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RESEARCH ARTICLE

Husbandry Protocols for Juvenile Loggerhead Sea Turtles (*Caretta caretta*) Based on Stress Response to Stocking Density and Dry-Dock Time

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ABSTRACT

When necessary, sea turtles are held captive for veterinarian care and research purposes. Protocols and basic guidelines have been described for husbandry of sea turtles with veterinarian needs but not considering physiological indicators of animal welfare. Because all sea turtle are imperiled species, monitoring their welfare is important. The aim of this study was to standardize husbandry protocols for loggerhead (*Caretta caretta*) juveniles held under seminatural conditions, based on circulating concentration of plasma corticosterone (Cort) and behavior. Two experiments were performed to analyze physiological and behavioral responses of the animals facing changes in stocking density and different dry-docking times. Cort analyses suggested that the number of animals per tank can be modified occasionally, without affecting their health and welfare. However, dry-docking time should be < 30 min, as indicated by the significant elevation of circulating Cort at ≥ 30 min, rising from 1.51- ng/ml to 5.28-ng/ml. Protocols tested did not affect behavioral responses, except for the breaths per move, which increased while Cort increased, despite differences exhibited by experimental animals in behavioral responses according to daily times (morning vs afternoon) and the sex of the animals.

KEYWORDS

Controlled conditions; welfare; Canary islands; North atlantic

Introduction

Reptiles represent a very diverse taxon, which includes more than 7000 species (Wilkinson, 2015), with the number of captive individuals increasing significantly over the last 20 years for companion animal (pet) trade, breeding and commercial industry, and for research purposes (Van Zanten & Simpson, 2021). Care and husbandry techniques of reptiles in captivity have changed significantly in the last 20 years. Traditionally, due to their low metabolic rates and apparent low activity, reptiles have been kept in small enclosures, paying little attention to their conditions and welfare (Van Zanten & Simpson, 2021). As general knowledge on reptiles has been increasing, care and husbandry techniques have been steadily improving (Wilkinson, 2015).

Regardless of the reason for keeping reptiles in captivity, all animals must have adequate conditions that fulfill their physiological and behavioral needs to ensure their welfare (Van Zanten & Simpson, 2021), that can vary depending on the perception that the animal has of a given situation (Whittaker et al., 2021). Most welfare protocols are based on four main principles: feeding, housing, health, and behavior, although some gaps remain in our knowledge of pain, stress, or natural

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behavior of reptiles (Whittaker et al., 2021). Specifically, there is a need to identify reliable indicators of health, including a transition from resource to animal-based indicators, and from group to individual assessment (Benn et al., 2019; Whittaker et al., 2021), using a combination of physiological, behavioral, and health indicators (Whitham & Wielebnowski, 2013), including a wide variety of parameters, such as behavior or homeostatic equilibrium after a stressful situation (Benn et al., 2019).

Exposure to stressful stimuli may induce a hormonal cascade mediated by the hypothalamic-pituitary-adrenal (HPA) axis commonly leading to the release of glucocorticoids from the adrenal cortex (Greenberg & Wingfield, 1987; Gregory & Schmid, 2001; Silvestre, 2014) to maintain or recover homeostatic processes (Greenberg & Wingfield, 1987). In reptiles, the main glucocorticoid secreted in the physiological regulation of the endocrine stress response is corticosterone (Cort) (Silvestre, 2014). Circulating Cort concentration increases in plasma within minutes to hours of exposure to a stressful stimulus (Greenberg & Wingfield, 1987).

It is widely accepted that measurement of products of the HPA axis, such as Cort, may serve as an indicator of acute stress (Greenberg & Wingfield, 1987). In the long term, however, the situation is less clear, as it has been observed that Cort concentration could increase or recover basal levels due to reasons unrelated to stress (Dickens & Romero, 2013; MacDougall-Shackleton et al., 2019). Additionally, the plasma Cort concentration could change according to the frequency of the stressor or to the type of stressor. Thus, in welfare studies it is important to include other indicators, such as the inhibition of growth and/or reproduction, as well as the feeding behavior.

