Plasma hormone levels in the green turtles *Chelonia mydas* during peak period of nesting at Ras Al-Hadd-Oman

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Abstract

Circulating estradiol (E_2), progesterone (Pro), testosterone, and corticosterone (B) levels were monitored in the green turtles *Chelonia mydas* during different nesting phases. Successful nesting includes emergence from sea, chamber and nest excavation, oviposition, burying the nest, and returning to sea. Unsuccessful nesting includes chamber and nest excavations but without oviposition. Blood samples were taken from the cervical sinus and collected within 5-min of capture to minimize stress. The samples were collected between 2000 and 0100 h during the peak season (May–October). High-performance liquid chromatography using a u.v. detection system coupled with tandem quadrupole mass spectrometry was used to measure B. Plasma B levels were significantly higher in successful and unsuccessful phases over emergence and excavation phases. However, B levels in successful versus unsuccessful or emergence versus excavation phases were not significantly different. Plasma steroid levels were measured by the Coat-A-Count RIA technique. Pro levels were significantly higher ($P<0{\cdot}005$) in successful over unsuccessful turtles and also successful turtles over turtles in the other phases ($P<0{\cdot}01$). The Pro levels immediately after nesting were found to be higher than that reported previously. Plasma testosterone values were higher in successful turtles but not significantly different from the turtles in other phases. Estrogen levels were undetected in all phases. Overall, the hormone values during different phases of nesting may play a major role in formulating the nesting behavior and physiology of the nesting activities in the green turtle.

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Introduction

Reproductive phases in sea turtles have been described in a few studies relative to their behavioral and physiological conditions. To date, relatively limited investigations have been conducted on the reproductive hormonal dynamics in green turtles (*Chelonia mydas*) relative to different phases of nesting. Circulating gonadotropins, gonadal steroids, and corticosterone levels associated with behavior and physiology of nesting activities have been investigated relative to breeding in some species of sea turtles (Licht *et al.* 1979, 1980, Wibbles *et al.* 1992, Jessop *et al.* 1999 on *C. mydas*; Rostal 1991 on *Lepidochelys kempi*; Licht 1982 on *Lepidochelys olivasea*; Wibbles *et al.* 1990, 1992, Guillette *et al.* 1991 on *Caretta carreta*; and Rostal *et al.* 1996 on *Dermochelys coriacea*). In addition, adrenocortical responsiveness to stress during nesting activities was examined in *C. mydas* (Jessop *et al.* 1999), in *L. olivacea* (Valverde *et al.* 1999) and in *C. caretta* (Gregory *et al.* 1996).

The physiology and behavior of sea turtles during nesting are poorly understood. Moreover, there are limited data on hormone dynamics during nesting with only a few studies monitoring the hormone levels at different phases of nesting (Licht *et al.* 1979, 1980, Jessop *et al.* 1999 on *C. mydas*; Licht 1982 on *L. olivacea*; Wibbles *et al.* 1990 on *C. carretta*).

The purpose of this investigation is to monitor sex-steroid levels during different stages of nesting of the green turtles at Ras Al-Hadd, one of the most populous nesting beaches in the world. In addition, B levels are monitored during nesting so the degree of stress can be assessed. The data will be used to formulate baseline information on the behavior and reproductive physiology of the species.

In addition, studying hormonal levels during nesting will permit us to relate these conditions to the species' reproductive potential and the data obtained in these studies can also be useful in the green turtle conservation program at Ras Al-Hadd, Oman.

Brief description of hormone dynamics in sea turtles during the reproductive cycle

Limited data are available on circulating hormone dynamics during the reproductive cycle in sea turtles. Figure 1 gives a general profile of the reproductive hormones and their influence during different phases of the cycle based on

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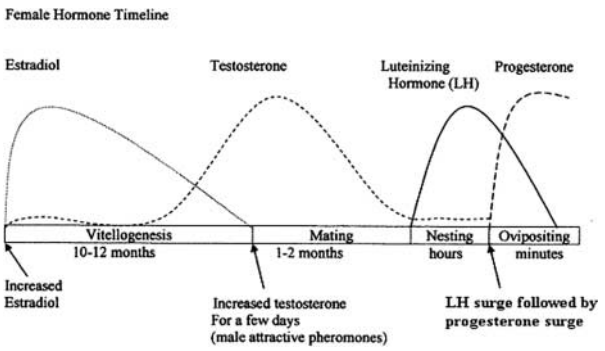

Figure 1 Female hormone timeline for green turtle. Based on data from previous investigations.

previous investigations on the sea turtles. In female green sea turtles, testosterone rises coincident with mating receptivity, and appears to last only a few days, suggesting that testosterone actually initiates breeding behavior in females (Licht *et al.* 1979). Moreover, sexually receptive females may produce male-attractive pheromones, and that testosterone may trigger their release, since pheromones are commonly under direct control of steroids (Owens & Morris 1985).

