

# Assessment of captive rearing conditions on loggerhead hatchlings: Effect of handling frequency and stocking density

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## Abstract

Frequently, stranded sea turtles require rehabilitation under controlled conditions. Currently, few publications have described the conditions under which rehabilitation is to take place, particularly with respect to the hatchling life stage. To address this paucity of data, we conducted some experiments to assist rehabilitating facilities assess their handling of hatchlings. While in captivity, hatchlings are routinely handled, for example, for data collection and cleaning. Standardization of handling and housing protocols is necessary to define the most adequate rearing conditions to maintain hatchling welfare. Accordingly, the aim of this study was to assess plasma circulating corticosterone (Cort) concentration and growth, as a biomarker for the stress of hatchling loggerhead sea turtles (*Caretta caretta*) under controlled conditions. We performed two experiments to analyze handling frequency and stocking density. In both, Cort was measured and correlated with variations in animal weight and length. In handling experiments, Cort exhibited no significant increase when hatchlings were handled once a week, whereas Cort was significantly elevated when hatchlings were handled once every 2 weeks, suggesting that hatchlings have the ability to acclimate to frequent handling. However, hatchlings exhibited similar growth and mortality, regardless of handling regime. In stocking density experiments, hatchling isolation induced a significant elevation of Cort, in comparison with hatchlings placed with conspecifics at increasing densities. Growth increased in singly housed hatchlings, while mortality increased in tanks with three or more hatchlings. The results obtained suggest that Cort, growth, and mortality should be measured to assess hatchling welfare when kept under controlled conditions.

## KEYWORDS

*Caretta caretta*, controlled conditions, handling protocols, hatchlings, loggerhead sea turtle, North Atlantic, welfare

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## 1 | INTRODUCTION

All sea turtle species are threatened by anthropogenic perturbations at sea (e.g., fisheries bycatch, litter, pollution) and on land (e.g., coastal development or destruction of nesting beaches) (Abreu-Grobois & Plotkin, 2008; Casale & Tucker, 2017; Mortimer & Donnelly, 2008; Seminoff, 2004; Wallace et al., 2013; Wibbels & Bevan, 2019). These threats increase pressure on the already high levels of natural mortality that take place on nesting beaches and at sea in the early life stages of these reptiles (Heithaus, 2013). Fortunately, conservation programs implemented globally, particularly geared at promoting hatchling production, have allowed the partial recovery of some sea turtle nesting populations over the past few decades (Shaver et al., 2005; Shaver & Rubio, 2008).

Besides increasing hatchling production, an activity that helps individual animals is the rescue, rehabilitation, and release of live stranded sea turtles (Caillouet et al., 2016; Innis et al., 2019). In these programs, eggs or hatchlings are collected from the wild (Heppell et al., 1996) and reared under controlled conditions for variable periods, ranging from months to years, before they are released back into the wild (Shaver & Wibbels, 2007). A 1-year period is considered appropriate because released animals are large enough to avoid most predators associated with hatchlings and posthatchling stages (Caillouet Jr et al., 1997; Shaver & Wibbels, 2007). Animals kept in rehabilitation facilities require standardized husbandry protocols, where several factors must be considered, depending on the characteristics of each species and the available facilities.

Animal health and welfare are important aspects to consider when holding sea turtle hatchlings under controlled conditions, including housing conditions, cleaning protocols, and feeding strategies, among others. General welfare and health during this time can be assessed through hatchling behavior, physiological biomarkers (e.g., stress biomarkers), or other indirect parameters, such as appetite or the absence of illness (Arena & Warwick, 1995). The homeostatic recovery of animals after changes in the environment or stressful, captivity-related situations is key to ensure animal welfare (Conte, 2004). Traditionally, circulating Corticosterone (Cort), the main glucocorticoid in reptiles, has been used as a biomarker of stress in sea turtles (Gregory et al., 1996; Milton & Lutz, 2003; Tokarz & Summers, 2011). Circulating Cort concentration increases in reptiles that face acute stressful conditions (Carbajal et al., 2018; Cockrem, 2013). Changes in circulating Cort of wild sea turtles have been studied under different field conditions. For example, in adult females during the nesting season, Cort variations have been linked to factors affecting reproductive success, for example, nesting density or shark attacks (Flower et al., 2018; Jessop, 2001; Jessop & Hamann, 2005; Jessop et al., 1999; Jessop, Sumner, Lance, et al., 2004; Rostal et al., 2001; Valverde et al., 1999; Whittier et al., 1997). Also, variations of Cort have been related to handling or recovery/illness in wild juvenile sea turtles (Aguirre et al., 1995; Gregory et al., 1996; Hunt et al., 2012; Jessop & Hamann, 2005; Jessop, Sumner, Limpus, et al., 2004), and with dispersal behavior in wild hatchlings (Hamann et al., 2007; Pereira et al., 2012). However, little is known about the variation of Cort concentration in hatchlings under controlled conditions, a key point to assess

rehabilitation programs aimed at promoting their health. Most established parameters to rear sea turtle hatchlings under controlled conditions are based on health and behavioral parameters, where the survival rate or the prevalence of diseases or injuries, under various temperatures and stocking densities, has been used (Fish and Wildlife Service, 2013).

