



Rapid stress response in post-nesting Kemp's ridley turtle (*Lepidochelys kempii*)

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Abstract. Vertebrate's stress response plays a significant role in helping individuals adapt to changing environments. One of the most challenging stages in female turtles' life cycle is during oviposition, especially when the turtles' nest during the daytime and have to face massive nesting events (arribadas), limited space on the beach, and high temperatures. The Kemp's ridley sea turtle (*Lepidochelys kempii*) is the smallest sea turtle, which has to withstand high temperatures during the nesting process due to its diurnal habits. Our objective was to determine corticosterone concentration as a response to induced stress and estimate its variations at the beginning, middle, and end of the nesting season. We measured the corticosterone concentration in serial blood samples (0, 20, 40, and 60 min.) in 22 Kemp's ridley sea turtles during the beginning, the middle, and the end of the nesting season. Our results show that the turtles had a significant increase in corticosterone levels at 20 min. after the onset of stress, and we found that corticosterone decreased at the end of the nesting season. The results point to a rapid response of the hypothalamic–pituitary–adrenal axis. We suggest that glucocorticoid levels return to baseline at the end of the breeding season, and modulation may allow successful completion of nesting throughout the entire seasonal nesting period.

Key words. Testudines, Cheloniidae, Carettinae, Corticosterone, Gulf of Mexico, nesting, sea turtles, stress protocol.

Introduction

During the sea turtle nesting period, nesting females face several environmental factors that may cause stress and increase their glucocorticoid levels; for example, the fluctuation of water temperature (HUNT et al. 2012) and the prolonged period of aphagia (CHEREL et al. 1988, WILLIAMS et al. 2008) documented in the green sea turtle, *Chelonia mydas* (LINNAEUS, 1758) (HAYS et al. 2002) and in the hawksbill turtle, *Eretmochelys imbricata* (LINNAEUS, 1766) (SANTOS et al. 2010) are frequent stressors. Sustained aphagia harms the females' body condition, deteriorating their physical condition and body mass (CHEREL et al. 1988). However, green sea turtles seem to tolerate periods of aphagia, demonstrating decreased corticosterone levels at the end of the reproductive season, suggesting that their nutritional status is not compromised (HAMANN et al. 2002).

The reproduction of marine chelonians depends on several factors, such as the fluctuation of corticosterone during the nesting season (VALVERDE et al. 1999, STEPHENSON et al. 2000). Nesting female sea turtles require hypothalamic–pituitary–adrenal (HPA) modulation to assist with sufficient energy to perform and complete oviposition successfully (*Lepidochelys olivacea* (ESCHSCHOLTZ, 1829): VALVERDE et al. 1999; *Dermochelys coriacea* (VANDELLI, 1761):

ROSTAL et al. 2001, AL-HABSI et al. 2006; *Chelonia mydas* and *Eretmochelys imbricata*: JESSOP 2001).

However, it is not clear whether this modulation of the HPA axis develops in the same way in all species of sea turtles. The Kemp's ridley sea turtle, *Lepidochelys kempii* (GARMAN, 1880), is the smallest sea turtle and due to its diurnal habits must withstand higher environmental temperatures during the nesting process than other species, which exhibit nocturnal nesting. This study aims to estimate corticosterone concentration in *L. kempii* as a response to induced stress and determine its variations at the beginning, middle, and end of the nesting season.

Materials and methods

The study was conducted on Santander beach, Alto Lucero municipality, Veracruz, Mexico (19°5'27.80" N, 96°31'34.49" W and 19°51'38.58" N, 96°27'46.47" W). We collected the Kemp's ridley plasma samples between April and June 2017 and during the same period in 2018. Plasma samples were collected from 22 turtles. Mean (\pm SD) of straight carapace length was 640.03 \pm 20.22 mm (range: 700.87–590.69 mm), and the mean mass was 3500.18 \pm 300.89 g (range: 4400.39–2700.34 g).

Stress protocol and sample collection

We carried out daytime surveys on the beach between 06:00 and 18:00 h. When we found a nesting female, we waited for oviposition to end and upon its completion immediately collected the first blood sample (0 min.). We then performed the stress protocol by laying the female on her shell for an hour and we collected 10 mm blood samples after 20 min., 40 min., and 60 min. (modified from VALVERDE et al. 1999). We collected blood samples by puncturing the cervical sinus using 21 g x 32 mm needles (OWENS & RUIZ 1980), after disinfection with iodine. Blood samples (10 ml) were placed in heparin tubes inside a cooler with cooling gels. In the laboratory, the plasma was centrifuged (3,000 rpm) for 10 min. The plasma was stored in Eppendorf tubes at -20°C (VALVERDE et al. 1999). We followed the Guidelines for Use of Live Amphibians and Reptiles in Field and Laboratory Research (American Society of Ichthyologists and Herpetologists 2004) were followed for the management of all animals used in the study. In addition, we adhered to the indications of the Mexican regulations for sea turtles, NOM-162-SEMARNAT-2012, and complied with protocols approved by the Secretariat of the Environment and Natural Resources of the Mexican Government (SGPA/DGVS/05014/17 and SGPA/DGVS/002776/18). We divided the nesting season into three periods: beginning (1–27 April), middle (28 April–25 May), and end (26 May–22 June).