In sea turtles, as well as other reptiles, the corpus luteum is the main site of progesterone (Pro) secretion (Klicka & Mahmoud 1972, Licht *et al.* 1979, 1980, Owens & Morris 1985, Nagahama 1987). Luteinizing hormone (LH), which induces ovulation, surges about 2 weeks after mating and just after nesting, as well as a massive release of Pro from preovulatory follicles and the corpora lutea (Owens & Morris 1985, Fig. 1). Moreover, Pro concentration in the green turtles increases only a few hours after oviposition (Licht *et al.* 1979, 1980).

Estradiol (E_2) is the primary stimulus to vitellogenesis and oviductal development in sea turtles (Owens 1976, Owens & Morris 1985, Ho 1987, Nagahama 1987). Owens & Morris (1985) reported that a peak in E_2 hormone in early spring may serve both behavioral and physiological functions (Fig. 1). It could induce the drive to migrate to nesting beaches, and cause final maturation of ovarian follicles just prior to first ovulation. Despite the huge number of eggs produced in a relatively short time at nesting beaches, E_2 levels in reproductively active female green turtles is very low or often undetectable by RIA technique (Licht *et al.* 1979, 1980, Wibbles *et al.* 1992).

Materials and Methods

Study sites

Ras Al-Hadd Reserve is located on the Gulf of Oman and the Arabian Sea between 22°32′ N and 59°45′ E and 22°14′ N and 59°48′ E. The northern 4 km of the reserve are located on the Gulf of Oman, while the rest of the Reserve is located on the Arabian Sea. The coastlines are mostly sheltered by rocky hills. These high-energy waves build a curb of sand that can get up to 2 m high. Approximately, 20 beaches with different lengths (50 m to 5·3 km) make up the Reserve. Most of these sheltered beaches are considered ideal nesting grounds for the green turtles.

Brief description of nesting exercises at Ras Al-Hadd

When ashore the nesting turtles undergo difficult and stressful physical exercises which, in the green turtles at Ras Al-Hadd, could last 2–3·5 h (AlKindi *et al.* 2003). Nesting exercises commence when the turtles emerge from the sea and move to a suitable nesting site, followed by excavating body and nest chambers, laying and burying eggs, and then returning to the sea (Hendrickson 1982, AlKindi *et al.* 2003).

The green turtles at Ras Al-Hadd frequently abandon several nesting sites before choosing a suitable site for oviposition (AlKindi *et al.* 2003). During the majority of time, nest abandonments are caused by lack of insufficient sand moisture leading to frequent collapse due to the lack of sand firmness (AlKindi *et al.* 2003). Rain at Ras Al-Hadd is very rare but during the peak nesting density (May–October) moist air comes out of the sea, which is of some help in reducing nest collapses and consequently fewer nest abandonments.

Mature female green turtles migrate to nesting beaches every 2–5 years (internesting period) to lay two to six clutches of eggs, at 12–15-day intervals (Ehrhart 1982, Miller 1997). Vitellogenesis in green turtle lasts between 10 and 12 months (Miller 1997). The 2–5-year interval between nesting episodes is considered an adaptation to the high-energetic costs of migrating between distant feeding and nesting locations (Miller 1997).

The nesting behavior of the green turtle at Ras Al-Hadd, which is related to this investigation, has been described in detail (see AlKindi *et al.* 2003). Courtship and mating occur during early March through June. The mating area is approximately 250 m from the nesting beaches. The description of courtship and mating behavior still in progress is a part of separate investigation from the present one.

Blood collection

A blood sample (10 ml) was taken from each turtle that was found active during one of the four nesting phases. The turtles sampled were healthy and without any physical defects or injuries. The nesting phases are defined as follows:

(1) Emergence. Turtles that had already emerged from the sea, and were on their way searching for suitable nesting sites.
(2) Excavation. Turtles that were actively excavating the body or nest chamber.
(3) Successful. Turtles that oviposited, buried, and camouflaged the nest site.
(4) Unsuccessful. Turtles that failed to oviposit their eggs after one to several trials and then returned to sea.