The aim of this study was to develop a method to assess protocols for the rearing program conducted in the Canary Islands (from 2006 to 2012), based on biomarkers of general health (Cort variation and growth) in relation to handling frequency and stocking density. To do this, we used hatchlings hatched and reared under controlled conditions to develop experiments to assess how varying levels of these factors affected the circulating Cort concentration of turtles, in addition to weight gain, body size increase, and mortality.

## 2 | MATERIAL AND METHODS

### 2.1 | Animal origin

Loggerhead hatchlings came from eggs collected from nesting beaches (*Ervatão*—16°02'29"N 22°41'52"W—and *Ponta Cosme*—16°02'00"N 22°42'28"W—beaches) at the “Reserva Natural das Tartarugas,” on southeastern Boa Vista Island (Cape Verde), which exhibits low natural hatchling productivity. Whole clutches were collected during oviposition, placed into plastic bags (one clutch per bag), and translocated to a hatchery where they were incubated, away from tides and natural predation. Egg collection and translocation were carried out by the staff and trained volunteers from the NGO Cabo Verde Natura 2000, following standard protocols (Abella et al., 2007). The experiments were conducted under the License n° 43/2013 and n° 25/2014 issued by *Direção Nacional do Ambiente (Cape Verde Government)* to NGO Cabo Verde Natura 2000, and blood samples were shipped to the Canary Islands under the License n°2/2015 from the *Direção Nacional do Ambiente (Cape Verde Government)*.

### 2.2 | Husbandry conditions

Experimental tanks were located at the Cabo Verde Natura 2000 indoor facilities, in Sal-Rei, Boa Vista Island (Cape Verde), under natural photoperiod. All tanks were identical, rectangular, 100 L capacity, made of glass, and placed together in the same room on different shelves. Each tank was equipped with its own external waterfall filter (50 L/h, renewal rate of 12 times/d) and filled with seawater collected from Cabral beach (Northern Boa Vista), a nesting beach close to the rearing facilities; seawater temperature was maintained at  $24.8 \pm 1.8^\circ\text{C}$ , within the natural thermal range in the wild (Mansfield et al., 2014). Filters were cleaned one to two times per week and food remains and feces were siphoned daily. Every 2 weeks, tanks were completely cleaned, and water renewed. Water pH,  $\text{NO}_2$ , and  $\text{NO}_3$  were monitored, being within natural ranges ( $\text{pH} = 7.67 \pm 0.01$ ,  $\text{NO}_2 = 1.94 \pm 0.21$  ppb, and  $\text{NO}_3 = 19.13 \pm 2.12$  ppb). Water turbidity was monitored visually, and if the water was estimated to have become cloudy before the cleaning day, it was replaced.

Each hatchling was marked with a number that was painted on their carapace with nail polish. The numbers were repainted every 2 weeks when hatchlings were handled to clean and collect biometric data and sampling. Handling consisted of a series of standardized protocols, including gently taking the animals out of the water, taking a blood sample (between 0.1 and 0.3 ml per sample, when programmed), measuring the carapace length with a caliper to the nearest 0.1 cm (Bolten, 1999), weighing them to the nearest 0.01 g, rubbing them softly with a wet cloth to remove algae, rewriting the identification number, and returning them to the water. The entire process took less than 2 min. Handling was always done on the same day of the week, at the same time, and following the same routine.

Hatchlings were fed five times a week (from Monday to Friday), leaving two fasting days (Saturday and Sunday). Hatchlings were fed the equivalent of 5% of their weight per feeding day, following feeding protocols developed by researchers from the University of Las Palmas de Gran Canaria (Spain), as part of the head-starting program in the Canary Islands from 2006 to 2012 (Liria-Loza, unpublished data), together with protocols established by Bluvias and Eckert (2010), for injured hatchlings. The amount of food (g) was recalculated for each hatchling after each weighing. Hatchlings were fed in the morning and the process consisted of cutting up fresh fish, shrimp, and/or squid with scissors in pieces smaller than their mouth, dispersing food items in the tanks. Feeding was monitored by direct observation, though it was not possible to monitor hatchlings continuously to prevent negative interactions. All hatchlings ate normally throughout the study period. No aggression behavior was observed, neither lesions derived from bites, but we cannot guarantee that aggression did not occur because hatchlings were not observed 24 h a day.

