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STANDARIZATION OF WELFARE PROTOCOLS BASED ON STRESS RESPONSE AND BEHAVIOR IN LOGGERHEAD SEA TURTLE *(Caretta caretta)*

Alejandro Usategui Martín
Las Palmas de Gran Canaria
2019

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

PhD Thesis

STANDARIZATION OF WELFARE PROTOCOLS BASED ON STRESS RESPONSE AND BEHAVIOR IN LOGGERHEAD SEA TURTLE (*Caretta caretta*)

"DESARROLLO DE PROTOCOLOS DE BIENESTAR BASADOS EN ESTUDIOS DE ESTRES Y COMPORTAMIENTO DE TORTUGA BOBA (Caretta caretta)"

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Doctorado en Acuicultura Sostenible y Ecosistemas Marinos
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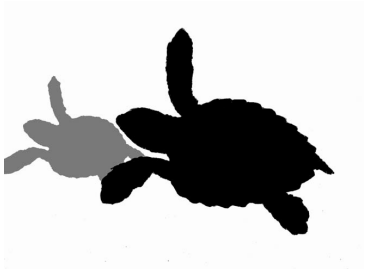
Las Palmas de Gran Canaria, Diciembre 2019



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D. DANIEL MONTERO VÍTORES COORDINADOR DEL PROGRAMA DE DOCTORADO EN ACUICULTURA SOSTENIBLE Y ECOSISTEMAS MARINOS DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA,

INFORMA,

Que la Comisión Académica del Programa de Doctorado, en su sesión de fecha 03 de diciembre de 2019 tomó el acuerdo de dar el consentimiento para su tramitación, incluyendo la mención internacional, a la tesis doctoral titulada:
"STANDARIZATION OF WELFARE PROTOCOLS BASED ON STRESS RESPONSE AND BEHAVIOR IN LOGGERHEAD SEA TURTLE (Caretta caretta)- DESARROLLO DE PROTOCOLOS DE BIENESTARBASADOS EN ESTUDIOS DE ESTRES Y COMPORTAMIENTO DE TORTUGA BOBA (*Caretta caretta*)"
presentada por el doctorando **D. ALEJANDRO USATEGUI MARTÍN** y dirigida por el doctor **DANIEL MONTERO VÍTORES**

Y para que así conste, y a efectos de lo previsto en el Artº 11 del Reglamento de Estudios de Doctorado (BOULPGC 7/10/2016) de la Universidad de Las Palmas de Gran Canaria, firmo la presente en Las Palmas de Gran Canaria, a 03 de diciembre de dos mil diecinueve

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UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
ESCUELA DE DOCTORADO

Programa de doctorado:

Doctorado en Acuicultura Sostenible y Ecosistemas Marinos

Título de la Tesis

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Tesis Doctoral presentada por D/Dª Alejandro Usategui Martín

Dirigida por el Dr/a. D/Dª. Daniel Montero Vítores

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Las Palmas de Gran Canaria, a **02** de **Diciembre** de 20**19**

Abstract

Sea turtles and humans have been sharing this planet for around 200 Ka and probably interactions between them have been occurred since the beginning, but the first register of this interaction dates from 5.000 BCE. Along human history sea turtles have been seen and used in many different ways. They have been considered as gods and viewed as a sign of fertility or longevity for many different cultures, but also have been seen, and consequently used, as a source of protein and raw material.

Meanwhile, sea turtle use has evolved along the time, from a traditional used, hunting and exploiting them with traditional methods, to an enormous fishing industry, which almost collapsed several sea turtle populations around the world. The indiscriminate consumption, together with other factors that sea turtles needed to face during the last decades, such as marine litter, marine pollution, habitat destruction and climate change, have produced a general decline in their populations, and consequently the necessity to include all the seven existing sea turtle species in the consideration as endangered species.

The worldwide situation of sea turtles makes necessary to launch and implement important conservation plans. “In situ” programs had been quickly implemented and are still running worldwide, mainly focused on protection and conservation of nesting habitats. For example, egg incubation is an important point to consider while carrying out conservation plans, since the incubation environment determine important phenotypic and performance characteristics of the hatchlings. Conservation programs have usually followed three types of incubation protocols: i) leave the nests directly on the natural area selected by the females, exposed to natural factors; ii) translocate natural nests to controlled incubation areas (hatcheries), to avoid high predation, floods or other

threats; or iii) incubate the eggs under controlled conditions in the laboratory. The last methodology has been also used for research, mainly to study sex determination and hatchlings performance and fitness. Traditionally, incubation under laboratory conditions has been done at stable temperatures, however constant temperature is rare under natural conditions, which typically oscillate over the course of incubation and little is known on how the alteration of the temperature pattern and/or regime during the incubation process could affect the hatchlings.