Samples were collected within 5-min capture to minimize the stress response caused by handling and blood sampling (Owens 1997). Blood samples were obtained from the cervical sinus using a modified procedure of Owens & Ruiz (1980) with a 21 gauge single-use needle and 20 ml syringe. All samples were collected between 2000 and 0100 h, during the peak season only (May–October) of 2002, which is the monsoon season at Ras Al-Hadd. During this time, the climatic conditions on the nesting beaches and the feeding waters near the nesting beaches are uniform without much variation in temperature or humidity. Beach and seawater temperatures were recorded at the time of observation. Blood samples were immediately stored in K3 EDTA Becton Dickinson Vacutainer tubes and put on ice until they were centrifuged within 3 h after blood collection. After centrifugation, the plasma was stored in liquid nitrogen in the field and transported to the lab, then permanently stored at −70 °C prior to analysis.

Analysis of plasma sex hormones

The Coat-A-Count (Diagnostic Products Corporation, Los Angeles, CA, USA) method was used to determine the amount of E_2, Pro, and testosterone in the plasma samples. Coat-A-Count is a no-extraction, solid-phase ^{125}iodine RIA designed for the quantitative measurement of plasma steroids in the serum. After incubation (3–4 h at room temperature), separation of bound from non-bound steroid was achieved by decanting. The tubes were then counted in a gamma counter (Beckman Gamma 5500 B counter). The quantities of steroids in the sample were determined by comparing the counts to a calibration curve. The sensitivity of the E_2, Pro, and testosterone assays were 8 pg/ml, 0·02 ng/ml, and 0·064 ng/ml respectively. All samples were analyzed in a single run, so there was no inter-assay variability.

Coat-A-Count progesterone (catalog no. TKPG1), estradiol (catalog no. TKE21), and testosterone (catalog no. TKTTI) were used to measure the amount of Pro, E_2, and testosterone in the serum. The three antisera were highly specific for Pro, E_2, and testosterone in the serum with very low cross-reactivity to other compounds that were present in the turtle's plasma. Cross-reactivities of the Pro antiserum with other steroids were 0·9% for corticosterone, 0·03% for cortisol, no detection for E_2, and 0·1% for testosterone. Cross-reactivities of the E_2 antiserum with other steroids were 0·29% for 17β-estradiol-3-monosulfate, 0·70% for β-estradiol 17-propionate, no detection for Pro, and 0·001% for testosterone. The cross-reactivities of the testosterone antiserum with other steroids were 0·002% for corticosterone, 0·005% for cortisol, 0·02% for cortisone, 0·02% for E_2, and no detection for Pro.

The validation of the tandem quadrupole mass spectrometric method was achieved by making a comparison of the extraction recoveries. The peak area of the chromatogram at 10 pg/µl standard solution, in water/acetonitrile, was compared with a standard, at the same concentration, of spiked turtle serum. The back-calculated concentration from the protein precipitation extraction method was used to evaluate the steroid assay in turtle serum.

It was shown that the efficiency was 98% (recovery) using the protein precipitation with detection of the highly specific method, tandem quadrupole mass spectrometry coupled to high-performance liquid chromatography (HPLC).

Analysis of plasma corticosterone by liquid chromatography–tandem mass spectrometry (LC–MS/MS)

HPLC conditions Water (+0·1% formic acid) and acetonitrile (+0·1% formic acid) were used as a mobile phase when analyzing the corticosterone by LC–MS/MS. A gradient was used at an initial time (0 min) of 50% water (+0·1% formic acid) which was ramped to 95% acetonitrile (+0·1% formic acid) over 3 min.

Mass spectrometric conditions The mass spectrometric conditions incorporated the use of atmospheric chemical ionization to generate the multiple reaction monitoring transition of 347·10 > 329·20 with a cone voltage of 45 V and collision energy of 14 eV. The corona current was set to 7 µA and the resolution settings on both quadrupoles were at unit mass resolution at base with ion energy of 1·0 V for both. The temperature for the source was 150 °C and the probe temperature was 600 °C. Throughout the experiment, the detector multiplier was set to 650 V and the argon gas pressure was $2{\cdot}70e^{-3}$ (mbar).