Throughout the experiments, dead hatchlings were not replaced due to permitting limitations in the number of animals used in our experiments. However, mortality was low for the overall study, so the lack of replacement should have had a minimal influence on final results in terms of growth and Cort concentration.

### 2.3 | Trial I: Handling protocol

In the first trial, 72 hatchlings were chosen from three different nests (24 hatchlings from each nest), hatched between October 31 and November 4, 2013. Two hatchlings from each nest (six hatchlings per tank) were reared for 6 months on each of the 12 tanks upon emergence.

Two different treatments were tested, with six replicates (six tanks per treatment). The first treatment (control group) consisted of handling the hatchlings once per week; in the second treatment, hatchlings were handled once every 2 weeks (low handling frequency [LHF]). Blood collection took place throughout the experimental period after each hatchling was pulled from the tank, at weeks 2 (w2), 10 (w10), 18 (w18), and 26 (w26). The weight (W; g) and minimum straight carapace length (SCLmin; cm) of each animal were also measured every time. Weight increase (WI) and carapace length

increase (LI) referred to the total increase of weight and length for each hatchling during the trial period, respectively. Mortality was also recorded during the entire experiment.

### 2.4 | Trial II: Effect of stocking density

In the second trial, 57 loggerhead hatchlings were chosen from three different nests (19 from each nest) and hatched between October 27 and 28, 2014. The number of hatchlings per tank was set up to evaluate the effect of stocking density. Trial II began just after hatchlings emerged from the nest and lasted for 6 months.

Four different treatments were tested, with three replicates (three tanks) per treatment. The first treatment (D1) consisted of one hatchling per tank (10 hatchlings/m<sup>3</sup>), where each hatchling came from a different nest. The second (D3) consisted of three hatchlings per tank (30 hatchlings/m<sup>3</sup>), with one hatchling from each tank come from a different nest. In a third treatment (D6), there were six animals per tank (60 hatchlings/m<sup>3</sup>), with two hatchlings from each nest. Finally, the fourth treatment (D9) consisted of nine hatchlings per tank (90 hatchlings/m<sup>3</sup>), with three hatchlings from each nest. None of the tanks presented any kind of environmental enrichment.

All hatchling data were treated using the same handling protocol as in the previous trial (handled once per week). Biometric data were collected following the same procedures described for Trial I. Hatchlings were weighed once per week and SCLmin was measured once every 4 weeks. WI and LI were calculated as in Trial I, and mortality recorded. Blood samples from each hatchling were collected just after emergence from the nest (w0), and then at weeks 7 (w7), 15 (w15), and 23 (w23).

### 2.5 | Blood collection and sample preparation

Blood volume in reptiles is about 5%–8% of their total body mass; it is recommended to extract a maximum of 10% to avoid harming the animal (Mader & Rudloff, 2006). Accordingly, we drew from 0.1 ml (when hatchlings weighed less than 37.5 g) to 0.3 ml (when hatchlings exceeded that weight) of blood. We drew blood samples from the dorsal cervical sinus, previously disinfected with an alcohol gauze, using 1 and 0.5 ml syringes with a 29 G/12.7-mm needle, and dispensed into 1 ml lithium heparin tubes. Samples were kept refrigerated until centrifugation at a relative centrifugal force of 3000 g for 5 min to obtain plasma. Blood plasma was pipetted into 1.5 ml Eppendorf® tubes and kept frozen at –30°C.

### 2.6 | Analysis of circulating Cort in plasma

Frozen plasma samples were sent to the Department of Animal Health and Anatomy (Veterinarian Faculty) from the Universitat Autònoma de Barcelona (Barcelona, Spain) to measure Cort

concentration. Assays were validated using competitive EIA kits (Neogen® Corporation Europe). Assay validation was conducted following the criteria for an immunological validation: precision, specificity, accuracy, and sensitivity (Buchanan & Goldsmith, 2004; Reimers & Lamb, 1991) using extracts from 20 samples.

Intra- and interassay coefficient of variation (CV) from all duplicated samples was calculated to assess the precision of the test. The specificity was evaluated with the linearity of dilution, determined by using 1:1, 1:2, 1:4, and 1:8 dilutions of a plasma pool with EIA buffer. Accuracy was assessed through the spike-and-recovery test, calculated by adding different amounts of pool to different volumes of pure standard Cort solution of known concentrations. Finally, the sensitivity of the test was given by the smallest amount of hormone that the assay could distinguish and measure.