A behavioral change could be an important indicator of disturbance, injury, and/or disease. Additionally, abnormal behaviors could be a proxy to identifying captivity-associated stress (Warwick et al., 2013). The behavioral complexity of reptilians (Burghardt, 2013) is evident when describing behaviors related to captivity/stress, which would depend on the animal class and species (Warwick et al., 2013).

Both physiological and behavioral responses contribute to the acclimation of the animal to disruptive environmental conditions (Jessop & Hamann, 2005; Sapolsky et al., 2000; Wingfield et al., 1998). When the animal cannot deal with that condition and the stressor continues over time (e.g., weeks), a condition of chronic stress ensues, where Cort concentration can recover normal basal levels, but other body functions are affected (Tokarz & Summers, 2011). Chronic stress in reptiles could inhibit their growth, may impair estrogen and vitellogenin production in females and testosterone in males,, and induce immunosuppression and significant behavioral changes, such as appetite suppression and inhibition of reproduction (Tokarz & Summers, 2011; Wilkinson, 2015).

Detailed husbandry protocols based on Cort, growth, and behavior variations according to different stocking densities, facility design (depth, shape, or environmental enrichment), temperature conditions, or food quality and quantity are available (Elsey et al., 1989, 1990; Tetzlaff et al., 2019; Van Zanten & Simpson, 2021; Zhang et al., 2017). Most of those protocols are for commercial species like crocodiles, alligators, or soft-shelled turtles (Van Zanten & Simpson, 2021), which are very well known due to their monetary value. In contrast, little is known about sea turtle husbandry and their environmental requirements (Songnui et al., 2017).

Detailed protocols and basic guidelines have been described for husbandry of sea turtles subjected to trauma (Bluvias & Eckert, 2010), including recommendations on holding conditions and handling during cleaning operations, health management, sampling procedures, and data collection. However, those guidelines are not based on physiological indicators of welfare (Bluvias & Eckert, 2010). Among the different aspects to consider during rehabilitation or research activities, housing conditions is one of the most important elements to consider to avoid deleterious effects on multiple individuals or social isolation (Arena et al., 2014), as it is sometimes unavoidable to house multiple turtles in a single tank.

As an example, loggerhead sea turtles (*Caretta caretta*) are mainly held in captivity when they are admitted into a rescue center for rehabilitation under veterinarian care (Phelan & Eckert, 2006). At rescue centers, animals stay in captivity for days to months, depending on the severity of their

injuries, or even years if they are considered unreleasable. Also, sea turtles could be held in captivity for research (Kawazu et al., 2018; Owens & Blanvillain, 2013). But since the loggerhead sea turtle is an imperiled species, monitoring its welfare while kept under captive conditions is paramount (Sherwen et al., 2018), and even more so if eventually the turtles are going to be returned to the ocean. Dry-docking (maintenance of the turtles out of the water), is necessary for cleaning purposes, data, or sample collection, or to administrate veterinarian treatments (Bluvias & Eckert, 2010). However, it is not clear how dry-docking affects sea turtles.

Accordingly, the aim of the present study was to optimize standardized husbandry protocols for loggerhead juveniles under seminatural conditions based on plasma Cort concentration and behavior response under different routine situations, such as separation/isolation situations or dry-docking time. To achieve this goal, two different experiments were performed. In the first one, changes in standardized stocking density were evaluated through Cort variation and behavior response. In a second experiment, a time-course study was conducted to evaluate variations of plasma Cort concentration in response to dry-dock time. The results allowed us to generate some recommendations when holding sea turtles in captivity in order to mitigate stress and promote positive animal wellbeing.

Material and methods

Animals included in the study

All animals used in this study come from eggs obtained in Boa Vista Island (Cape Verde) and incubated in the Canary Islands, and were reared under seminatural conditions, under the Canary Islands Government authorization (Nº reg: REUS/20330/2014), and individual Convention on International Trade in Environmental Species of Wild Flora and Fauna (CITES) permits. A total of 12 animals were included in the experiments, where their growth and health were monitored (weighed periodically and lesions/infections surveyed) during the entire rearing program. Growth data from the preceding 5 years were used to compare growth rates before and after been included in the experiments. All turtles were sexed when they were 2 years old through laparoscopy (Divers, 2010; Innis, 2010; Wyneken et al., 2007), resulting in six females and six males.