Analysis of corticosterone (CORT) concentration

We analyzed plasma CORT concentration in the laboratories of the Faculty of Medicine and the Brain Research Center of the Universidad Veracruzana using the Corticosterone (Human, Rat, Mouse) ELISA kit from IBL International. All samples were duplicated (100 µl of solution per sample) in polystyrene plates with flat bottom wells. Accordingly, plasma samples and controls were conjugated with 200 µL of horseradish peroxidase signal amplifier and then mixed for 10 sec., and we incubated at room temperature for one hour. The plates were washed three times with diluted washing solution (400 µl per well). We added 100 µL of substrate solution (Tetramethylbenzidine: TMB) to each well. We incubated the mixture for 15 min. at room temperature and stopped the enzymatic reaction by adding 50 µL of stop solution to each well. We read the solution using the microtiter plate reader 10 min. after adding the stop solution. We calibrated the curve with the Myassays program by using four logistic parameters (4PL), as indicated in the kit. Analytical validation was obtained using ANOVA with parallelism to the standard curve with two samples in serial dilution. The curves were parallel to the standard curve ($P > 0.05$). We obtained an intra-assay coefficient of variation ranging from 6 to 12.8 % ($n = 2$), with the repetition of one sample in the same assay. The coefficient of variation between trials ranged from 11 to 20% ($n = 2$) measuring the same sample and different trials. Analytical validations demonstrated the accuracy and precision of the assays.

Data analysis

We determined normality using Shapiro-Wilk tests. We performed a repeated measures Friedman test to determine whether non-zero CORT concentration ratio times differed, and applied Dunn's post hoc tests. We used ANOVA or Kruskal-Wallis to determine whether the beginning, the middle, and the end of the nesting season differed statistically. All statistical analyses were performed in R version 3.6.2 (R Core Team 2013).

Results

We recorded significant differences between the CORT concentrations from the 22 samples taken at different times (Friedman, $F = 30.6$; $df = 3$; $p < 0.001$). The mean concentration of corticosterone at: T-0 was 6.02 ± 3.21 ng/mL, T-20 was 8.20 ± 3.36 ng/mL, T-40 was 9.74 ± 4.26 ng/mL and T-60 was 12.22 ± 4.57 ng/mL (Fig. 1). The post hoc results showed significant differences in the increase in CORT in all treatments with respect to time zero, after 20 min. ($p = 0.0345$), 40 min. ($p < 0.0034$) and 60 min. ($p < 0.001$). The highest CORT concentration was at T-60 ($p < 0.01$).

The CORT concentration differed significantly between the beginning, middle and end of the nesting season ($\chi^2 = 7.05$, $gl = 3$, $p = 0.07$). A significant increase in T-60 ($p = 0.031$) compared T-0 was observed (Fig. 2a). T-0 and T-60 differed significantly during the middle season ($\chi^2 = 13.8$, $df = 3$, $p = 0.00319$), with a significant increase at 60 min. ($p = 0.0275$) (Fig. 2b). At the end of the season, differences were found between the times of the samples in CORT concentration ($\chi^2 = 17.914$, $df = 3$, value of $p = 0.0004581$), with a significant increase occurring after 20 min. of testing ($p = 0.0456$) (Fig. 2c).

We found a significant decrease in CORT concentrations at the end of the nesting season ($F = 5.6126$; $df = 2$, p -value = 0.01423), with differences between the beginning and the end of the nesting season ($p = 0.0206571$), and the middle

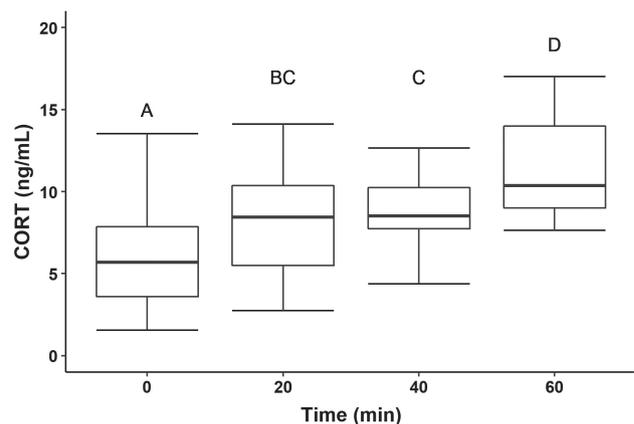


Figure 1. Variation of corticosterone concentration according to sample times of stress protocol in *Lepidochelys kempii* nesting season. Different letters indicate significant differences.

and the end of the nesting season ($p = 0.0327$). We did not find differences in CORT concentrations between the 60-min. records at the beginning, middle, and end of the nesting season ($\chi^2 = 1.1098$, $df = 2$, p -value = 0.5741) (Fig. 3).