One important "ex situ" conservation plan, which have been developed to try to avoid the depletion or the extinction of some populations, was the sea turtle mariculture, where sea turtles were held under controlled conditions to meet the demand of sea turtle products and to avoid taking them from the wild. Fortunately, this kind of projects was developed for some years but has been disappearing in recent times. However, in the last decades, a new tool for sea turtle conservation has appeared, the head starting programs, which could be considered as a reinterpretation of sea turtle mariculture. These programs consist on rearing hatchlings under controlled conditions during determined periods, ranging from a few days to some months or even years, aiming to enhance their recruitment into wild populations once they are released in better conditions (larger, stronger, harder shell, etc.). One-year period is considered the most appropriate, because they are larger enough to avoid most of the predators associated with post hatchling stage. The head starting program made with Kemp's ridley turtles *(Lepidochelys kempii)* at Padre Island National Seashore (PINS) (Texas, USA) is the most known and recognized, and it could be considered that this project established the bases to hold sea turtles under controlled condition.

Apart from direct population reinforce with head starting programs, keep animals under controlled conditions may provide insights about some life stages that are very difficult to observe and study in the wild, mainly on species with very complex life cycles, which consequently are partially unknown. The protocols to rear sea turtles under controlled conditions have been improved over time, but not only in the context of head starting programs when animals are held for relatively long periods of time, also for short periods of time when they are held for recovery or for research purposes. In general, most of the established parameters to rear sea turtle hatchlings under controlled conditions are based in the own experience of each facility focused on health parameters, survival rates, or

the prevalence of diseases or injures, under certain environmental, handling and housing conditions. However, very few have considered direct indicators of welfare.

There is very little information available on welfare aspects on sea turtles, and even less on welfare of sea turtles held under controlled conditions. Given that sea turtles are endangered species, it is a priority that protocols to hold these species under controlled conditions must be standardized, not only in terms of health, but also based on welfare parameters.

Animal welfare can be assessed through behavior, levels of physiological indicators (i.e. stress indicators) or other indirect parameters like absence of illness or the proper appetite. Traditionally, blood-circulating corticosterone (CORT) has been used as a stress response and subsequently a welfare physiological indicator, as this is the main glucocorticoid produced by reptiles to face and adapt to stressful conditions. Among the different species of sea turtles, CORT variation has been widely studied. In adult females during the nesting season, CORT variations have been linked to reproductive success, high density nesting processes, season period, and handling or predation stimulus. The studies conducted with wild juveniles have been related to handling or recovery/illness processes, and in wild hatchlings with dispersal behavior. However, little is known about CORT oscillation when these reptiles are held under controlled conditions or during the human – sea turtle interaction in the wild for data collection or particular researches (tagging programs, genetic studies, feeding behavior, etc.).

Considering sea turtles spend most part of their life on marine ecosystems, most of the “in situ” conservation plans are focused on nesting females because they are more accessible during the nesting process. During the last years, tourism has also been included in the turtle conservation “toolbox” aiming to increase the awareness about sea turtles, as well as an alternative and sustainable resource for local communities.

The acquisition of baseline data is crucial to measure successes or failures of the conservation programs. In addition, having baseline data enables conservationists to assess the impact of management strategies and conservation programs and it is also necessary to improve and implement standardized protocols for eco tourist “turtle tours”. However, all the interactions human – sea turtles, whether for conservation, protection, research or ecotourism goals, should be done considering the welfare of the animals, to

prevent possible induction of stressful situations, which subsequently could impact on sea turtle behavior or reproductive success.

From a global point of view of the interaction between humans and sea turtles, from the incubation of the eggs to adult stages, there is a generalized lack of information about how our presence, interaction or handling affect the welfare of these endangered reptiles. The aim of the work presented here was to establish and improve standardized protocols on the different life stages of loggerhead sea turtle *(Caretta caretta)* hold under controlled conditions or during their interaction with humans, based on welfare indicators. To achieve it, several specific objectives were proposed, achieving them through different studies.

The first study aimed to determine the effect of different standardized incubation protocols on specific quality parameters of reproductive success on loggerhead sea turtle, such as incubation time, hatching duration, hatching and emergence success, hatchlings phenotype and shelf-righting time (as fitness indicator). Results showed that incubation temperature regimes (high or low) influenced all parameters studied more than temperature patterns (constant or variable). An optimal range of incubation temperatures was determined for the Northeast Atlantic population, assessing favorable values for hatchlings, with lower temperatures on 27°C, similar to the Northwestern Atlantic populations (Florida rookery), and higher limits greater than 31ºC, which is the upper limit established on the Florida (USA) rookery, showing that Cape Verde rookeries seems to be more adapted to higher incubation temperatures.

The second study was focused on improving standardized husbandry protocols for loggerhead hatchlings in terms of welfare response. To achieve it, CORT variations were monitored under different rearing conditions such as stocking density, seawater temperature and culture-related handling processes. Results demonstrated that adequate welfare conditions to held loggerhead hatchlings under controlled condition were: i) handling standardized protocols applied regularly (once per week); ii) holding temperatures ranging from 26 ° to 28 °C; and iii) hatchling isolation during the first 6 months must be refused, as this holding situation can induce a chronic elevation of plasma corticosterone.