Husbandry conditions

Animals were kept under a natural photoperiod (outdoor tanks), in three 5,000 L rectangular tanks (4.00 m x 1.25 m x 1.00 m) with continuous seawater flux (1,500 L/h, renewal rate of 7.3 times/day) directly from the Atlantic Ocean. To keep rearing conditions as natural as possible, seawater was neither filtered nor chemically treated, and no temperature control treatment was applied. Consequently, the water had the same conditions as the nearby ocean, with temperatures ranging between 18° and 23°C throughout the year, with 2 seasons: warm (temperatures >21°C, from June to November) and cold (temperatures <21°C, from December to May).

All animals were handled following the same standardized protocol throughout the trials. Tanks were completely cleaned twice a week (Monday and Friday). When tanks were drained, feces and algae were removed with a scrub brush, then rinsed with fresh water, and finally filled up again with seawater. Every 4 weeks the animals were weighed (kg) and biometric data recorded (in cm), according to Bolten (1999) (minimum, standard, and maximum curve carapace length, CCLmin, CCLst and CCLmax, respectively) to monitor their growth during the entire period. At the time of measurement, animals were also scoured to remove epibiota.

All animals were fed three times per week (Monday, Wednesday, and Friday), leaving four fasting days (Tuesday, Thursday, Saturday, and Sunday). Quantity of food per animal was 2–4% of their body weight, established according to their weight, age or life cycle stage, and water temperature seasonality, recalculated after every weighing period. This protocol was developed by researchers at

the University of Las Palmas de Gran Canaria (ULPGC; Liria-Loza A., unpublished data), based on feeding protocols established by Bluvias and Eckert (2010) for injured sea turtles, and adapted to healthy animals.

Animals were fed with a mix of different fishes and squid from local fisheries, fresh or frozen, depending on availability. The feeding process consisted of cutting the whole fish (bones, scales, and gut included) into smaller pieces, weighing, and dispersing it into the tanks. The amount of food ingested by each turtle was monitored by direct observation. Food intake of all the animals was seemingly adequate and no aggression between them was observed. No lesions were observed in any of the animals during the study.

This study has established the animal density of three turtles per tank as control density or control conditions, based on previous observations conducted in the Canary Islands, such as:

1.Growth rates (CCLmin $y^{-1} \pm$ SE, ranged from 4.95 ± 1.43 cm y^{-1} to 8.31 ± 1.35 cm y^{-1}) were comparable to the natural growth rate ranges estimated for wild loggerheads from the Northwest Atlantic rockery (Braun-McNeill et al., 2008) and to those estimated for neritic individuals of the Mediterranean rockery (Casale et al., 2011).

2. No aggression among animals or stereotypies (compulsive and/or repetitive behaviors) were observed during the 5 years prior to the beginning of the study, when the 12 animals included in the experiment were kept in the same conditions described in the preceding section (5,000 L outdoor tanks) with a three animals per tank density, allocated according to their weight. These two factors suggested that animals grew properly and exhibited no signs of chronic stress at a density of three individuals per tank. Additionally, the mean growth of the animals the year before and after the current study was calculated to evaluate any possible effect of the applied treatments over the growth rates.

Trial I. Effect of stocking number variation

We used nine animals (four females and five males) to assess the effect of transient stocking number variation of sea turtles on plasma Cort concentration. We used three identical outdoor tanks (5,000 L, rectangular tanks), same longitudinal orientation (270° W), and same sun/shade ratio. We assayed two different treatments: (i) decreasing the number of three turtles per tank (D3; control treatment) to one turtle (D1); (ii) increasing the number from three to five individuals per tank (D5). Each experiment lasted 9 days and was replicated three times. In this experimental design, we therefore used a "time" for "space" replication substitution (Tuya et al., 2001). Between replications, turtles were placed back at three animals per tank (control density) for 7 days, to let them recover. The process to distribute the animals between the different treatments and the recovering periods was random and different at each replication, with the aim of avoiding potential individual effects.

We drew blood samples from turtles at three points in the experiment: one just before starting the trial (day 0), then at day 4 (day 4) and the last just before finishing each replicate at day 9 (day 9).

