC GTA TGC CAG GTT A-3') developed by Encalada *et al.* (1996). These primers were designed to target an area of 510 basepairs of the 5' end of the control region. It is necessary first to calculate the molar extinction coefficient of the primer at 260 nm. The molar extinction coefficient is equivalent to the absorbance at 260 nm (A260) of a 1 M solution of primer. Dividing the A260 of the primer stock solution by the molar extinction coefficient will give the molar concentration of the primer.

6.2.2.2. Analysis of PCR products:

PCR products consist of fragments of DNA which are normally of a length defined by the boundaries of the PCR primers. PCR products are generally less than 10 kbp in length. For identification of PCR products, the most commonly used method is electrophoresis of an aliquot of the PCR on an agarose gel (0.25 mg Agarose in 30 ml 1xTBE buffer) and visualization by staining with ethidium bromide, which is a fluorescent dye that intercalates into the DNA. After staining, ultraviolet transillumination allows visualisation of the DNA in the gel which can be recorded by photography.

6.2.2.3. Sequencing PCR products:

Dideoxy sequencing is the most commonly used method for sequencing the PCR products. Sequencing is carried out using a DNA polymerase to extend a primer along a single-stranded template in the presence of the four dNTPs. The DNA to be sequenced can be double- or single- stranded. If it is double-stranded DNA, then the strands have to be separated by thermal denaturation. Single-stranded DNA can be used directly in the sequencing reaction. Amplified double-stranded mtDNA was purified with Amicon centricon centrifugal microconcentrators (Centricon-100 and 30). The sequencing reaction is terminated in a random fashion by the incorporation of a dNTP analog, a dideoxynucleoside triphosphate (ddNTP), producing DNA chains of varying length that all terminate with the same 3' base. These are separated by high-resolution polyacrylamide gel electrophoresis. In order to detect these chains, the most common methods employ the incorporation of a radiolabel or a fluorescent label.

6.2.2.3.1. Sequencing Protocol of Single Stranded PCR products:

For sequencing by radiolabelling, the following steps were followed.

1-) For each DNA sample to be sequenced make up an eppendorf tube containing:

6 µl single strand PCR product (6x concentrate)

1 µl primer (10 µM); use opposite primer to the one used in PCR

1 µl DMSO (Dimethyl sulfoxide)

2 µl 5x sequenase buffer

place samples in a a heating block which is heated >80 °C and allow to cool slowly to room temperature.

2-) Make up labelling mix (enough for all samples +1), for each sample

1 µl DTT	Enzyme mix (for each sample +1)
2 µl G-mix or I-mix	1.625 µl enzyme dilution buffer
2 µl enzyme mix	0.25 µl Sequenase
0.25 µl ^{35}S-dATP*	0.125 µl PPase

* this was added last, since after this point the labelling mix is radioactive.(double gloves, safety glasses)

3-) 2.5 µl of each ddNTP dispensed into a 96 well plate, one set of four for each sample for both G-mix and I-mix. A plastic adhesive film was placed over the wells. 20 ml of stopping dye was put in a well nearby and stored at -20 °C until needed.

4-) 5.25 µl labelling mix were added to each sample tube and spun down briefly(5 sec.) in a microfuge, then left to sit for 3 minutes at room temperature. The 96 well plate of ddNTPs was placed onto the platform in the water bath which was preheated to 42 °C.

5-) 3.5 µl of labelling mix was dispensed into the four wells containing ddNTPs and covered with adhesive plastic, and lead brick, and incubated at 42 °C for 3 min.

6-) 4 µl of stopping dye was dispensed into each well, from the local stocks already established covered and labelled with Radioactive tape and then stored at -20 °C. When the gel was ready (50 °C) and the water bath was at 90 °C, it was incubated for 3 minutes in the water bath and 3 minutes on ice and then 3 µl of the samples was loaded. For Acrylamide gel; 40 g Urea, 28 ml dH_2O, 12 ml Acrylamide, 8 ml 10xTBE were mixed until urea dissolved and then 320 µl of 25% APS and 40 µl TEMED added. Then the gel was poured, the comb inserted backwards and clamped to the top and left overnight or for a minimum of 3 hrs.