The third study was also aimed to improve husbandry protocols based on welfare but focused on juvenile loggerheads hold under controlled conditions for long time.

CORT response and spontaneous behavior were studied under different routine situations such as changes in standardized stocking density and different dry-docking times. Results established that the number of juvenile loggerheads per tank could be punctually modified for short periods (until 9 days), without affecting their welfare. However, time of dry-docking must be no higher than 15 min during examination or cleaning procedures, as indicated by elevation of circulating corticosterone. Results also showed that standardized protocols tested, used in the Canary Islands facilities, did not affect behavior parameters measured.

The fourth and last study evaluated how different protocols used on data collection of nesting female loggerheads could affect their welfare, and consequently compromise their reproductive success, in order to establish more accurate standardized field methodologies. To achieve it, several nesting females were sampled before and after the application of different data collection protocols, always after laying eggs and when females were coming back to the ocean, even in failure attempts. Results showed that loggerhead nesting females presented low CORT levels, but inversely related with their size, where the larger the female, the lower the CORT levels. Results also showed a low hormonal response when different protocols were applied, but, even when no effect on CORT concentration has been observed, an especial care must be taken while interacting with nesting females on the beach as behavior can be also modified by human presence and subsequently welfare can be affected. The standardized protocols from conservation programs in nesting areas does not allowed, based on welfare and conservation parameters, the interaction with nesting females before the oviposition process, hence, the effects of people presence or interaction before eggs laying could not be analyzed.

Animal welfare parameters should be considered while developing or establishing handling and housing protocols for the conservation, protection or recovery of endangered species, in order to avoid the possible deleterious effects of human interaction could have on these species.

Resumen

Las tortugas marinas y los seres humanos llevan compartiendo el planeta alrededor de 200.000 años y posiblemente llevan interactuando entre ellos el mismo tiempo, pero el registro más antiguo que demuestra esta interacción data de 5.000 años antes de la era común. A lo largo de la historia de la humanidad las tortugas marinas han sido y siguen siendo vistas y usadas con diferentes propósitos. Se las ha considerado como deidades y también como un símbolo de fertilidad y longevidad, además de como una fuente de proteínas o materia prima.

El uso y el consumo de tortugas marinas ha evolucionado a lo largo del tiempo, desde su explotación y caza con métodos tradicionales, hasta el desarrollo de una industria pesquera dirigida a las tortugas marinas que casi consigue el colapso de algunas de sus poblaciones. El consumo indiscriminado, junto con otras amenazas a las que las tortugas marinas llevan sometidas en las últimas décadas, como la basura marina, la contaminación, la destrucción del hábitat, o el calentamiento global, han provocado el declive de sus poblaciones y, por consiguiente, que las siete especies de tortugas marinas existes hoy en día estén consideradas como especies "*en peligro de extinción*".

La situación en la que se encuentran las tortugas marinas a nivel global exige el desarrollo y la puesta en marcha de importantes planes de conservación en todo el mundo. Los programas de conservación "*in situ*" se pusieron en marcha rápidamente hace años y mucho de ellos siguen funcionando actualmente, principalmente aquellos centrados en la protección y conservación de los hábitats de nidificación. Por ejemplo, en tortugas marinas el proceso de incubación de los huevos es un aspecto muy importante a tener en cuenta en el desarrollo de programas de conservación, debido a que los factores ambientales que afectan al nido durante la incubación, principalmente la temperatura y la humedad, determinan importantes aspectos fenotípicos de las crías, así como sus condiciones físicas que marcarán su supervivencia. Este tipo de programas de conservación han seguido tres

modelos de incubación: i) la incubación natural directamente en el lugar seleccionado por la propia hembra, y expuesto a los factores naturales; ii) la translocación de los nidos para su incubación en áreas de incubación controlada (vivero / *hatchery*), evitando las altas tasas de depredación, las inundaciones u otras amenazas naturales; y iii) la incubación de los huevos de forma artificial en laboratorio. El último tipo de incubación también ha sido utilizado en investigación, principalmente en estudios de determinación sexual por temperatura y en estudios del efecto de la incubación en el desarrollo y crecimiento de las crías. Tradicionalmente los estudios de incubación realizados en laboratorio se han desarrollado con temperaturas de incubación constantes, contrariamente a lo que ocurre en el medio natural, donde la temperatura de incubación oscila a lo largo del proceso de incubación. Actualmente se conoce poco sobre cómo esta alteración en el patrón de la temperatura de incubación podría afectar a las crías de tortuga marina.