To study the behavioral response of the animals, each tank was separately studied by videotaping one hour in the morning before the feeding and cleaning process (from 9:00 to 10:00 am) and one hour in the afternoon (from 16:00 to 17:00 pm), during the experimental period, to assess whether the time of day had any effect on behavior. Cameras set-up and video analysis are described in the "Behavior analysis" section below.

Trial II. Effect of dry-dock time

To study the time course of circulating Cort concentration during dry-dock time, we placed eight animals (six females and two males) in 5,000 L rectangular tanks, with two turtles per tank. We assayed four different treatments. The basal protocol for all four treatments was the same. Following cleaning and data collection routines, this consisted of: (i) taking the animals out of the tank; (ii) measuring and weighing them (<5 min); and (iii) putting them back in the tank. The four treatments

differed in the time that animals were exposed to the dry-dock protocol. In the first treatment (T0), animals were sampled immediately after the standardized routine handling protocol and did not spend any time in dry-dock period. In the second (T15), third (T30), and fourth (T60) treatments, animals were dry-docked for 15, 30, and 60 min, respectively. Every Friday during a month, we repeated the experiment to avoid possible effects of individual responses and pseudo-replication, where the eight animals experienced all the treatments.

We drew two blood samples per turtle, the first just after taking the animal out of the water, prior to the standardize routine handling protocol, and the second just before putting it back in the water and after the respective dry-docking time. We considered the first draw a control. We obtained mean Cort concentration after dry-dock protocol.

Behavior was analyzed using video cameras to record the animals for one hour after putting them back in the water following dry-dock experiments. We conducted the analysis of the video as described in the "Behavior analysis" section below.

Blood collection and plasma extraction

We collected 2 ml of blood per animal, which is less than 10% of total blood volume recommended to avoid harming animals (Mader & Rudloff, 2006). Blood collection followed official permits established by the *Spanish Ministry for the Ecological Transition and the Demographic Challenge* for handling and sampling sea turtles. We collected blood samples from the dorsal cervical sinus, previously disinfected with an alcohol gauze, using a 5 ml syringe with a 21 G/38 mm needle, and dispensed into 2 ml lithium heparin tubes. We kept samples in a cooler with ice packs until centrifugation at relative centrifugal force of 3000 g for 5 min to obtain plasma. We pipetted plasma into 2 ml Eppendorf® tubes and kept frozen (−30°C) until analysis.

Analysis of circulating Cort in plasma

Frozen plasma samples were analyzed in the Department of Cell Biology, Physiology, and Immunology at Universitat Autònoma de Barcelona (Barcelona, Spain) to measure Cort concentration using double antibody in equilibrium radioimmunoassay (RIA). Briefly, Cort radioimmunoassay used [125I] Cort – carboxymethyloxime – tyrosine – methylester (ICN – Laboratorios Leti, Barcelona, Spain) and synthetic Cort (Sigma, Barcelona, Spain), as the standard and an antibody raised in rabbits against Cort – carboxymethyloxime – bovine serum albumin. The range of the standard curve was between 6.25 pg and 1,600 pg of Cort per tube. The antibody used had a cross-reactivity of 2.3% with progesterone, 1.5% with desoxycorticosterone, and less than 0.1% with any other steroid tested. All samples that were statistically compared were run in the same assay (Scorrano et al., 2014).

Behavior analysis

We used GoPro5 cameras to study the behavior of the animals in response to the treatments (density variation and dry-dock). We installed cameras above the tanks to record the animals around the entire tank. The cameras were controlled remotely to avoid the interference of the people presence in the behavior of the animals. Different behavioral responses were analyzed: (i) breathing rate (BR; breaths/min), defined as the number of times the animal took its nostrils out of the water per minute (dividing by 60 the total breaths observed in 1 hr); (ii) swimming activity (SA; swims/hr), defined as the total number of swimming strokes in 1 hr; (iii) breaths per movement (b-SA; breaths/SA), defined as the average of breaths registered per swimming activity; (iv) swimming duration (SA-d; min/SA), defined as the average of swimming activity's duration (in minutes) conducted by the animal in 1 hr; (v) activity rate (AR; %), defined as the total time the animal was active swimming in the tank, expressed as a percentage.