7-) The gel was prepared for loading and the comb placed combwells downwards. 1xTBE buffer was put to the top and watched for linkage to the bottom and bubbles were removed by injecting the buffer. Some stopping dye was added into every second well and

omitted from the linking wells and the loading order was noted on to the glass. The gel was run with program 3 (90 Watt) until the gel temperature goes up to 50 °C and after loading, with program 2 (60 Watt).

8-) After the 3 or 7 hours run, 2 filter papers were cut the same size as the gel. After taking off the upper glass, one of the filter papers was put onto the gel and pressed gently until the gel stuck to the paper. Another paper was also put to the underneath of the gel and the extra sides were trimmed. The gel was covered with plastic and dried for 2 hrs. in a vacuum dryer.

9-) After drying, the gel was placed in a tray and put with a film under the red light and left for 3 days, and then developed on the developing machine.

For sequencing by fluorescent labelling, the following steps were followed.

1-) By using the 6 times concentrate double-strand PCR was prepared by Centricon 100, 2.8 µl distilled water, 3.2 µl primer (1 µM), 6 µl PCR product and 8 µl terminator ready reaction mix added and one drop of mineral oil was added and then run for 24 cycles PCR.

2-) Tubes were prepared by adding 2.0 µl 3M Sodium acetate and 50 µl 95% ethanol and the entire PCR product transferred into these tubes and then left on ice for 10 minutes. Then it was centrifuged for 15 minutes at maximum speed in a microcentrifuge.

3-) The ethanol was aspirated and the pellet was rinsed by adding 250 µl 70% ethanol and then aspirated again. The pellet was dried in a vacuum centrifuge for 8 minutes.

4-) The pellet was resuspended with only enough loading solution (5:1 deionised formamide and 50 mM EDTA to bring the sample volume up to a final volume of 4-5 µl) and then centrifuged briefly.

5-) The samples were heated at 90 °C for 2 min. and incubated on ice until loading to a 4.5% acrylamide gel and analysed with an automated DNA sequencer.

Two green turtle and one loggerhead turtle samples were sequenced by following the radiolabelling protocol, and other samples were sequenced with fluorescently labelled primers and analysed with an automatic DNA sequencer (Applied Biosystems model 373) and individual sequences were then aligned by eye.

6.3. Results and Discussion:

The sequences that were obtained, for each species, can be seen in Figure 6.1. All the samples belong to one species showed the same pattern of sequence from both type of

sequencing method used. The samples were aligned for 487 bases from the 5' end of the control region. There were no polymorphisms among the 17 green turtle samples. This finding lowers the estimated genetic diversity for the green turtle population nesting on Cyprus and fails to detect any genetic exchange with the Atlantic population. Encalada *et al.* (1996), in the study of phylogeny and population genetic structure of Atlantic and Mediterranean green turtle populations, found 18 haplotypes (accession numbers in Genbank, Z50124-Z50140) among 147 individuals from nine nesting populations by sequencing the mtDNA control region. All my green turtle samples showed haplotype XIII of Encalada *et al.* (1996). It would be interesting to look at the samples from the green turtles nesting on the Turkish beaches in order to look for other possible haplotypes in the Mediterranean, since both my samples and those of Encalada *et al.*, (1996) were only from Cyprus.

Schroth *et al.* (1996), in their study of DNA sequence analyses of the mitochondrial region of loggerhead turtle in the Eastern Mediterranean, found 11 haplotypes (GenBank accession number U72747) by sequencing 518 base pairs from 30 individuals including from Cyprus.They also reported briefly that there are haplotypes which were limited to geographically well-defined areas, but they did not report the haplotype distribution within the Mediterranean.Their study also suggested that there are genetic differences not only between coastal areas (for example, Greece and Turkey), but also between colonies from adjacent nesting beaches. The sequences of 10 samples in this study also showed a single haplotype. Encalada *et al.* (Unpublished data from Genbank; accession numbers are AJ001074 to 83) have found 10 haplotypes among the loggerhead samples from all around the world. The results of this study match the haplotype B (AJ001075) of Encalada et al. (unpublished work) and Haplotype III of Schroth *et al.* (1996) reported with accession number U72747, except with the variation at locus of 297 (317 in Table 6.1) with "G". The accession number (U72747) of Schroth *et al.* (1996) matches the haplotype "C", except for the length of sequences (accession numberof Encalada *et al.* (unpublished work). Variations with the other reported loggerhead turtle mtDNA control region sequences (U22261 of Bowen *et al.*, 1995; U40435 of Dutton *et al.*, 1996; L35254, L35255 of Laurent *et al.*, unpublished data) are also shown in bold letters in the sequences (Table 6.1).