Un plan de conservación *"ex situ"* que se desarrolló con el objetivo de evitar el colapso o la extinción de ciertas poblaciones de tortugas marinas, ha sido la maricultura de tortugas marinas, cuyo objetivo era criar tortugas marinas en condiciones controladas con el fin de satisfacer la demanda de productos derivados de estas sin tener que recurrir a animales salvajes. Este tipo de proyectos estuvieron en marcha algunos años, pero afortunadamente han ido desapareciendo poco a poco. Sin embargo, en las últimas décadas se estableció una nueva herramienta de conservación de tortugas marinas, los programas de "iniciación" o *"head starting"*, que pueden ser considerados como una reinterpretación de la maricultura con tortugas marinas. Estos programas consisten en el mantenimiento de crías de tortugas marinas en condiciones controladas por un periodo de tiempo determinado, desde unos pocos días hasta varios años, antes de liberarlas al medio natural, con el objetivo de incrementar su reclutamiento en las poblaciones salvajes, dadas sus mejores condiciones físicas (mas grandes, mas fuertes, con el caparazón mas duro, etc.). El intervalo de tiempo más adecuado para un programa de este tipo es de un año, ya que es el periodo durante el cual las tortugas marinas alcanzan el tamaño suficiente para evitar el gran número de predadores naturales asociados a sus primeros días / meses de vida. El programa de "*head starting*" realizado con la tortuga Kempi *(Lepidochelys kempii)* en *Padre Island National Seashore* (PINS), Texas (Estados Unidos de América) es el más conocido y está considerado como el proyecto que estableció las bases para el mantenimiento de tortugas marinas en condiciones controladas.

El mantenimiento de tortugas marinas bajo condiciones controladas no solo sirve para reforzar las poblaciones a través de los programas de *"head starting"*, sino que también permite el estudio de ciertas fases del ciclo vital de las tortugas que en el medio natural son difíciles de observar y de estudiar, debido a la gran complejidad que presenta el ciclo de vida de las tortugas marinas, y que, en consecuencia, todavía es muy desconocido. Los protocolos para el mantenimiento de tortugas marinas en condiciones controladas han mejorado a lo largo del tiempo, no solo en el marco de los programas de *"head starting"*, donde los animales son mantenidos durante largos periodos de tiempo, sino también en otros programas en los que las tortugas se mantienen durante periodos más cortos, como la recuperación de animales heridos o enfermos, o determinados proyectos de investigación. En general, la mayoría de los parámetros que se siguen para el adecuado mantenimiento de tortugas marinas en condiciones controladas, tanto crías como juveniles, se basan en la propia experiencia de cada instalación, que a su vez se fundamentan en parámetros de salud, como las tasas de supervivencia o la prevalencia de ciertas enfermedades o lesiones, bajo determinadas condiciones ambientales, de manejo, o de hacinamiento. Sin embargo, muy pocos tienen en cuenta los indicadores directos de bienestar animal.

La información disponible en la literatura con relación al bienestar de las tortugas marinas es escasa y todavía menos sobre bienestar de tortugas marinas en cautividad. Además, teniendo en cuenta que las tortugas marinas se encuentran en peligro de extinción, debería ser prioritario que los protocolos para el mantenimiento de estas especies bajo condiciones controladas se estandarizaran no solo en base a la salud de los animales, sino también, en base a indicadores de bienestar que puedan ser monitorizados en todo momento.

El bienestar animal se puede medir a través del comportamiento, del nivel de indicadores fisiológicos (Indicadores de estés) o de otros factores indirectos como pueden serlo la ausencia de enfermedades o un apetito inadecuado. Tradicionalmente en reptiles, la concentración de corticosterona (CORT) presente en sangre ha sido utilizado como indicador fisiológico del bienestar, ya que es el principal glucocorticoide producido por los reptiles para afrontar y adaptarse a situaciones de estrés. En las tortugas marinas las variaciones de CORT han sido ampliamente estudiadas. Por ejemplo, en las hembras adultas durante la época de nidificación, las variaciones de CORT han sido relacionadas con el éxito reproductivo, con procesos de nidificación de alta densidad, con la época del

año, la manipulación e incluso con la presión de los predadores. Por otro lado, en juveniles salvajes se han estudiado estas variaciones durante su manipulación y recuperación, así como en crías, durante su dispersión tras emerger del nido. Sin embargo, se conoce poco sobre las variaciones de CORT cuando estos reptiles están bajo condiciones controladas o cuando existe una interacción con los humanos en el medio natural para la recogida de datos o para otras actividades de conservación o investigación.

Teniendo en cuenta que las tortugas marinas pasan la mayor parte de su vida en el ecosistema marino, la mayoría de los planes de conservación "in situ" se centran en las hembras nidificantes, ya que durante el proceso de nidificación usan el hábitat terrestre y son mucho más accesibles. Durante los últimos años, el desarrollo de actividades ecoturísticas con tortugas marinas también se ha considerado como una herramienta de conservación, debido a la creación de conciencia sobre estos animales, así como al desarrollo de un medio de vida alternativo y sostenible para las comunidades locales que viven o vivían de la explotación de las tortugas como recurso alimenticio.