Discussion

The stress protocol shows that Kemp's ridley sea turtles can increase corticosterone concentration within 20 min. in response to stress. This response was similar at the beginning, middle, and end of the nesting season and corticosterone levels were highest when exposure to intense

stimuli reached one hour. The rapid response we found in *L. kempii* is faster than the response reported for *L. olivacea* during a similar stress protocol, where the response occurred 120 min. after the stressor was applied (VALVERDE et al. 1999). The results are in line with our hypothesis; the Kemp's ridley sea turtle modulates stress response differently to other sea turtles species when faced with a stressful stimulus, considering that Kemp's ridley sea turtle, is the smallest sea turtle and has diurnal habits that force them to withstand higher environmental temperatures during the nesting unlike other species which exhibit nocturnal nesting. We therefore, found differences in the stress response time between a diurnal nesting species and a nocturnal nesting species of sea turtle.

The increases in CORT concentration in response to stress are similar to those found in immature *L. kempii* individuals during capture (GREGORY & SCHMID 2001). Immature individuals showed a low response to stress at 13 h of transported (7.05 ± 2.82 ng/ml), while individuals transported at 26 h had a mean increase in corticosterone of 11.56 ± 3.42 ng/ml (HUNT et al. 2016). The corticosterone concentration reported by HUNT et al. (2016) is similar to the concentrations we report (12.61 ± 3.39 ng/ml). Our data suggests that in adult females Kemp's ridley, the response time and glucocorticoid concentration are similar to immature individuals when they face stressors factors different.

The high concentration of glucocorticoids suggests that nesting Kemp's ridley sea turtles have a different and faster response capacity than olive ridley turtles to stressors. Different stressors occur during daytime nesting than at nighttime nesting and could be affecting the increase in CORT concentration, such as sun exposure and overheating of the sand (SPOTILA & STANDORA 1985), as well as a more significant human presence and increased number of predators (WINGFIELD et al. 1998). In addition, *L. kempii* is the smallest marine turtle (MÁRQUEZ 1996, PRITCHARD & MORTIMER 2000), and therefore has a lower resistance

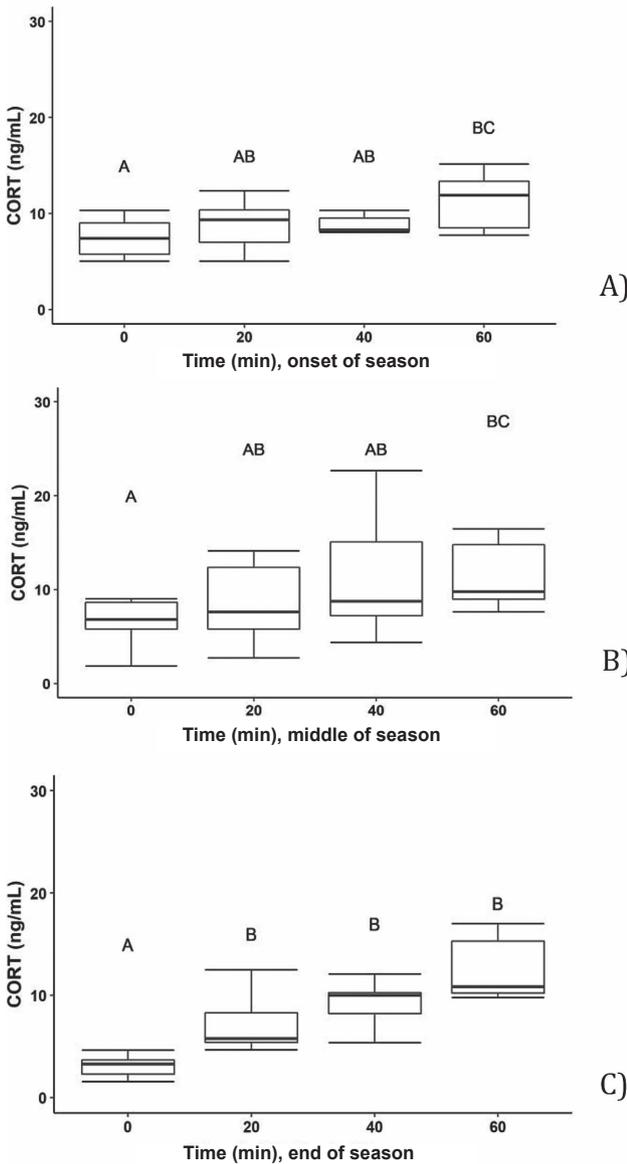


Figure 2. Differences between sample times in Kemp's sea ridley turtles nesting season: A) onset, $p = 0.0311$; B) middle, $p = 0.0275$; and C) end, $p = 0.0456$. Different letters indicate significant differences.