C. mydas 1	TACCTTTGAC	ACAGGGAATA	AAAGTGTCCA	CACAAACTAA	CTACCTAAAT	TCTCTGCCGT
61	GCCCAACAGA	ACAATACCCG	CAATACCTAT	CTATGTATTA	TCGTACATCT	ACTTATTTAC
121	CAATAGCATA	TGACCAGTAA	TGTTAACAGT	TGATTTGACC	CTAAACATAA	AAAATCATTG
181	AATTTACATA	AATATTTTAA	CAACATGAAT	ATTAAGCAGA	GGATTAAAAG	TGAAATGACA
241	TAGGACATAA	AATTAAACCA	TTATACTCAA	CCATGAATAT	CGTCACAGTA	ATTGGTTATT
301	TCCTAAATAG	CTATTCACGA	GAAATAAGCA	ACCCTTGTTA	GTAAGATACA	ACATTACCAG
361	TTTCAAGCCC	ATTCAGTCTG	TGGCGTACAT	AATTTGATCT	ATTCTGGCCT	CTGGTTAGTT
421	TTTCAGGCAC	ATACAAGTAA	CGACGTTCAT	TCGTTCCCCT	TTAAAAG - - -	- - - - - - - GCC
481	TTGGGTTGAA	TGAGTTCTAT	ACATTAAATT			

*C.caretta*1	CCAATTAAAC	TACCCTTTGA	CGCAAAAGAA	GCGCCAACAT	GTAAATTTAC	CTATATTCTC
61	TGCCGTGCCC	AACAGAATAA	TATCCATAAT	ACCTATCTAT	GTATTATCGT	ACATCAACTT
121	ATTTACCACT	AGCATATGAT	CAGTAATGTT	GTCGATTAAT	CTGACCTTAA	ACATAAAAAC
181	T - ATTAATTT	TGCATAAACT	GTTTTAGTTA	CATGACTATT	ATACAGGTAA	TAGGAATGAA
241	ATGATATAGG	ACATAAAATT	AAACCATTAT	TCTCAACCAT	GAATATCGTT	ACAGTAATAG
301	GTTATTTCTT	AGTTCAGCTC	ATCACGAGAA	ATAAGCAATC	CTTGTTAGTA	AGATACAATA
361	TTACCAGTTT	CAAGTCCATT	AAG TCATGTC	GTACATAACT	GATCTATTCT	GGCCTCTGGT
421	TGGTTTTTTC	AGGCACATTA	AGGCAGTAAA	GTTCATTCGT	TCCTCTTTAA	AAGGCCTCTG
481	GTTGCAAGTA	AATGAGTTCT	ATACATTAAA	TTTATAACCT	GGCATACGGT	GGTTTTAC

Figure 6.1. The mitochondrial DNA control region sequences of the samples from Northern Cyprus. (Bold letters indicate the polymorphic sites found in the literature; Hyphens ("-") within the sequences represent "gaps" which are added in order to align and allow for the presence of base "insertions" in other sequences at corresponding positions).

An inverse relationship between nesting population size and mtDNA diversity is apparent for other populations (Lahanas *et al.*, 1994; Encalada *et al.*, 1996). However, some small populations with very low diversity have been observed (e.g. Aves Island, Guinea Bissau). These results help to confirm the idea that the Mediterranean populations of green turtles and loggerhead turtles were founded recently by migration of a very small number of females from the Atlantic. This may have occurred after the last glacial period (Bowen *et al.*, 1993b). On the other hand there is also evidence of diversity of loggerhead populations within the Mediterranean and this may be because of the short post-glacial history of Mediterranean loggerhead colonies (Schroth *et al.*, 1996). Since post-glacial immigration, the natal homing behaviour must have caused demographic isolation between different populations within the Mediterranean, leading to the genetic differentiation in the nuclear and mitochondrial genome (Schroth *et al.*, 1996). All the loggerhead samples from Cyprus showed the haplotype "B". In order to detect other haplotypes in the Mediterranean, many more samples from a wider variety of locations would be necessary.