Statistical analysis

We conducted all statistical analyses using R version 3.1.2 (R Department Core Team 2014). In both trials, we analyzed the effect of the different treatments over Cort concentration using a generalized linear mixed-effects model (GLMM) with a "negative binomial" family error structure and a "logit" link function, to account for the non-independence of grouping/repeated measures, allowing both intercepts and slopes to differ between groups, with the treatments and sex as fixed factors. We considered sampling days in the first trial, and replicated times in both experiments, as random factors.

We analyzed the effect of the treatments from both trials over the different behavioral responses in the same way, using GLMMs with the same family error structure and a logit link function and the same fixed factors, treatment and sex. We included another fixed factor for trial I: daytime (morning vs afternoon). The different replicated times were random factors in both trials, as well as the sampling days for the first trial. For all models, we checked model assumptions of homogeneous variance and normality of error through visual inspection of residuals and quantile–quantile plots (Harrison et al., 2018). We implemented mixed models using the "lme4" package (Bates et al., 2015).

We performed correlation tests (Pearson's) between Cort concentration obtained in the first trial and the spontaneous behavior measured to relate both responses. We did the same with the second trial, but also included the correlation with the Cort difference between pre and post treatment. We considered differences in results significant at $p < .05$.

Results

Trial I. Effect of variation on stocking density

There were no significant differences in mean Cort concentration among the three studied densities (D1: $z = -0.651$, $p = .52$, D5: - = -0.095, $p = .92$; see Table 1). The possible effect of secondary factor was also analyzed, where no significant differences were found between sexes (male: $z = 0.625$, $p = .53$; see Table 1), neither on the interaction of both fixed factors ("density" x "sex") (D1-male: $z = -0.217$, $p = .83$, D5-male: $z = -0.421$, $p = .67$), so individual sex did not significantly affect differences in mean Cort concentration, which ranged from 0.19 to 2.14 ng mL^{-1}. Random factors accounted for very low variance (replicates: variance explained = 0.00; sampling times: variance explained = 0.023), showing that these two factors had a minor effect over mean Cort concentration during the trial.

In relation to behavioral response, BR was not affected by changes in the density of animals per tank. Instead, both secondary factors, sex and daytime, induced significant differences in BR (morning: $t = -3.35$, $p = .02$, male: $t = -3.36$, $p = .01$), where animals exhibited higher BR in the afternoon than in the morning, and females exhibited higher BR than males (see Table 1). The same occurred with SA, which was not significantly affected by treatments (D1: $t = 0.18$, $p = .87$, D5: $t = 1.86$, $p = .11$), moreover, animals swam significantly more times during the afternoon ($t = -4.54$, $p = .02$), and females swam significantly more than males ($t = -3.20$, $p = .001$) (see Table 1). Mean SA-d was only significantly affected by daytime ($t = 4.31$, $p = .001$), where each movement was longer in the morning than in the afternoon (see Table 1) but was not affected by variation in animal density (D1: $t = 0.63$, $p = .58$, D5: $t = -1.97$, $p = .08$), nor by the sex of the animals ($t = 1.52$, $p = .20$). AR was also only significantly affected by the daytime ($t = 2.56$, $p = .04$), swimming longer in the morning than in the afternoon (see Table 1). Also, none of the variables significantly affected b-SA, being around 10 breaths/SA for all variables explored (see Table 1). Additionally, the two random factors induced very low variance (replicates: variance explained range = 0.22–0.00; sampling times: variance explained range = 0.04–0.00), showing that these two variables did not have a significant effect over the different behavioral responses. Finally, no correlation was found between Cort concentration and the different behavioral responses.

Table 1. Mean corticosterone concentration and behavioral response across stocking density, sex, and day-time.