La obtención de datos basales sobre las tortugas marinas es de vital importancia para evaluar si lo planes de conservación están siendo exitosos o no. Además, con estos datos los conservacionistas e investigadores pueden evaluar el impacto de los programas de gestión y conservación, así como mejorar e implementar los protocolos que permitan una mejor gestión del ecoturismo con tortugas marinas. Sin embargo, todas las interacciones entre humanos y tortugas, ya sea para objetivos de conservación, protección, investigación, o incluso ecoturismo, debe hacerse siempre considerando el bienestar de los animales, para así prevenir posibles situaciones de estrés que puedan alterar el comportamiento de las tortugas o su éxito reproductor.

Observando la relación entre tortugas marinas y humanos en conjunto, desde la incubación de los huevos hasta los estadios adultos, se aprecia que hay una falta generalizada de información sobre cómo la presencia, la interacción o la manipulación por parte de los humanos, puede afectar al bienestar de estos reptiles en peligro de extinción. El objetivo de esta tesis doctoral es establecer y mejorar los protocolos estandarizados de manejo de tortuga boba (*Caretta caretta*), bajo condiciones controladas o en otros tipos de interacción con los humanos, en las diferentes etapas de su ciclo de vida, basados en indicadores de bienestar. Para conseguirlo, se han propuesto diferentes estudios para responder a una serie de objetivos específicos.

El primer estudio esta orientado a determinar el efecto de protocolos estandarizados de incubación para huevos de tortuga boba sobre diferentes parámetros de su calidad reproductiva, como son el tiempo de incubación, la duración de la eclosión, el éxito de eclosión y emergencia, además del fenotipo de las crías y su tiempo de volteo (como indicador de actividad). Los resultados mostraron el régimen de temperatura (alta o baja) tiene un mayor efecto sobre los parámetros estudiados que el patrón de temperatura durante la incubación (constante o variable). Los resultados permitieron identificar un rango de temperatura óptimo (de 27°C a 32°C) para la incubación de huevos de la población de tortuga boba del Noreste del Atlántico, además de encontrar cierta diferencia con el límite superior del rango óptimo de incubación, con respecto a las colonias del Noroeste del Atlántico.

El segundo estudio se centró en mejorar los protocolos estandarizados de mantenimiento de crías de tortuga boba en condiciones controladas, en base a parámetros de bienestar. Para lograrlo, se midieron las variaciones de CORT durante diferentes situaciones de cría, tales como el número de tortugas por tanque, la temperatura del agua, y la frecuencia en el manejo de las crías. Los resultados demostraron que las crías de tortuga boba bajo condiciones controladas presentaron unas mejores condiciones de bienestar cuando los protocolos de manejo se aplican de forma regular (una vez por semana); se disponen en temperaturas del agua de entre 26 ° y 28°C; y durante los primeros 6 meses de vida, se evita el aislamiento de los individuos ya que puede provocar un aumento crónico de los niveles de corticosterona en sangre.

El tercer estudio se centró en la mejora de los protocolos de manejo de juveniles de tortuga boba, tras largos periodos de mantenimiento bajo condiciones controladas, en base a indicadores de bienestar. A través de los resultados obtenidos, tanto en niveles de corticosterona como en el comportamiento espontáneo de los animales, se ha podido establecer que: el cambio puntual del número de tortugas por tanque, por un periodo determinado (hasta 9 días) por necesidades de espacio y/o logísticas, no afecta a su bienestar; sin embargo, para operaciones de limpieza o de chequeo, los animales no deben de pasar más de 15 minutos fuera del agua, debido a que esto provoca un aumento progresivo en los niveles de corticosterona. Por otro lado, el comportamiento espontaneo de las tortugas durante los distintos protocolos no se vio afectado por las diferentes condiciones de mantenimiento.

En el cuarto y último estudio se evaluó si los protocolos utilizados en la obtención de datos de hembras de tortugas boba durante el proceso de nidificación pueden afectar a su bienestar, pudiendo llegar a comprometer su éxito reproductor, con el objetivo de establecer unos protocolos estandarizados más adecuados para el trabajo de campo, basados en el bienestar. Para ello, se tomaron muestras de sangre de hembras nidificantes, antes y después de llevar a cabo diferentes protocolos de recogida de datos. Las muestras se tomaron siempre después de la puesta de los huevos y durante el regreso de la hembra al mar, incluso si se trataba de intentos fallidos de nidificación. Los análisis mostraron que las hembras nidificantes presentan niveles bajos de CORT en sangre, inversamente relacionados con su tamaño, cuanto más grande es la hembra más bajos son sus niveles de CORT en sangre. Los resultados también evidenciaron la ausencia de respuesta hormonal ante los protocolos aplicados, pero, aunque dichos protocolos no induzcan ningún efecto sobre los niveles de CORT, no hay que dejar de tener una especial atención cuando se interactúa con las hembras en las playas, ya que la simple presencia de humanos podría alterar su comportamiento y en consecuencia su bienestar e incluso su éxito reproductor. Los protocolos establecidos por los programas de conservación en las zonas de nidificación no permiten, bajo criterios de conservación y bienestar animal, la interacción con las hembras antes del proceso de puesta de los huevos, por lo que la valoración sobre la incidencia de la presencia humana en esta fase del proceso de nidificación no ha podido ser evaluado.