The green turtle population nests only on the beaches of Cyprus and southeast beaches of Turkey (Baran and Kasparek, 1989; Groombridge, 1990). The coasts of Cyprus and Turkey represent the only significant nesting habitat remaining for green turtles in the Mediterranean Sea. Extinction here would nearly extirpate the green turtle population from an entire sea basin, and this is reason enough to merit a very high conservation priority. This nesting population is threatened with imminent extinction by habitat degredation, incidental fishery mortality, and development for tourist industries.

Perhaps the primary conservation value of these data lies in the appreciation of the need for thorough natural history studies for wildlife management. Literature suggests that conservation initiatives based on incomplete natural history information can be disastrous (Frazer, 1992; Bowen *et al.*, 1994), and seemingly esoteric aspects of organismal biology or ecology (such as temperature dependent sex determination) make the difference between success and failure in wildlife management programmes. The preprogrammed female reproductive behaviour makes it unlikely that the loss of breeding habitats can be compensated for by emigration to other colonies; that is, the loss of nesting sites is accompanied by the loss of specific genotypes (Schroth *et al.*, 1996). Thus, to preserve the genetic diversity of the sea turtle population in the Mediterranean one needs to protect individual nesting sites.

Chapter 7. Heavy metals in turtle eggs and hatchlings

This chapter considers the heavy metal concentrations in loggerhead turtle eggs and hatchlings.

7.1. Introduction:

The carnivorous loggerhead turtle occurs in the Mediterranean Sea, Atlantic, Indian and Pacific Oceans (Dodd, 1988). Recent reports showed that sea turtles are considerably affected by marine pollutants, such as debris, tar balls and toxic chemicals (Carr, 1987; Gramentz, 1988; Sakai *et al.*, 1995) and suggested the need to monitor chemical pollutants in an effort to preserve their populations. All species of sea turtles are regarded as endangered. Therefore, killing sea turtles is prohibited except for research purposes, for which only very limited samples are available. Therefore, if possible, eggs should be used as indicators for monitoring heavy metal levels in turtles. There are two possible sources of heavy metals for the eggs; either from the subtratum or from the mother. The loggerhead is long lived, taking at least 25-30 years to reach maturity, and during its life time it may built up concentrations of trace metals in its tissues. Sea turtles and their eggs have been analysed for heavy metals and organochlorine compounds (Hillestad *et al.*, 1974; Stoneburner *et al.*, 1980; Witkowski and Frazier, 1982; Davenport and Wrench, 1990; Rybitski *et al.*, 1995; Bishop *et al.*, 1995; Sakai *et al.*, 1995). The heavy metal concentration of loggerhead eggshells differs significantly between Florida and Georgia/ South Carolina rookeries (Stoneburner *et al.*, 1980), and heavy metals are found at higher concentrations in liver than in eggs of loggerhead turtles (Sakai *et al.*, 1995).

The purpose of this study was to document heavy metal concentrations in the eggs of loggerhead sea turtles nesting along the southwest coast of Turkey, as this was suggested as a method of heavy metal monitoring by Sakai *et al.*, (1995). This study was conducted to assess the inter-clutch and beach and intra-clutch (top and bottom eggs in the nest) variations in heavy metal concentrations in eggs.

7.2. Materials and Methods:

Samples (eggshells, yolk and liver) were collected on four beaches of Turkey (Dalyan, Fethiye, Patara and Kizilot) in two ways. One was just before hatching, some eggs were removed for sex determination purposes and the eggshells, yolks and livers of these embryos were used for metal analyses. Another source was about one week after the last hatching track was seen the nest was excavated and the same samples were collected from dead-in-shell embryos.

The concentrations of cadmium, lead, iron, copper and zinc were analysed as follows;

1-) All the samples were removed from the eggs and then air dried for about 10 days and then dried in oven at 50 °C for 4-7 days to a constant weight.

2-) A 0.050 mg -0.500 mg of dried tissue was weighed out and placed in a 50 ml glass round-bottomed flask.

3-) Samples were digested by adding 10 ml concentrated Nitric acid, and placed on a hot plate in a fume cupboard and left overnight until samples were digested. Samples appear clear when digested.

4-) After cooling, samples were diluted to 10 ml using distilled water.