	CORT	BR (breaths/min)	SA (swims/hr)	b-SA (breaths/SA)	SA-d (min/SA)	AR (%)
Main factor						
Density						
control	0.74 ± 0.51	0.60 ± 0.19	3.30 ± 1.92	11.07 ± 6.13	12.27 ± 10.15	55.88 ± 22.19
D1	0.47 ± 0.42	0.61 ± 0.22	3.29 ± 2.37	9.65 ± 6.74	12.67 ± 11.55	61.52 ± 24.35
D5	0.64 ± 0.39	0.57 ± 0.20	3.55 ± 1.71	10.16 ± 5.70	10.01 ± 8.25	49.65 ± 22.95
Secondary factor						
Sex						
Female	0.62 ± 0.43	0.62 ± 0.19^{A}	3.78 ± 1.93^{A}	10.27 ± 5.91	10.19 ± 8.52	54.23 ± 22.49
Male	0.68 ± 0.43	0.55 ± 0.20^{B}	3.17 ± 1.77 ^{B}	10.52 ± 5.98	11.61 ± 9.80	51.41±.59
Daytime						
Morning	-	0.53 ± 0.18^{A}	2.94 ± 1.73^{A}	10.21 ± 5.59	13.26 ± 10.56^{A}	56.85 ± 24.31^{A}
Afternoon	-	0.64 ± 0.20 ^{B}	3.97 ± 1.86 ^{B}	10.58 ± 6.25	8.73 ± 7.12 ^{B}	48.60 ± 21.15 ^{B}

Corticosterone concentration is given in ng/ml (±SD). Different capital letters denote significant differences ($p < 0.05$) among groups.

Trial II: Effect of dry-dock time

The results obtained showed that Cort concentration significantly increased after 30 min in dry dock (T30) ($z = 2.195$, $p = .026$), and even more after 60 min (T60) ($z = 6.806$, $p < .001$), when compared with T0 and T15 dry-dock times (see Figure 1). The possible effect of secondary factors has been also analyzed, where no effect was found in relation to sex ($z = 0.396$, $p = .70$), nor in relation to the interaction of both dry-dock time and sex (see Table 2). The random factor (replicated times) induced a very low variance in the models, so the effect of this variable over Cort (variance explaining = $8.9e^{-10}$) was not relevant.

In relation to behavioral responses, only b-SA was significantly higher after T60 treatment ($z = 4.176$, $p = .0001$) (see Table 2). In addition, this behavioral variable was the only one showing significant correlation with Cort concentration, where the higher the Cort concentration, the more breaths per movement. The variance induced by the random factors on the different models was very low (BR: variance explained = $1.43e^{-9}$; SA: variance explained = $5.54e^{-17}$; b-SA: variance explained = 0.00; SA-d: variance explained = 0.00; AR: variance explained = 0.00), so the effect over response variables was minor.

Testing the possible effect of treatments

Growth analysis was performed to evaluate the possible effect of treatments on the individuals, where no significant differences ($p > .05$) were found between the mean growth (± *SD*) of the animals the year before the current study (5.7 cm y-1 ± 1.60), and the mean growth after treatments (4.9 cm y-1 ± 1.07).

Discussion

Husbandry protocols for sea turtle recovery recommend one animal per tank to avoid possible infection spread or aggression among animals (Bluvias & Eckert, 2010; Higgins, 2003), based on health aspects derived from housing conditions of stranded animals coming from the wild. Moreover, no information has been reported on the stocking density for healthy sea turtles held under captive conditions for long periods, which is an important aspect to consider from the welfare point of view (Arena et al., 2014).

Husbandry protocols for sea turtles need to determine not only the adequate number of animals per tank, but also the effect of short-term variations in housing conditions, such as change in density derived from husbandry requirements, including cleaning or health treatments (Bluvias & Eckert, 2010). Results from the present study showed that, in loggerhead juveniles, short-term changes, such as individual isolation or increase in density of animals for nine days, did not alter the mean