Statistical analysis

Results are reported as the mean ± S.E.M. Moreover, one-way ANOVA was used followed by Bonferroni's multiple comparisons to detect any statistical differences in measurements of various parameters among nesting stages. $P \leq 0{\cdot}05$ was considered significant among values. All statistical analyses were performed by SPSS statistical package for windows (version 11·0), while the figures were made on Sigma Plot (version 8.0).

Results

Mean plasma Pro concentration was approximately twice as high in successful turtles (4·3 ± 0·71 ng/ml, $N=22$; Fig. 2) as it was in emerging, excavating, or unsuccessful turtles (1·99 ± 0·26 ng/ml, $N=21$; 1·74 ± 0·21 ng/ml, $N=12$; 1·75 ± 0·47 ng/ml, $N=16$) respectively (Fig. 2).

These results were significant (successful versus emerging, $P=0{\cdot}005$; successful versus excavating, $P=0{\cdot}01$; successful versus unsuccessful, $P=0{\cdot}004$). No significant differences were detected between emerging, excavating, and unsuccessful turtles.

Mean plasma (testosterone) concentration was also somewhat elevated in successful turtles (0·42 ± 0·040 ng/ml, $N=22$;

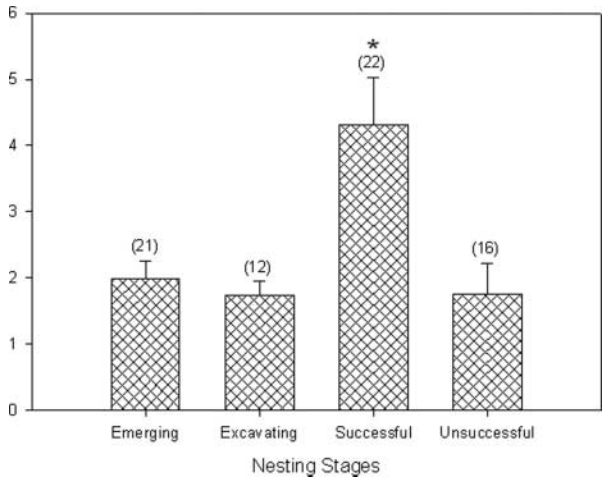

Figure 2 Mean plasma progesterone concentrations (±S.E.M.) of female green turtles at different nesting stages. The numbers on the bars indicate the sample size. Asterisk (*) indicates significant difference ($P<0{\cdot}05$).

Fig. 3), but was not significantly different from emerging, excavating, or unsuccessful turtles (0·30±0·039 ng/ml, $N=21$; 0·29±0·026 ng/ml, $N=12$; 0·29±0·045 ng/ml, $N=16$; Fig. 3).

Estrogen (E_2) concentrations were undetected in all the nesting stages. The relative humidity on the nesting beaches fluctuated between 85 and 95% because of the misty condition generated by the southwest monsoon. The sand and water temperatures based on 65 observations were 30·403±0·475 and 24·648±0·385 °C respectively.

Plasma B mean levels (ng/ml) were: emerging ($N=27$, 0·65±0·16), excavating ($N=12$, 0·44±0·37), unsuccessful ($N=24$, 1·06±0·14), and successful ($N=47$, 1·13±0·01). There were significant differences between unsuccessful versus emerging, $P=0{\cdot}03$; unsuccessful versus excavating, $P=0{\cdot}01$; successful versus emerging, $P=0{\cdot}004$; and successful versus excavating, $P=0{\cdot}002$. However, plasma B levels were not statistically significant between successful versus unsuccessful (Fig. 4).

Discussion

This investigation is based on a thorough study of green turtles, *C. mydas*, captured from a natural population during nesting activity. Sex steroids and corticosterone levels were monitored at different nesting phases and conditions to add a new dimension to overall understanding of hormone dynamics in sea turtles.