5-) Concentrations of metals in the tissues were measured by flame atomic absorption spectrophotometer (Phillips PU 9200 Spectrophotometer, Pye Unicam Ltd., Cambridge). The instrument is an automated analytical machine which incorporates advanced processing facilities and operates under a central control microprocessor. An array of sensors provides input and output data to the processing system which constantly monitors the instrumental conditions and provides analytical results. The instrument burns a mixture of air and acetylene gases and uses one hollow cathode lamp and one (deuterium) background correction lamp. Lamp current and wavelength were changed automatically depending on the chosen metal. The standard solutions of metals were prepared by diluting standard solutions for atomic absorption with distilled water. After calibration of the instrument using standards, several standards were repeated throughout each set of analyses. The concentration of the metal is calculated from the reading from the Spectrophotometer by multiplying by 10 which is the dilution factor and dividing by the dry weight of the tissue.

The concentration of mercury was analysed as follows;

1-) A 0.050 mg -0.500 mg of dried tissue was weighed out and placed into a flat-bottomed conical flask.

2-) Samples were digested by adding 10 ml concentrated Nitric acid, covered with a large marble and placed for 1 hour in a water bath which was already set at 90 °C.

3-) Samples were removed from the water bath and placed on a hot-plate and boiled gently for 1 hour.

4-) Samples were removed and flasks left for cooling.

5-) 1 ml Hydrogen peroxide was added to each flask and these were then placed for another 1 hour in the water bath.

5-) After cooling the flasks, the solution was transferred into a 50 ml volumetric flask. The conical flask was rinsed with distilled water, and rinsings were also added to the volumetric flask. The volume was made up to 50 ml with distilled water.

6-) The reducing agent was prepared by mixing 20 g tin(II) chloride, 100 ml concentrated hydrochloric acid and 900 ml distilled water. A blank solution was also prepared by mixing 200 ml concentrated nitric acid and 800 ml distilled water. Three standards were also prepared by dilution of 1 ppm stock solution.

7-) Prior to analyses, the reducing agent was aerated for 30 minutes and then the samples were analysed.

7.3. Results and Discussion:

Heavy metal concentrations in the yolk, liver and eggshell of loggerhead turtles are shown in Table 7.1. Cd, Pb, Fe, and Cu concentrations were highest in the liver, whereas Zn was highest in yolk. Hg concentrations in eggshell and yolk of loggerhead turtles were below the detection limit, therefore not all the samples were analysed. Sakai *et al.* (1995) found, in the study of heavy metal monitoring from sea turtle eggs using samples from Japan, higher concentrations of Fe, Zn, Cd, and Hg in the yolk, Cu concentrations in the shell, and no detectible Pb concentrations in shell, albumen and yolk of the eggs.

Table 7.1. Heavy metal concentrations (ppm or $\mu g/g^{-1}$, mean±SD) in liver, eggshell and yolk of loggerhead turtle.

	Fe	Zn	Cu	Cd	Pb	Hg	Reference
Embryo Liver (n=22)	35.83±9.98	23.84±3.10	21.21±2.62	1.26±0.43	2.48±0.46	0.51±0.046	This study
Adult Liver (n=7)	649±385	27.9±4.35	17.9±8.17	9.29±3.30	below detec. lim.	1.51±2.93	Sakai *et al.*(1995)
Eggshell (n=22)	17.75±7.00	5.00±2.20	5.29±0.48	0.649±0.131	0.633±0.162	below detec.lim.	This study
Eggshell (n=5)	10.6±2.20	2.17±0.59	5.57±0.77	< 0.01	below detec. lim.	0.0040±0.0013	Sakai *et al.*(1995)
Yolk (n=22)	15.79±3.62	57.21±2.23	0.928±0.102	0.359±0.135	1.307±0.228	below detec.lim.	This study
Yolk n=15-27	71.27 to 74.67	73.5 to 80.5	4.96 to 6.60	0.02 to 0.19	1.13 to 2.18	0.41 to 1.39	Stoneburner *et al.* (1980)
Yolk (n=5)	25.1±2.18	34.4±3.18	1.57±0.07	0.026±0.007	below detec. lim.	0.0121±0.0034	Sakai *et al.*(1995)

The concentrations of essential elements (Fe, Zn, Cu) in yolks fluctuated to a lesser extent than those of Cd, Pb and Hg in this study in accordance with other studies

(Stoneburner *et al.*, 1980; Sakai *et al.*, 1995). There were no significant differences between Fe, Zn and Cu concentrations from samples from different beaches or from different levels in the nest (one-way ANOVA, $P>0.05$ in all cases). On the other hand, significant differences were found between the concentrations of Pb in eggshells from different beaches (one-way ANOVA, $F_{3,19}=3.53$, $P<0.05$), Cd in liver samples from the top and bottom parts of the nest (one-way ANOVA, $F_{1,20}=5.86$, $P<0.05$) and Hg in liver samples between the different parts of the nest and between the beaches (one-way ANOVA, $F_{3,63}=4.12$, $P<0.05$).