El bienestar animal debe de ser siempre tenido en cuenta en el momento de desarrollar y aplicar protocolos de manejo de especies amenazadas para su protección, conservación o recuperación, para así evitar los posibles efectos negativos que nuestra interacción pueda tener sobre estas especies.

Acronymes

ANOVA	Analysis of the variance
BCE	Before the Common Era
CCL	Curve carapace length
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CORT	Corticosterone
CTF	Cayman Turtle Farm
CV	Coefficient of variation
EIA	Enzyme immunoassay
GLM	Generalize linear model
GLMM	Generalize linear mix model
HPA	Hypothalamic - pituitary - adrenal axis
IH	Increase high
IL	Increase low
incCORT	Corticosterone increase
ka	Kilo annum – thousand years
L	Length
LI	Length increase
Ma	Mega annum – million years
NGO	Non-governmental organization
NMFS	National Marine Fisheries Service
PINS	Padre Island National Seashore
PIT	Passive integrated transponder
PNPA	Port of Nagoya Public Aquarium
postCORT	Post corticosterone

preCORT	Pre corticosterone
PT	Pivotal temperature
RIA	Radioimmunoassay
rpm	Revolutions per minute
SCL	Straight carapace length
SD	Standard deviation
SH	Stable high
SL	Stable low
TRT	Transitional range of temperature
TSD	Temperature-dependent sex determination
UAB	Universitat Autònoma de Barcelona
UICN	International Union for Conservation of Nature
US	Unite States of America
W	Weight
WI	Weight increase

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Acknowledgements

The realization of this thesis has been possible thanks to the help, support and hard work of many people. So, I would like to dedicate some lines in this manuscript to thanks all of them:

First, I would like to thank all the people from the NGO Cabo Verde Natura 2000 who worked hard collecting data, eggs and samples for this thesis during night patrolling at the beaches. I know very well how hard it can be work surrounded by swarms of mosquito, or with these capeverdian hot and humid conditions, and also the exhausted walks up and down through the soft sand of Ponta Cosme beach. Off all people from Natura, a special thanks to Judit Pinós, because without her initiative and effort this thesis could never has been done, Judit thanks a lot for launch and leading all the experiments and all your effort sampling hatchlings and nesting females in Cape Verde. In the Canary Islands version, thanks to the NGO ADS Biodiversidad, for give me the opportunity to start and continue my scientific career with these fantastic dinosaurs, and even trust me to take care of them.

Also, thanks to my supervisor Daniel Montero, first to accept the direction of this thesis even without been related with your study area, thank you very much for your guidance, advice and support during all this process, and also for your confidence.

Ana Liria, the "*Queen of Sea Turtles*", there are not enough words to write how much I own you. Back in 2015 you received me with open arms in the "*Independent republic of the sea turtles*" and during this five years you do not only have taught me all I know about sea turtles, you also have taught me how to love these animals and this job through your passion and devotion. I am also grateful for your help and support during this thesis and also out of it. During these five years you have been more than a boss, we have been colleagues and friends.

Patricia Ostiategui, thank you for placing the sea turtle´s seed on me. Probably if you never had gone to Greece to work with sea turtles, I would not be here, writing the acknowledgements of a thesis about sea turtles.

I would also thanks to my friends, who have been there supporting me and making this journey easier and funnier, our battles with mackerels Andrea; Asier, thank you for all the surfing days and also for the climbing days together with Victor; Juankri, thanks for the night of darts games. Victor, also thank you for all your help with the sea turtles. Thank you to all of you and also to the ones who have not been named, for be there and help me to clear my head during all this time.

A special thanks to you, Cristina, for been at my side this last year, the most difficult one. Thank you for encouraging and supporting me when I need it the most.

Agradecer a toda mi familia, que desde la distancia siempre me habéis apoyado, a mis hermanos por sus visitas a la isla y consejos. El mayor y más importante de los agradecimientos es para mis padres, Inmaculada y Ricardo, siempre habéis estado a mi lado incluso estando a más de 2000 km, siempre me habéis apoyado en el rumbo que he llevado en mi carrera y gracias una y mil veces por darme desde pequeño lo necesario para poder cumplir mis sueños. Gracias, os quiero.