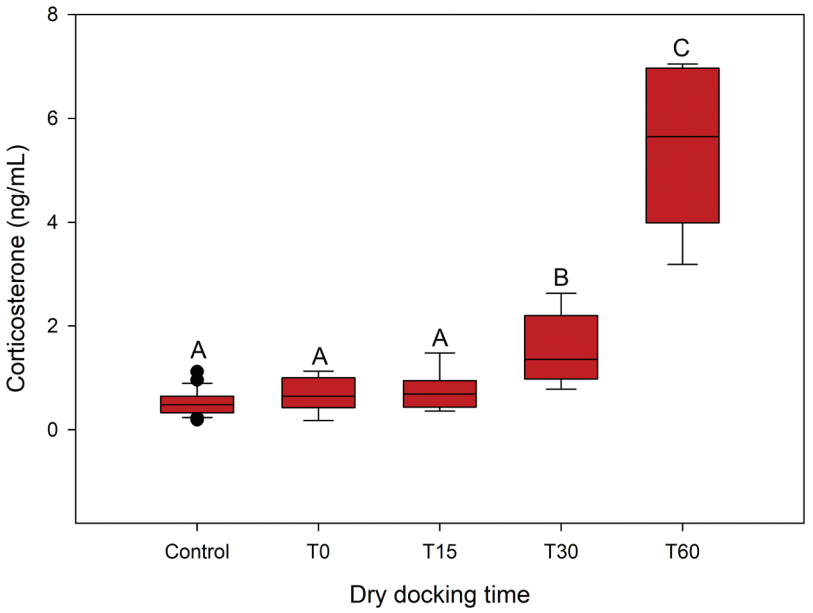

Figure 1. Mean circulating corticosterone concentration for the control group and the different dry-dock time treatments. Different capital letters above the boxes denote significant differences ($p < .05$) among groups. Big dots are outliers and middle lines represent the median.

circulating Cort concentration of the animals, and that no aggression between individuals was recorded, suggesting that welfare of individuals was not affected in the short term. No other studies on individual density variations have been conducted on captive sea turtles. Moreover, our results are similar to those reported for harvest-size saltwater crocodiles (*Crocodylus porosus*) housed on a farm in communal or individual pens, where no differences in Cort concentration were found between the animals allocated to individual and in communal tanks (Isberg & Shilton, 2013; Isberg et al., 2018). Other reptiles showed different Cort responses to changes in density, such as the Eastern Box turtle (*Terrapene carolina carolina*) subjected to one hour of isolation and confinement, which exhibited elevated Cort concentrations (West & Klukowski, 2018). Shorter periods have been reported to induce relatively slow or non-significant Cort elevations in tortoises (Ott et al., 2000). The lack of effect of the different densities tested may be an indication of the ability of loggerhead juveniles to withstand changes in the number of animals per tank under seminatural conditions.

Besides turtle density, handling time associated with cleaning protocols or animal care must also be analyzed and standardized. For instance, such protocols may involve dry-dock periods for transport, cleaning routine, or other husbandry practices related to sea turtle care (Bluvias & Eckert, 2010). In this regard, Flower et al. (2015) described a significant Cort increase after 6 min of handling wild juvenile loggerheads from Jekyll Island (Georgia, USA), reaching the highest peak of circulating Cort after 30 min, being those values 8-fold higher than resting values. Gregory et al. (1996) found a similar increase of 7.2fold at 30 min after handling wild loggerhead juveniles from the Florida population. In the present study, the 5-min handling protocol tested (for biometric data collection) did not induce significant changes in mean circulating Cort concentration when compared to control values, in captive loggerhead turtles. Moreover, dry-dock protocols induced a significant increase of mean circulating Cort only after 30 and 60 min, with 3-fold and 10-fold increases, respectively. These results showed a similar Cort response when compared to previous loggerhead sea turtle studies (Flower et al., 2015; Gregory & Schmid, 2001; Gregory et al., 1996). Briefly, results of the present study suggest that the maximum time a juvenile loggerhead should be

Table 2. Mean behavioral responses across different dry-dock treatments and sex and mean corticosterone concentration by sex.

	BR (breaths/min)	SA (swims/hr)	b-SA (breaths/SA)	SA-d (min/SA)	AR (%)	
Main factor						
Treatment						
control	-	-	-	-	-	
To	0.33 ± 0.22	3.16 ± 2.68	5.76 ± 4.21	33.69 ± 24.82	92.26 ± 13.82	
T15	0.24 ± 0.16	2.17 ± 1.64	4.97 ± 3.59	30.33 ± 24.76	69.58 ± 31.24	
T30	0.33 ± 0.18	2.51 ± 1.80	7.58 ± 5.93	32.36 ± 27.29	70.16 ± 34.27	
T60	0.39 ± 0.19	1.33 ± 0.48	20.36 ± 12.35*	42.12 ± 20.69	91.30 ± 17.43	
Secondary factor						
Sex						CORT
Female	0.36 ± 0.20	2.53 ± 2.07	11.26 ± 11.92	31.92 ± 24.86	76.93 ± 29.12	1.28 ± 1.68
Male	0.20 ± 0.08	1.60 ± 0.76	8.75 ± 3.66	42.72 ± 19.08	92.50 ± 14.12	1.71 ± 1.99

Corticosterone concentration is given in ng/ml (±SD). Asterisk (*) denotes significant differences ($p < .05$) among groups.

out of the water without triggering an adrenal response is less than 30 min, because at 30 min Cort concentration was already significantly elevated. This suggested husbandry protocol is more restrictive than that previously proposed by Higgins (2003), who suggested a dry-dock time of 30 min to prevent carapace desiccation and peeling, which could provoke incidence of opportunistic pathogens.