The only significant concentration differences found between the top samples (last laid) and bottom samples (first laid) within the were with Cadmium and Mercury. All other metals were not influenced by the egg-laying order, as reported previously (Sakai *et al.*, 1995). The concentration differences found between the Pb samples in eggshells on different beaches can either be explained as due to different levels of Pb pollution on different beaches, or different groups of females present on different beaches in the Mediterranean as genetically reported recently (Schroth *et al.*, 1996). The source of Pb can be tested whether from the mother or from the beach by directly taking the eggs during the laying for metal analysis. Similar results were reported previously (Stoneburner *et al.*, 1980) for Western Atlantic loggerhead population which is composed of demes. Probably these results also can be treated as different groups of females that are nesting on different beaches, and they may use different feeding grounds where the pollution levels may differ. The data presented by Stoneburner *et al.* (1980) and Sakai *et al.* (1995) and this study are quite different in some elements in particular, Sakai et al. (1995) report very high concentrations of iron in livers of adults whereas levels in this study were measured in embryo livers and were very much lower. It is unclear whether this is a difference related to turtle age, or between sites or due to analytical methods. Although, some differences in concentrations of heavy metals in different populations would be expected, there is need for more analysis of metals in turtle eggs with modern analytical equipment and quality controlled laboratory procedure.

Chapter 8. General Discussion:

From work done in this study, it can be concluded that the temperature of sea turtle nests in the eastern Mediterranean is between 24 and 35 °C and rises by up to 9.6 °C during incubation. Such data have not been reported previously for the Mediterranean population, or for other populations.

Although a few studies mentioned that there may be some temperature differences within the nest and that this may be due to metabolic heating (Hendrickson, 1958; Bustard and Greenham, 1968; Ackerman *et al.*, 1985; Thompson, 1988), there were no detailed work on the temperature differences within the nest. My data showed that top eggs were warmer and bottom eggs were cooler than the middle ones in the first third of incubation; but later in incubation middle temperatures become the same as the temperature of the top eggs or even sometimes warmer due to metabolic heat.

Temperature differences within the nest have two consequences. First due to temperature differences at different levels of a nest, different sex ratios prevail at different levels. There is a majority of females at the top level of a nest and males from the bottom of a nest. The wide differences in temperature within a nest mean that one finds both sexes of hatchlings from most nests. The second outcome of the temperature difference within a nest is that, since the temperature affects the rate of development (Miller, 1985), the hatching and emergence of hatchlings from that nest may take more than a single night. The wider this temperature difference within that nest the longer the time required to complete the hatching of all hatchlings. Hatching intervals of green turtle nests were shorter than at loggerhead turtle nests, since smaller temperature variations were observed in green turtle nests which are deeper than loggerhead turtle nests. Emergence asynchrony has been reported previously (Hendrickson, 1958; Witherington *et al.*, 1990; Hays *et al.*, 1992; Gyuris, 1993; Kaska, 1993; Peters *et al.*, 1994), but developmental differences that occured due to temperature differences within the nest were never investigated previously.

The duration of incubation is related to species of turtle, but temperature exerts a significant effect on the duration of incubation and the rate of development within a species. Low temperatures increase the period of incubation and slow the rate of development whereas high temperatures decrease the period and increase the rate of development. For example, in *C.mydas* the longest period of successful incubation in which temperature was monitored was 99 days at 23-25 °C (Ackerman and Prange, 1972) and the shortest was 47-49 days at 32 °C (Bustard and Greenham, 1968). The