## 2.7 | Statistical analysis

We conducted all statistical analyses in R version 3.1.2 (R Department Core Team 2014). For both experiments, we analyzed the effect of the different treatments over Cort concentration using a generalized linear mixed-effects model (GLMM), to handle grouping/repeated measures, allowing both intercepts and slopes to differ between groups. In both experiments, we analyzed mortality in relation with treatments and time using a generalized linear model (GLM), with a binomial family error structure and a logit link function, where hatchlings were considered as dead (0) or alive (1), for each treatment and time. In the second experiment, we divided hatchlings into two groups after completion of the experiment: those that survived the entire experimental period and those that underwent some mortality. With this setup, using a GLMM we analyzed differences in weight and Cort between the two groups, together with the different treatments (D1, D3, D6, and D9), with the time as a random factor. For all models, we checked model assumptions of homogeneous variances and normality of errors through visual inspection of residuals and quantile-quantile (QQ) plots (Harrison et al., 2018). We implemented mixed models using the “lme4” package (Bates et al., 2015).

We conducted Pearson's correlation tests between Cort concentration and weight, as well as between Cort and weight increase and length increase. Finally, we conducted a one-way ANOVA to analyze any possible effects of the different treatments over the weight and length increase at the end of the experiment. We considered results significantly different at  $p < 0.05$ .

## 3 | RESULTS

### 3.1 | Biochemical validation of the EIA

Mean intra- and interassay coefficient of variation for Cort was 10.3% and 11.9%, respectively. In the dilution test, obtained and expected Cort concentrations were significantly correlated ( $r = 0.99$ ,

$p < 0.05$ ), with a mean percentage error of 6.4%. In the spike-and-recovery test, hormone standard spiked with the pool presented a mean recovery of  $103.1\% \pm 1.66$  (mean  $\pm$  SE). The sensitivity of the Cort assay was 34 pg/ml plasma. These results demonstrated that the EIA Kit used was precise, specific, accurate, and sensitive in measuring Cort concentration in plasma of the loggerhead sea turtle.

### 3.2 | Hatchlings

In 2013, a total of 216 eggs hatched from the 236 incubated in the hatchery, hatchlings having a mean mass of  $18.20 \pm 0.19$  g and straight carapace length of  $44.08 \pm 0.19$  mm. In 2014, 207 eggs hatched out of 255 incubated; hatchlings from this cohort had a mean mass of  $16.17 \pm 0.13$  g and straight carapace length of  $42.20 \pm 0.13$  mm. All nests from both years presented a mean emergence success of 86.2% (range 95.5%–67.4%).

### 3.3 | Trial I: Handling protocols

Handling frequency affected neither the total weight increase ( $F = 0.073$ ,  $p = 0.789$ ) nor the SCLmin increase ( $F = 0.029$ ,  $p = 0.993$ ) of loggerhead hatchlings. The mean ( $\pm$ SE) carapace length increase (LI) during the entire trial was  $82.53 \pm 3.1$  mm for hatchlings handled frequently (control group) and  $81.52 \pm 2.1$  mm for hatchlings handled less frequently (LHF). The mean ( $\pm$ SE) weight increase (WI) was  $292.05 \pm 23.5$  g for hatchlings handled frequently (control group) and  $290.98 \pm 14.8$  g for hatchlings handled less frequently (Table 1).

Through the 6 months, nine hatchlings out of 72 died during the trial. According to the GLM results, this mortality was not significantly explained by different handling frequencies (Table 1). Cort concentration did not change over the course of the trial for control and LHF hatchlings, as there was no significant effect of the different sampling times (variance = 0.795,  $SD = 0.89$ ). However, LHF hatchlings exhibited significantly higher circulating Cort concentration than those of the control group ( $t = 6.93$ ,  $p < 0.05$ ; Figure 1).

Finally, no correlation between Cort concentration and growth was found, neither between Cort and weight ( $\rho = -0.10$ ,  $p > 0.05$ ), Cort and WI ( $\rho = -0.08$ ,  $p > 0.05$ ), nor with an increase in carapace length LI ( $\rho = 0.19$ ,  $p > 0.05$ ).

### 3.4 | Trial II: Effect of stocking density

The different stocking densities had no significant effect on the total WI ( $F = 1.90$ ,  $p > 0.05$ ) and on the SCLmin increase ( $F = 2.45$ ,  $p > 0.05$ ), even though hatchlings under treatment D1 appeared to grow slightly faster. The mean ( $\pm$ SE) carapace LI ranged from  $40.1 \pm 2.0$  mm to  $50.2 \pm 0.7$  mm and the mean ( $\pm$ SE) WI ranged from  $86.4 \pm 7.0$  g to  $117.1 \pm 4.5$  g (Table 2).