The hormone levels were measured from freshly captured turtles to avoid stress and to ensure that the values were closely related to natural conditions. It has been confirmed that captive reptiles are sensitive to stress and thus studies on captive animals may differ from those obtained under natural conditions. Stress may have a profound effect on hormonal levels and overall reproductive activities in turtles (Lance 1994, Mahmoud & Licht 1997). Moreover, the data in this study were gathered under a uniform climatic condition with a narrow variation in temperature and humidity of the nesting beaches during the peak period. In addition, the blood samples were also taken from the nesting turtles during a certain time of the night in order to exclude the possible influence of diurnal variation on hormonal levels. These conditions may reflect uniformity and consistency of the data as indicated in the results. Wide variations in climatic

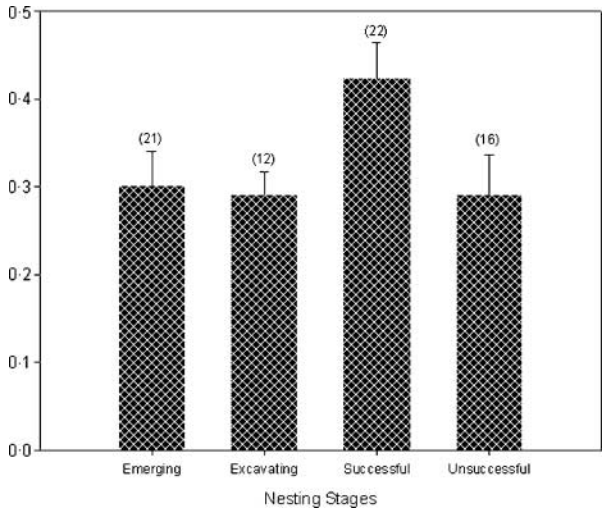

Figure 3 Mean plasma testosterone concentration (±S.E.M.) of female green turtles at different nesting stages. The numbers on the bars indicate the sample size. There were no significant differences between nesting stages.

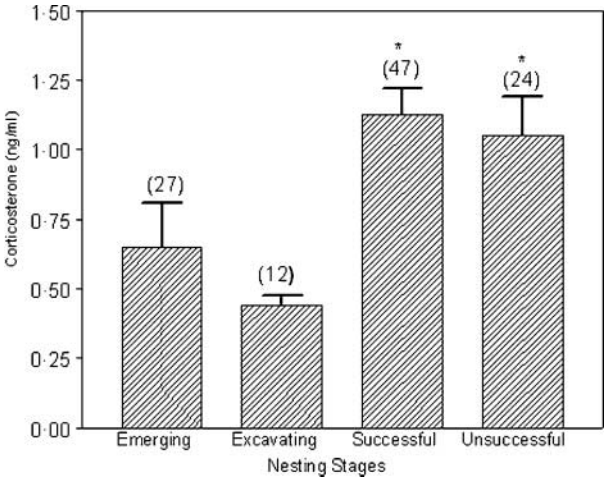

Figure 4 Mean plasma corticosterone concentration (±S.E.M.) of female green turtles at different nesting stages. The numbers on the bars indicate the sample size. There were significant differences between some of the nesting stages. Asterisk (*) indicates significant difference ($P<0{\cdot}05$).

conditions and inconsistency in sampling time may influence the hormone levels (Mahapatra *et al.* 1987, Mahmoud & Licht 1997).

In reptiles, it has been suggested that E_2 is the primary stimulus for vitellogenesis (Ho 1987). In this study, it is unclear why E_2 was undetectable during the process of nesting in green turtles, but this has been observed previously (Licht *et al.* 1979, 1980, Wibbles *et al.* 1992). One possible explanation for the low E_2 levels in green turtles is that E_2 may play a minor role during the nesting process. Moreover, the follicles are already in a mature state for the next clutch and probably for all clutches. Thus, a high titer of E_2 is no longer needed at this time, and relatively low concentrations of E_2 are sufficient to support follicle function. Owens & Morris (1985) suggested that low E_2 in this species may be related to hormone receptor affinities, thus far less hormone is required for activation compared with any species having low-affinity receptors. Furthermore, it is possible that E_2 is not the major circulating estrogen in green turtles and we are missing much higher levels of a more important molecule (Owens & Morris 1985, Coufal & Whittier 2003).

The low level of testosterone during nesting is also reported in other sea turtles (Licht *et al.* 1979, 1980, Wibbles *et al.* 1992). Like E_2, testosterone may not play a major role in triggering nesting behavior. On the other hand, low levels of testosterone may be sufficient to stimulate the nesting behavior in this species.