duration of natural incubation varies between beaches and between seasons at a beach (see Hirth, 1980). Cooler conditions associated with the monsoon season can lengthen the incubation period to about 70 days compared to 54 days during the dry season (Hendrickson, 1958). Mrosovsky and Yntema (1980) reviewed data from controlled temperature incubation of green and loggerhead turtles to establish an estimated factor useful for predicting the change in the duration of incubation caused by a 1 °C change in temperature. A 1 °C difference would inversely alter the length of incubation by 5 days over the entire period, but in the natal beach a 1°C decrease adds approximately 8.5 days to the incubation period (Mrosovsky and Yntema, 1980; Mrosovsky, 1980). The mean incubation temperatures of nests can only be used for estimating the incubation periods for nests, and suggest that 1 °C temperature variation within the clutch causes a 4 day delay in both hatching and emergence of hatchlings. Therefore, differences in incubation period can be expected. Therefore nests should not be excavated right after the first bont of hatchling emergence, since there may be some recently hatched embryos in the nest.

Emergence of hatchlings was closely correlated with the cooling of sand temperatures at 15-20 cm depths above the nest in accordance with previous work (Hays *et al.*, 1992; Gyuris, 1993), but the time of emergence was not correlated with any fixed absolute temperature.

Within the 8 °C range of successful development temperatures (25-33 °C), temperature also has a determining impact on the sex of the hatchlings (Yntema and Mrosovsky, 1980; Morreale *et al.*, 1982; Mrosovsky, 1994). In general, higher temperatures produce female hatchlings and lower temperatures produce male hatchlings. The critical period of sexual determination of turtles is the middle third of incubation period (Spotila and Standora, 1985; Mrosovsky, 1994). The mean temperatures during the middle third of the incubation period were found to be closely correlated with the percent sex ratio of nests, but mean temperatures over the entire incubation period provide a poor prediction of sex ratio. Although there was a female biased sex ratio in general, there was a single male dominated loggerhead nest from Fethiye. That nest was on the part where there are small stones (rocks) present on the surface of the beach, probably this caused the cooling of the nest site and therefore a male dominated sex ratio was produced. For both species the overall difference in numbers of males and females between top and bottom of nests was significant.

The pivotal temperature for all sea turtle species are reported to lie within a 1 °C range (28.6 - 29.7 °C) (Mrosovsky, 1994). The results of sex ratios and pivotal

temperatures reported from constant temperature experiments were closely correlated with the sex ratio and the mean temperatures in this work. Therefore, it can be concluded that the pivotal temperature of Mediterranean sea turtles is also around 29 °C, since one loggerhead turtle nest produced a 44% female sex ratio with a mean temperature of 28.1 °C during the middle third of incubation.

Sand temperatures were also monitored on the beaches and it was found that there was decreasing temperature with increasing depth. Sand temperature differences on different beaches or on the same beach have been reported previously (Morreale *et al.*, 1982; Hays *et al.*, 1995), and similar sand temperature differences were also observed on different beaches. Sand temperature differences were observed at different distances from the sea on the same beach and at the same distance from the sea on different beaches. Therefore sand or air temperatures were not closely correlated with nest temperatures. Therefore assumptions made on sex ratio using sand or air temperatures may not be as reliable as the middle third temperature of the incubation period.

One of the most challenging issues facing the conservation management of sea turtles will be the long-term effect of the rise of sea level and temperature as part of the greenhouse effect (Mrosovsky *et al.*, 1984; Davenport, 1989). For example, the predicted increased athmospheric temperatures will result in increased sand temperatures which in turn will affect the sex ratio of hatchlings, with more females being produced.

Predation patterns of loggerhead and green turtle nest were found to be different on the beaches of Northern Cyprus. Although loggerhead nests suffered whole and part predation during the entire incubation period, green turtle nests suffered most predation just before or during hatching. As differences in sex ratio at different levels of nests were found, probably predation pattern will also affect sex ratio of a nest, since most the partial predation occurs at the top level of a nest where the majority of hatchlings would be female. On the other hand, predation during hatching may occur randomly but the hatchlings hatched and emerged late are most likely to suffer predation. Therefore one can speculate that potential female loggerhead hatchlings are in danger of part predation during the incubation period, but potential male green turtle hatchlings are more likely to be predated during hatching. While this was the case, loggerhead turtle nests may suffer part predation since they are shallower, and access is easier for a predator, than green turtle nests. During the hatching time the smell of the first group of hatchlings may give a clue for predators about the location of a nest, regardless of species.