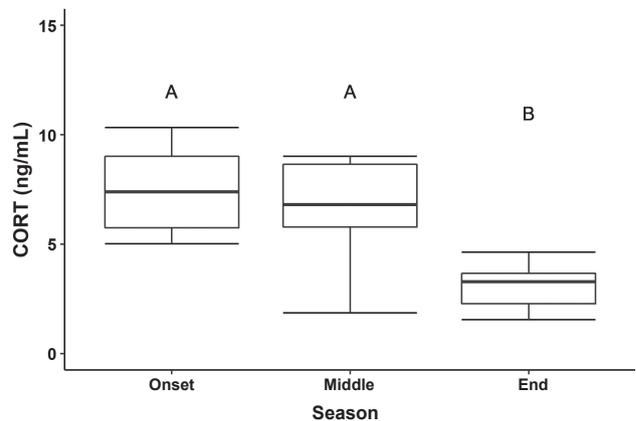


Figure 3. Differences in corticosterone concentration in T-0 during Kemp's ridley sea turtles nesting season: onset vs. end ($p = 0.0206571$), middle vs. end ($p = 0.0327514$). Different letters indicate significant differences.

to acute stress because they have fewer relative energy reserves compared to larger species (WINGFIELD et al. 1995); this could explain the rapid response to stress, generating a high concentration of CORT.

The stress response recorded in *L. kempii* was faster than other species of sea turtles. For example, suppression of hormonal response has been recorded for nesting females of *C. mydas* and *E. imbricata* suppression, as they do not show high CORT levels after capture (JESSOP 2001), in contrast to the significant increase found in Kemp's ridley nesting females. A stress modulation response has been observed in species such as *Lepidochelys olivacea* (VALVERDE et al. 1999) and *Dermochelys coriacea* (ROSTAL et al. 2001, AL-HABSI et al. 2006), and the modulation of the HPA axis allow them to maximize reproductive investment (JESSOP 2001). However, our results suggest that this characteristic is not shared by *L. kempii*, which has a lower sensitivity threshold to stressors than other sea turtles because it shows rapid activation in the HPA axis. This strategy may aid survival, improving their ability to stay alert, as being a uniquely diurnal nesting species they are exposed to greater risk factors during the nesting season.

In nesting Kemp's ridley sea turtles CORT levels decreased between 0.78 and 7.1 times to the end compared to the beginning of the stress protocol. Immature individuals CORT concentration increase 7.2-fold after 30-min. retention (GREGORY et al. 1996), and they increased 15 times in the face of acute stress during net capture (GREGORY & SCHMID 2001). In both afore mentioned studies, CORT's levels increase in the stress test's final was greater than our investigation. Those results were consistent with other sea turtle species where immature individuals show higher HPA axis activation than nesting females (AGUIRRE et al. 1995, VALVERDE et al. 1999, MOORE & JESSOP 2003, HUNT et al. 2012). We recorded differences in CORT levels at the beginning, middle, and end of the nesting season. We expected an increase in CORT levels as the nesting season progressed due to the possible loss of energy reserves and deterioration of the female's body condition during the period of aphagia; however, contrary to expectation, we observed a significant decrease in CORT concentration at the end of the nesting season. Similarly, WHITTIER et al. (1997) found CORT concentrations of *Caretta caretta* (LINNAEUS, 1758) to decrease significantly during the nesting season, suggesting that basal CORT level stabilize, as has been observed in immature individuals. Nesting female *C. mydas* did not reach critical body condition levels after the nesting season, suggesting that the body does not perceive prolonged periods of aphagia as a stressor (HAMANN et al. 2002). Sea turtles display seasonal physiological changes that function to regulate annual reproductive patterns, and at the end of the nesting season, body deterioration can be minimized, allowing the females to reach the feeding sites (ROSTAL et al. 1998). The variation in corticosterone concentration in our study and other species of marine turtles demonstrates the need for further studies that take environmental conditions and the individuals' intrinsic conditions into account. In conclusion, nesting fe-

male Kemp's ridley sea turtles respond quickly to a stressor stimulus, and at the end of the nesting season they manage to reduce the concentration of CORT with respect to the beginning of the season, so the modulation of the HPA axis helps them to successfully conclude all nesting events during the season.

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