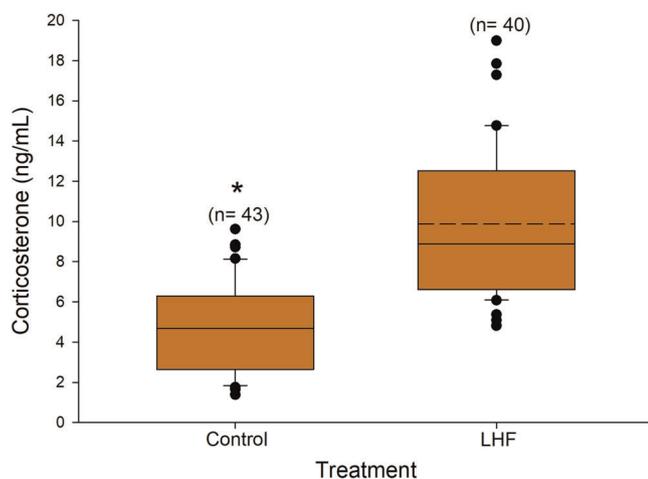
During the 6 months study, 27 hatchlings out of 57 died, most of them (18) from the D9 treatment, and none from D1. Nevertheless,

**TABLE 1** Length increase (LI, mm), weight increase (WI, g, mean  $\pm$  SE), mortality of hatchlings (in absolute numbers), and percentage and Cort concentration (ng/ml, mean  $\pm$  SE) of the hatchlings from Trial 1, according to the standardized handling protocols, as well as according to the different sampling times (week) for the Cort concentration

	Control	Low handling
N	36	36
Length Increase (LI)	82.53 $\pm$ 3.1	81.52 $\pm$ 2.1
Weight Increase (WI)	292.05 $\pm$ 23.5	290.98 $\pm$ 14.8
Mortality	4 (11.1%)	5 (13.9%)
Corticosterone		
Week 2	4.96 $\pm$ 0.66	11.18 $\pm$ 1.07
Week 10	4.66 $\pm$ 0.49	8.74 $\pm$ 1.12
Week 18	4.01 $\pm$ 0.79	10.16 $\pm$ 1.03
Week 26	4.84 $\pm$ 0.54	9.00 $\pm$ 0.70
General	4.68 $\pm$ 0.31	9.88 $\pm$ 0.51*

Note: Asterisk denotes significant differences ( $p < 0.05$ ).

according to the GLM results, mortality was not significantly different among treatments (Table 2). Furthermore, the between-groups GLMM test indicated that there were significant differences in weight between the group of survivors and the group of dead hatchlings, with the survivors being heavier ( $t = 4.36$ ,  $p < 0.05$ ), as well as between the different treatments, with hatchlings from treatment D1 being significantly heavier than others (D3:  $t = -6.12$ ,  $p < 0.05$ ; D6:  $t = -6.03$ ,  $p < 0.05$ ; D9:  $t = -8.40$ ,  $p < 0.05$ ). Additionally, the survivors were significantly heavier in all the treatments (Figure 2).



**FIGURE 1** Circulating corticosterone concentration in loggerhead hatchlings handled once a week versus those at low handling frequency (LHF). Dots represent outliers. Solid line within the box is the median. Dashed line represents the mean. Asterisk denotes significant differences ( $p < 0.05$ ) among handling treatments [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

The within-subject GLMM test indicated that there was a significant time effect for all treatments (D3:  $t = 403.6$ ,  $p < 0.05$ ; D6:  $t = 175.1$ ,  $p < 0.05$ ; D9:  $t = 164.6$ ,  $p < 0.05$ ), indicating that hatchlings in all treatments, gained weight over time. Moreover, the interaction of time and group (survivors and nonsurvivors) was significant for all the treatments, except for D1, which did not have any dead animal (D3:  $F = 17.21$ ,  $p < 0.05$ ; D6:  $F = 5.29$ ,  $p < 0.05$ ; D9:  $F = 1.74$ ,  $p < 0.05$ ). This means that each group changed over time, but they changed in different ways. As seen in Figure 2, the lines of mass increase were not parallel and progressively moved farther apart over time. However, there were no significant differences in Cort concentration between the group with all survivors and those with mortality.

Cort concentration did not change over the course of the study, so there was no significant effect due to the different sampling times (variance = 2.187,  $SD = 1.479$ ). Moreover, the number of hatchlings per tank exhibited a statistically significant effect ( $t = 6.47$ ,  $p < 0.05$ ) on the circulating Cort concentration. Hatchlings from treatment D1 had higher circulating Cort concentration in blood (5.46  $\pm$  0.78 ng/ml) compared to hatchlings from the other treatments (D3, D6, and D9; Table 2, Figure 3;  $p > 0.05$ ).

As with Trial 1 experiments, no correlation between Cort concentration and growth parameters was found, with no significant correlation between Cort concentration and mass ( $\rho = 0.05$ ,  $p > 0.05$ ), WI ( $\rho = 0.00$ ,  $p > 0.05$ ), nor with LI ( $\rho = -0.12$ ,  $p > 0.05$ ).