Gracias a todos,

Alejandro

1

Photo: Mimi Carr

Introduction

1.1 Sea turtles and humans

1.1.1. A convergence history

The oldest known marine turtle belonging to the pan-chelonidoidea clade, was swimming more than 120 Ma ago on an ancient sea allocated where Colombia is located nowadays. This primitive turtle, *Desmatochelys padillai* (Fig 1.1), it is already extinct and far away related to modern sea turtles. While *D. padillai* was classified in the Protostegidae family, the seven sea turtle species extant nowadays in our seas are classified in another two different families (Cadena & Parham 2015). Cheloniidae family encompasses six of the seven current species and the older record of this family is from 24 Ma ago (Cadena *et al*. 2018). The remaining specie belongs to the Dermochelyidae family which appeared in the Paleocene 59 Ma ago (Parham & Pyenson 2010, Cadena & Parham 2015).

Fig 1.1. Representation of the oldest known marine turtle, the Desmatochelys padillai. (Cadena & Parham 2015).

The origin of Humans (*Homo sapiens*) is situated around 200 ka ago (Stringer 2016) which means that we have been sharing this blue planet with sea turtles since the beginning. Even if humans and sea turtles has been interacting for longer time, older records of this interaction dates from 5000 BCE according to some human settlements found with sea turtle bone remains in Dalma Island (Arabian Peninsula) (Mendonca *et al.* 2016). Therefore, this interaction dates from more than 7000 years and evidences are abundant and diverse, where many remains of humans and sea turtles have been found together all around the world, from the Mediterranean Sea to Oceania (Frazier 2003, 2004, Allen 2007, Mendonca *et al.* 2016).

The interaction human - sea turtles is related to different causes. Sea turtles have been seen, and still are being seen in some places, as a source of proteins or raw material, to develop tools, as well as an important part of the zoolatry of many cultures, been considered as gods by Mayans, Chinese or Africans cultures, among others. In addition, since ancient times sea turtles have symbolized certain feelings and concepts, such as creation, endurance, fertility, longevity and so on (Bassie-Sweet 1999, Frazier 2003, Stookey 2004, Moran & Yu 2005, Mendonca *et al.* 2016) (Fig 1.2).

Fig 1.2. Left: A cave painting near in Australia depicting a sea turtle. The age of the painting is estimated at 1600 to 1900 years old (Griffith University). Right: Aegina, the greek silver stater with a sea turtle, considered one of the first coins from the 6th century BCE (Ancient Art & Numismatics).

1.1.2. From food to conservation

The high consumption and demand of sea turtles around the world resulted in an unsustainable capture of sea turtles from the wild (Fig 1.3) (Witherington & Frazer 2003, Hamann *et al.* 2010). To face that problem, the rearing of sea turtles under controlled conditions was proposed as an alternative that was done for centuries, mainly collecting the animals directly from the wild and rearing them until they reach the desired weight (Hendrickson 1974). Given the rapid depletion of sea turtle populations, mariculture was also suggested as a conservation tool, with the idea of replacing the animals extracted from the wild with sea turtles coming from rearing systems, imitating their life cycle under controlled conditions (Ehrenfeld 1974, Stickney 2000). Besides, the intensive production of sea turtle was developed in many places around the world, where the green turtle (*Chelonia mydas*) was the main reared sea turtle, but other species were also successfully reared, including the loggerhead (*Caretta caretta*) (Higgins 2003). The main turtle farms were located in Torres strait (Australia), in Reunion Island (France), Bahamas (USA) and in Gran Caiman Island (United Kingdom) (Ehrenfeld 1974, Hendrickson 1974). Finally, none of them resulted profitable, due to the high economic costs, the poor results obtained in terms of production, the difficulty to reproduce the life cycle of sea turtles, together with changes in the CITES regulation, which forbid the consumption of sea turtle products, and induce a shift in the perception of mariculture of sea turtles in favor of research and reintroduction (Stickney 2000, Higgins 2003).

Fig 1.3. Unloading green sea turtles in Grand Cayman during the latest 50s (Ronald Mitchell/The National Archives UK).

1.2. Controlled conditions as a conservation tool

The indiscriminate consumption together with other factors that sea turtles have needed to face during the last decades, such as marine litter, pollution and habitat destruction or climate change, have produced a general decline on their populations, and consequently the necessity to include all the seven existing sea turtle species in the consideration as endangered species (Fig 1.4) (Seminoff 2004, Abreu-Grobois and Plotkin 2008, Mortimer and Donnelly 2008. Wallace *et al.* 2013, Casale and Tucker, 2017, Wibbels & Bevan 2019). The worldwide situation of sea turtles makes necessary to develop important conservation plans worldwide (Hamann *et al.* 2010).

Fig 1.4. Loggerhead sea turtles entangled in marine debris (Teo Lucas).

Direct sea turtle conservation on nesting beaches started back in the 1950s with a few programs, which during the last 60 years have increased to thousands of programs all around the world. Conservation plans have also included the implementation of fishing laws to avoid the by-catch as well as the creation of marine protected areas. During last years a new conservation strategy has appeared based on local communities and tourism, in order to increase the awareness about sea turtles as well as offered an alternative way of life for local communities based on sea turtles (Fig 1.5) (Hamann *et al.* 2010).