The standardized protocols tested in this study (i.e., variation in turtle density and dry-dock times) did not induce significant changes in the behavior of the experimental animals, except for b-SA. Indeed, after 60 min of dry-dock, mean b-SA increased significantly, which correlated with an increase in mean circulating Cort concentration. Changes in circulating Cort have been associated with changes in reptile behavior (Denardo, 2006; Silvestre, 2014), including enhancement of anti-predator responses (Thaker et al., 2009), reduced aggressive behavior (Tokarz, 1987), and increased defensive behavior (Stepanek et al., 2019). Additionally, Cash and Holberton (1999) showed a significant increase in locomotor activity within 48 hr after Cort implant in the Red-Eared Slider Turtle, *Trachemys scripta elegans*. Those authors suggested that the effects of Cort on behavior may be context-dependent (e.g., whether the turtles can find food) and Cort concentration-dependent (Cash & Holberton, 1999). However, in the present study, we did not test food deprivation, nor exposure to a predator. Regarding the change in mean b-SA, Cort, and dry-dock time, it has been shown that Cort has the capability to induce an increase in metabolic rate in squamates, salamanders, and even in birds (DuRant et al., 2008; Jimeno et al., 2018; Preest & Cree, 2008; Wack et al., 2012). It is possible that the increase in mean b-SA we observed was a direct effect of Cort, although we cannot explain why we did not observe a concomitant increase in BR, while differences in BR between sexes were observed, which contrast with findings in crocodilians where no differences in BR between sexes were found (Huggins et al., 1969).

In the density trial, females exhibited higher mean b-SA and SA than males, irrespective of Cort concentration or density treatment; however, we have not found an explanation for this divergent behavior between males and females, since at juvenile stage sexual dimorphism has not been reported in sea turtles. Nevertheless, this statistical difference could reflect the difference in sample size between males and females. Further investigation in this area is needed.

Daily cycles in diving and swimming behavior have been described for loggerhead sea turtles (Cardona et al., 2005; Revelles et al., 2007). According to Sakamoto et al. (1990), wild female loggerheads increased their diving depth and swimming activity from mid-afternoon/evening until sunrise, and the rest of the time they stayed at the surface. In our behavioral analysis, we observed an increase in mean SA during the afternoon, as well as an increase in mean BR, consistent with Sakamoto et al.'s (1990) observations. These behavioral responses to density were concomitant with a reduction of mean SA-d and mean AR. Thus, more studies would be needed to determine if those

behavioral differences between morning and afternoon are due to endogenous (e.g., daily hormonal cycles) or exogenous processes (e.g., presence of people or treatments themselves).

In the long term, growth rates of the animals included in the study were comparable to the natural growth rate ranges (Braun-McNeill et al., 2008; Casale et al., 2011; Lenz et al., 2016; Petitet et al., 2012), and growth rates were the same before and after the study. Also, no aggression among animals was observed during after the study, suggesting that the animals grew properly and exhibited no signs of chronic stress (Tokarz & Summers, 2011; Warwick et al., 2013Wilkinson, 2015).

In conclusion, our results show that husbandry protocols of isolation or increasing the number of animals per tank can be applied for short periods (nine days) without affecting loggerhead sea turtle welfare. However, we recommend keeping dry-dock protocols to less than 30 min, as indicated by the elevation of circulating Cort concentration.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Author contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Alejandro Usategui-Martín, Ana Liria-Loza, Lluis Tort, and Fernando Tuya. The first draft of the manuscript was written by Alejandro Usategui-Martín and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability statement

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

Ethics approval

This is an observational study, so an ethical approval is not required, and no human participant are included in this research.

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