The most interesting result in this investigation is the unexpected high Pro levels shortly after oviposition. Pro studies indicated that the Pro rise is associated with LH surge (Licht *et al.* 1979, Licht 1982, Wibbles *et al.* 1992) which usually occurs 24–48 h after nesting. Lance *et al.* (1979) reported similar observations in that plasma progesterone and LH levels were significantly higher during nesting conditions than non-nesting conditions (cruising in open water).

In this investigation, the early rise in Pro levels may be associated with early stages of ovulation immediately following oviposition and consequently the formation of new active corpora lutea. This cannot take place unless there is an early surge in LH shortly following oviposition (Licht *et al.* 1979, 1980, Licht 1982, Owens & Morris 1985). We cannot conclude with certainty the cause of such high Pro values, since the gonadotropins were neither measured in this study nor have we any record of corpora lutea formation and development in this species.

It has been reported that the retention of eggs in the uterus remains in effect as long as there are active corpora lutea producing high amounts of Pro (Ho 1987). Moreover, the high levels of Pro may also inhibit LH and consequently ovulation of follicles for the next clutch (Klicka & Mahmoud 1977). In other words, egg retention remains in effect as long as the corpora lutea are active, which usually last 2 weeks in green sea turtles (Licht *et al.* 1979). In addition, there are other factors that may be investigated relative to the high Pro values in the green turtles from Ras Al-Hadd. There are environmental, behavioral, and genetic factors that may differ from the other populations of this species.

In our study, B plasma levels in green turtles were slightly higher than the values reported on B levels previously by Jessop *et al.* (1999) for the green turtles in Australia. This small difference may be due to the use of the tandem quadrupole mass spectrometry in this study which is more sensitive and selective for the analysis of corticosterone than the RIA that was used. In addition, the green turtles at Ras Al-Hadd spend more time on the beach than the green turtles in Australia because of the frequent nest collapse due to low sand moisture. Therefore, the turtles at Ras Al-Hadd may be subjected to a higher degree of stress. Moreover, genetic variation between the two geographic populations may also play a role in how the turtles cope with stress during nesting.

Valverde *et al.* (1999) suggested that the ovipositing olive ridley (*L. olivacea*) turtles in Costa Rica have adopted a high-sensitivity threshold associated with diminished sensitivity of hypothalamo–pituitary–adrenal axis during a chaotic mass nesting (arribada). During nesting, the olive ridleys showed low values of corticosterone during all phases of nesting. They concluded that the olive ridleys have adopted neurophysiological mechanisms that reduce the stress response to disturbance, so the process of oviposition can be completed successfully.

Some vertebrates including sea turtles are able to tolerate environmental and social stresses temporarily in order to achieve breeding successfully as a tradeoff of reproduction success for potential survival and therefore in many species of reptiles, glucocorticoid hormones remain unchanged when animal is subjected to stress (see review by Wingfield & Sapolsky 2003).

During the peak nesting period at Ras Al-Hadd, the corticosterone values remained relatively stable despite stress, such as exhaustive and laborious exercises and crowding conditions. The corticosterone levels maintained the same pattern associated with each nesting phase. For example, in successful nesting turtles, B levels were significantly higher than in emerging turtles. This trend remained unchanged throughout the sampling period (May–October).

Based on these results, it can be concluded that the nesting green turtles were subjected to some degree of stress, and the magnitude of stress is associated with time and energy spent on the nest.

Although the nesting green turtles had gone through difficult and stressful episodes, B values were relatively low which is comparable to other sea turtle studies. Overall, the green turtles at Ras Al-Hadd have adopted the same magnitude of tolerance to stress during nesting as the other sea turtles, so breeding can be facilitated successfully.

The nesting season at Ras Al-Hadd is year round (AlKindi *et al.* 2003) while courtship and mating extend over several months, a unique condition found in the populations of Ras Al-Hadd. Moreover, the mating area is only about 250 m from the nesting beaches, which is different from other studies where the mating area is not close to the nesting area

(Owens 1980). It is hard to conclude if the close proximity between nesting beaches and mating has any influence on the hormone levels of the nesting turtles at Ras Al-Hadd.

There is a strong indication, based on the tag-recapture and the genetic marker studies (unpublished data) that there is more than one population in the area. The result of this investigation on hormone dynamics during phases of nesting will add an important information and a new dimension to the overall understanding of the reproductive physiology of the green turtles at Ras Al-Hadd. Future research will include other phases of life history, such as vitellogenesis, courtships, mating, and migration.

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