The most common causes of mortality in developing eggs and hatchlings were nest predators and inundation. Screening is one effective way of protecting nests against predation, but this screen has to be built from inflexible and rather strong material. Relocation of nests on the night of laying, that are most likely to be inundated, to a safer area increased the hatching success. The movement of sea turtle eggs to protected areas is a common practice (Hendrickson, 1958; Simon, 1975). It has been demonstrated that rough handling during such egg movement will significantly reduce hatching success (Limpus *et al.*, 1979; Parmenter, 1980). In discussing the effect of handling on eggs, Hendrickson (1958) reported an extended incubation through the emergence period (54.3 days compared to 51.4 days) and a reduced hatching success (40% compared to 46.7%) for eggs that had been moved the morning following oviposition. The movement of sea turtle eggs during the critical period (12 hr to 14 days) may significantly reduce the hatching success. However, at a temperature which approaches the minimum threshold in accordance with the slowed rate of development at low temperatures, the movement sensitive critical period was shifted by nearly 36 hours (Limpus *et al.*, 1979). The formation of the large sub-germinal space and the consequential rising of the blastodisk to a position next to the shell membrane dictate that the egg cannot be moved without risk of rupturing the vitelline membrane. Therefore, the relocation should be done if possible very soon after laying or on the night of laying.

The sand characteristics of the beaches were similar among 10 beaches. Because nest gases must diffuse through the column of sand above the clutch, both nest depth and sand conditions (grain size, organic content, and moisture) control the flux of gas to and from the eggs. A detailed discussion of the interactions concerning embryonic respiration in sea turtle nests can be found elsewhere (Ackerman and Prange, 1972; Ackerman, 1977, 1980, 1981a,b). Nest moisture levels affect both the size and weight of hatchlings (Packard *et al.*, 1980, 1981; Tracey *et al.*, 1978; Mcgehee, 1990; Kam and Lillywhite, 1994). The moisture content of the beach varies between 3.1 and 11.2 % moisture by weight while eggs in natural nests are incubating. Bustard and Greenham (1968) reported the lowest moisture content at the bottom of an egg chamber to be 3.8 % for successful incubation and 2.0% for unsuccessful incubation. Wetter conditions, such as inundated nests, may restrict proper air flow around the eggs and cause death by suffocation (Tracy *et al.*, 1978). The moisture content, and the volume of air filled space of sand samples from the beaches in this work showed similar results. Therefore these beaches are quite suitable for nesting of sea turtles in the Mediterranean.

Post-ovipositional development begins within hours of oviposition. At first the only indication is the formation of a white spot close to the upper-most pole of the egg. The changes in the gross morphology of the embryos, after examining 1882 loggerhead embryos and 1169 green turtle embryos, are found to be rapid at first and slow as incubation proceeds. These changes have been grouped and the embryos staged into 24 stages (6-30), and a short and practical staging table has been developed for Mediterranean sea turtles. These stages were closely correlated with those described by Miller (1985). Some abnormalities were also observed among these samples. The most common abnormalities were supernumerary and subnumerary scutes, albinos, head abnormalities, jaw abnormalities and twinnings.

Although the essential elements did not significantly differ in concentrations, Pb levels in eggshells were found to be different among the samples. As the heavy metal concentrations may differ for each nesting group of females (Stoneburner *et al.*, 1980), lead samples from this study and the genetical study of Schroth *et al.* (1996) suggest that there are different groups of females nesting along the Mediterranean beaches. Since the genetical samples taken during this study were only from the west coast of Northern Cyprus, only single haplotypes for each species were found. This result helps to confirm that the Mediterranean populations of green and loggerhead turtles were founded recently by migration of very small numbers of females from the Atlantic, but additional haplotypes can probably obtained by sampling a wider variety of locations in the Mediterranean.

The goal of sea turtle conservation effort is the continued survival of turtles, and this can only be reached by including the adult and nesting phase together with the development phase. The nesting grounds have to be protected. Some nesting beaches should have restricted public access (such as Dalyan) but others should be open and public involvement encouraged. Such involvement should be in the context of a management programme which protects the nesting areas and the female during oviposition. The loss of nesting sites is accompanied by the loss of genetic diversity. Therefore individual nesting areas need to be protected in order to preserve the genetic diversity of sea turtle populations in the Mediterranean.

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