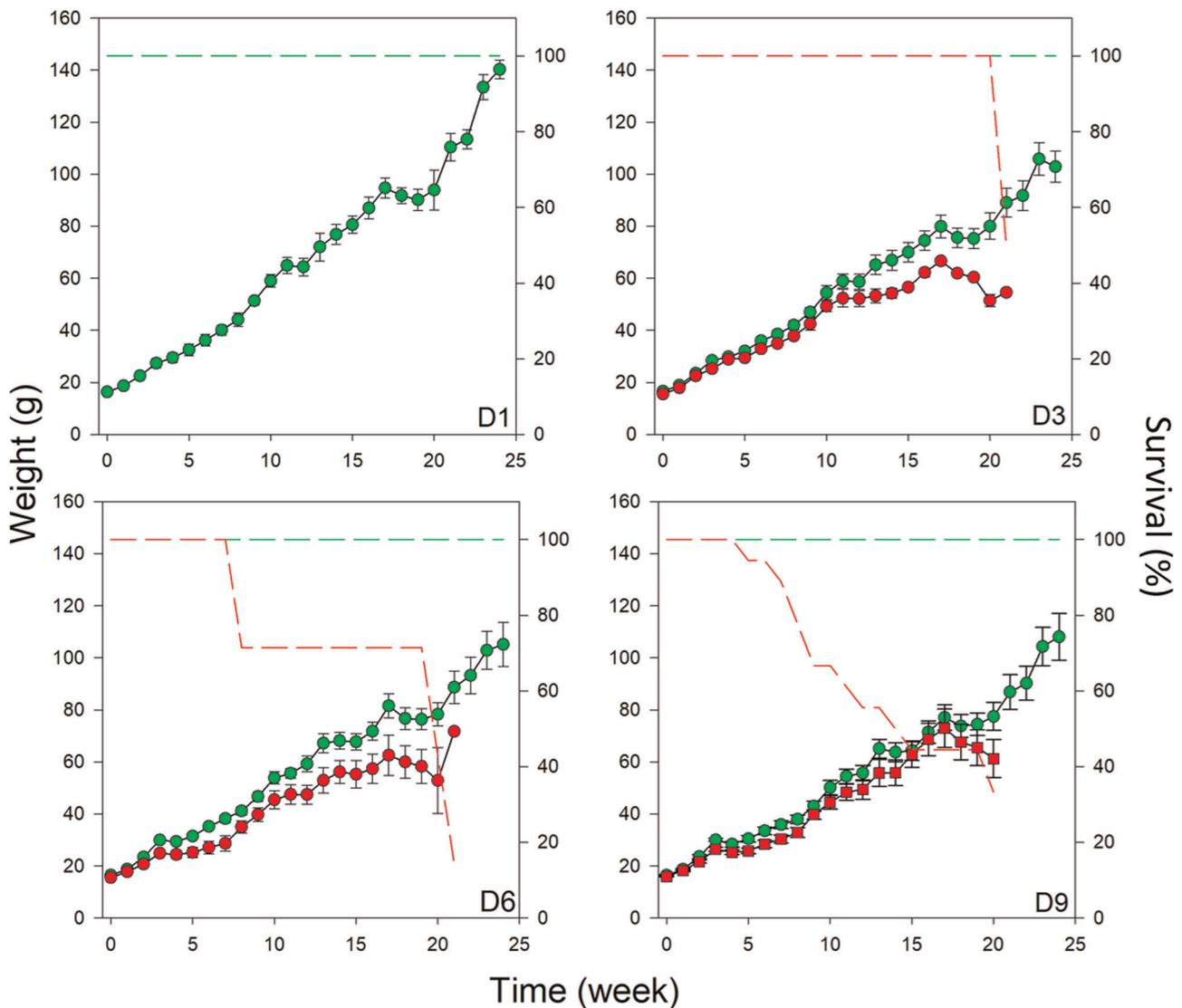
## 4 | DISCUSSION

When sea turtle hatchlings are reared under controlled conditions for short periods, either for conservation or research purposes, or for longer periods in head-start programs, different procedures, such as weighing, measuring, or cleaning, are conducted on a regular basis (Hamann et al., 2007). Although different studies have examined how some of those procedures can affect hatchling behavior, none of them have dealt with how controlled conditions, including handling procedures, could modify physiological functions that may be used as biomarkers of animal welfare and health. In our study, we attempted to use Cort as a biomarker of stress and health on loggerhead hatchlings. The reason for this is that sea turtles, like other vertebrates, respond to unexpected physical stimuli (i.e., capture and handling; Wingfield et al., 1997) with an elevated circulating concentration of Cort. This increased Cort has been demonstrated in Kemp's Ridley, Olive Ridley, loggerhead, and Green sea turtles (Gregory & Schmid, 2001; Gregory et al., 1996; Hunt et al., 2016; Jessop et al., 2000; Valverde et al., 1999). Our hypothesis was that if rearing conditions were not optimal, circulating Cort concentration would increase significantly. An important caveat is that Cort is not a stress hormone per se; rather, Cort is a hormone with profound metabolic effects that are part of an integrated response to stress (MacDougall-Shackleton et al., 2019). As such, Cort elevation may be due to multiple factors (Dickens & Romero, 2013; MacDougall-Shackleton et al., 2019). Our results are consistent with our hypothesis in that LHF hatchlings handled with low frequency (i.e., every 2 weeks) exhibited a significantly elevated

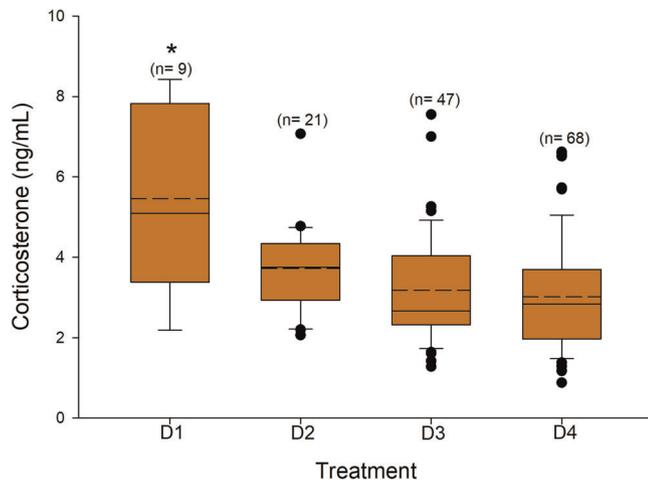
	D1	D3	D6	D9
N	3	8	18	27
Length Increase (LI)	50.2 ± 0.7	40.1 ± 2.0	40.6 ± 1.9	41.9 ± 2.0
Weight Increase (WI)	117.1 ± 4.5	89.3 ± 6.3	86.4 ± 7.0	88.0 ± 7.0
Mortality	0 (0%)	2 (22.2%)	7 (38.8%)	18 (66.6%)
Corticosterone				
Week 0	4.57 ± 1.95	4.07 ± 0.66	3.59 ± 0.43	3.46 ± 0.35
Week 7	6.05 ± 0.95	3.32 ± 0.35	2.80 ± 0.29	2.63 ± 0.23
Week 15	3.82 ± 0.15	4.44 ± 0.13	3.31 ± 0.36	3.02 ± 0.31
Week 23	7.83 ± 0.08	3.48 ± 0.33	2.70 ± 0.25	2.60 ± 0.32
General	5.46 ± 0.78*	3.72 ± 0.24	3.18 ± 0.20	3.01 ± 0.17

Note: Asterisk denotes significant differences ( $p < 0.05$ ).

**TABLE 2** Length increase (LI, mm) and weight increase (WI, g, means ± SE), mortality of hatchlings (in absolute numbers), and percentage and Cort concentration (ng/ml, mean ± SE) of the hatchlings from Trial II, according to different stocking densities, as well as according to the different sampling times (week) for the Cort concentration



**FIGURE 2** Change in mass (g) and mortality (%) in the four rearing density treatments in loggerhead hatchlings raised in captivity. Dashed line represents the survival and the dotted line the hatchling mass. Red dots correspond with the hatchlings that exhibited mortality, and green dots to those with no mortality [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**FIGURE 3** Circulating corticosterone concentration in loggerhead hatchlings reared at four density treatments, D1 (one hatchling), D3 (three hatchlings), D6 (six hatchlings), and D9 (nine hatchlings). Dots represent outliers. Solid line within the box is the median. Dashed line represents the mean. Asterisks denote significant differences ( $p < 0.05$ ) among density treatments [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

Cort concentration relative to hatchlings handled once a week. Indeed, the concentration attained by these animals was similar to that of juvenile Loggerhead sea turtles in response to handling (Gregory et al., 1996), and to other species of turtles such as the red-eared slider turtle (*Trachemys scripta*) (Cash et al., 1997), and the Kemp's ridley sea turtle (*Lepidochelys kempii*) (Hunt et al., 2016), though all these studies dealt with acute stress, unlike ours. Our results suggest that once-a-week handling provides hatchlings with enough physical stimulation to habituate to routine handling activities. Habituation is a phenomenon by which vertebrates stop responding with elevated Cort concentration to repeated noxious stimuli (Cyr & Romero, 2009; French et al., 2008; Romero & Wikelski, 2002). Despite the elevated Cort concentration observed in LHF hatchlings, we did not observe a concurrent decrease in growth (neither in length nor in mass, nor increased mortality when compared to control hatchlings. Presumably, Cort elicited effective compensation of physiological disruption caused by LHF conditions, such that these hatchlings were able to exhibit normal growth, similar to those handled more frequently. Considering our results, we recommend that monitoring mortality, Cort concentration, and growth should be included in the assessment of the welfare of hatchlings reared under controlled conditions, as the information derived from these measurements seem to be complementary. It is important to remember that the duration of this study was only 24 weeks (6 months). Perhaps, this period was too short to identify deleterious effects of LHF conditions on growth in response to handling and rearing conditions in our study. We recommend extending the duration of future similar studies to 1 or 2 years, ages at which animals are typically released into the wild (Shaver & Wibbels, 2007). In short, handling the hatchlings once per week or once every two weeks produced similar gains in growth, at least during the first 6 months of life. However, if Cort concentration

somehow reflects suboptimal conditions that may divert some energy from growth and other physiological process in a longer timeframe, then handling the animals once a week may be advisable.

Regarding the effect of stocking density, our results showed that hatchling isolation induced a significant elevation of Cort concentration in loggerhead hatchlings. Indeed, when single hatchlings were placed in separate tanks in the absence of any environmental enrichment, Cort concentration exhibited a significant rise relative to the other densities studied. Having at least three hatchlings per container showed the same significantly lowered Cort response, in comparison with single hatchlings. Isolation in other vertebrates has been demonstrated to change their behavior (Riley et al., 2017), and similar changes have been described in other reptiles. For example, hatchlings of veiled chameleon (*Chamaeleo calypttratus*) raised in isolation were less sociable and bold (Ballen et al., 2014). Additionally, water snakes (*Natrix maura*) exhibited lessened social activity when they were incubated in isolation (Aubret et al., 2016). Interestingly, when sea turtle hatchlings reach the sea, they swim strongly offshore, away from the coastline, to avoid predators. In the case of Florida loggerheads, hatchlings swim until finding floating Sargasso aggregations, where they obtain refuge from predators, food, and thermal benefits (Mansfield et al., 2014). This refuge allows hatchlings to grow until they reach a size that offers protection against most predators (Mansfield et al., 2014). The differences in Cort concentration found in this study between hatchlings kept in isolation, and those sharing the tank with other hatchlings, could be due to the physiological response to isolation, as hatchlings may miss the protection given by enrichment structures, such as floating seaweeds, or to the absence of conspecifics. The latter raises the possibility that the aggregation by the design of sea turtle hatchlings early in life increases the health of the hatchlings as they disperse toward the safety of deeper waters. This would not be truly surprising, since the clutch of eggs is laid virtually at once and it is well known that hatchlings work together upon hatching to dig their way out of the nest and crawl—as a group—to the ocean. This synchronous group work is crucial to safely climb out of the nest and to reduce predator-associated mortality on crawling hatchlings. Single hatchlings in a tank devoid of any environmental enrichment may be too exposed to feel safe. Future studies including environmental enrichment on isolated hatchlings could be conducted to gain an improved understanding of the physiological and behavioral responses to isolation on loggerhead hatchlings, as suggested by Case et al. (2005).

For the high stocking densities experiment, we partitioned the Cort and biometric data of the hatchlings between two groups: one with all survivors and another one that included all nonsurvivors. Although there was no increased Cort concentration relative to that of isolated hatchlings, the mortality rate was considerably higher (range 22%–66.6%), whereas we did not observe mortality for the treatment with single hatchlings. Interestingly, hatchlings began to die earlier in the treatments of higher stocking densities: the higher the density, the earlier the hatchlings began to die. Along with the increased mortality in tanks with higher stocking density, we noted that the hatchlings that died were losing weight in all high-density

treatments before the moment of death, relative to those that survived over the 6 months of treatment. So, even though there was no significant difference in Cort concentration as a function of higher densities other factors, such as competition for food, spread of diseases, aggressive behavior, individual genetics, and so forth, may have affected the hatchlings, causing important mortality and decreased growth. Thus, assessment of stocking density and other environmental conditions, such as handling frequency, must include not only Cort measurements but also growth and survival rates as complementary measures of health. This is because stress may manifest in subtle and nearly undetectable ways, and multiple biomarkers in addition to circulating Cort concentration, may be necessary to ascertain hatchling health (Dickens & Romero, 2013; MacDougal-Shackleton et al., 2019), as done in this study.

In conclusion, according to our results, when loggerhead hatchlings need to be held under controlled conditions, standardized protocols should be applied, using regular handling (once per week), as growth was not affected, and Cort concentration remained low, suggesting that these were better conditions than subjecting hatchlings to lower frequency handling protocols (every 2 weeks). The stocking density of loggerhead hatchlings could be as high as 60 hatchlings per m<sup>3</sup> (three to six hatchlings per 100-L tanks) during the first 6 months of rearing under controlled conditions, to promote low mortality rates. Isolation of individual hatchlings is not recommended, as our results suggested a significant elevation of plasma Cort, though growth appeared to be better, and mortality was not observed. Finally, experiments with environmental enrichment need to be conducted on isolated hatchlings to confirm if this effect is caused by the lack of structures inside the tanks that may serve as a refuge.

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## CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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