1.2.1. keeping sea turtles under controlled conditions

Rear sea turtles under controlled conditions have been used as a conservation tool in many places and with different species. Loggerhead sea turtle is one of the species that

have been successfully reared under controlled condition along their entire life cycle, for long periods in the context of head starting and reintroduction programs (Higgins 2003), or for relatively short periods for recovery or veterinarian treatments (Fig 1.6) (Bluvias & Eckert 2010) and for research purposes (Stokes *et al.* 2006).

Fig 1.5. Tourists around a loggerhead female during the oviposition process in Ervatao beach, Cape Verde (Dr. Ana Liria Loza).

Fig 1.6. Loggerhead juvenile during the recovery process at the Wild Fauna Recovery Center facilities in Gran Canaria (A. Usategui-Martín)

The head starting programs are important conservation tools, focused to enhance the recruitment of sea turtles within wild populations (Bell *et al.* 2005). Those programs consist on collect neonates from the wild after hatching (Heppell *et al.* 1996), or even the eggs during oviposition to incubate them artificially (Caillouet Jr 2000), to rear hatchlings under controlled conditions during determined periods, ranging from a few days to some months or even years, and released them into the wild, strongly increasing its survival rates (Shaver and Wibbels 2007). One-year period is considered the most appropriate, because they are larger enough to avoid large number of predators associated with post hatchling stages (Fig 1.7) (Caillouet Jr *et al.* 1997, Shaver and Wibbels 2007).

Fig 1.7. Loggerhead sea turtle release after one year of head starting. Cofete beach, Fuerteventura, Spain (Dr. Ana Liria Loza).

1.2.2. Transition from farms to conservation centers, looking for the way to standardize protocols

The head starting programs have been conducted with most of species of sea turtles (Owens & Blanvillain 2013), but two species have concentrated almost all the efforts: Kemp's ridley turtles (*Lepidochelys kempii)* at Padre Island National Seashore (PINS), Texas (USA) (Fontaine *et al.* 1990, Caillouet *et al.*, 1992, 1993; Klima & McVey, 1995), and green

turtles (*Chelonia mydas)* in Florida (USA) (Huff, 1989) and the Cayman Islands (United Kingdom) (Wood & Wood, 1993). These programs could be considered a reinterpretation of the sea turtle mariculture, where sea turtles were held under controlled condition to meet the demand of sea turtle products without taking them from the wild. The most important and the first mariculture center, Mariculture Ltd, was stablish in 1968 on Grand Cayman Island in the British West Indies, later called Cayman turtle farm (CTF) Ltd (Cayman Turtle Centre nowadays). These facilities were mainly focused on produce green sea turtles for human consumption (Fig 1.8) (Stickney 2000).

Fig 1.8. Green sea turtles in the meat production tank at the facilities of the CTF (World Animal Protection, 2011)

In 1977 the main Kemp's ridley nesting colony, near Rancho Nuevo, Mexico, hosted less than 800 nesting females, so the Mexican fishing department together with different US institutions implement one of the most important head starting programs for the conservation of sea turtles. A reintroduction program has been developed focus on establish a new nesting colony at PINS, where eggs coming from Rancho Nuevo were incubated and imprinting, and some of the hatchlings where sent to National Marine Fisheries Service (NMFS) Galveston Laboratory to be reared for one year before releasing them into the Gulf of Mexico. (Caillouet Jr 2000). To face that program, the rearing of the loggerhead sea turtles was firstly proposed to get experience before to start the culture

of Kemp's ridleys. Holding facilities were the same for both species, where hatchlings were held isolated in individual plastic pots allocated in a common tank. Isolation was stablished in other to avoid biting, injuries and infections. Holding plastic pots increased in size as sea turtles grew. Kemp's ridleys were released into the wild when they were raised for 9-11 months, while loggerheads stayed in the NMFS laboratories for two years before released them, so larger tanks were disposed (Caillouet Jr 2000).

In that program, preliminary husbandry conditions were determined based on flow-through system, where seawater was taken directly from the Gulf of Mexico and filtered before reaching the facilities through different well points, accumulated in a sump to allow particulates to settled and pumped to fiberglass reservoirs, where, if needed, seawater was heated up with immersion of electric heaters. Rearing tanks received the water at 23-31 °C (mortality of sea turtles was observed at temperatures below 20°C) and salinity between 20 to 39 ppt. Rearing tanks were cleaned from food and turtle wastes three times per week and deeply once per month to remove algae and similar from the tanks, animals where fed with floating pellets manufactured by Purina Mills. Inc (Caillouet & Landry 1989). All the animals ate every day, half of the portion in the morning and the remaining in the afternoon, the amount of food for hatchlings was 2% of their body weight, while for one-year animals was a 1%, even though food amount could be modified in order to control the growth rate. All the handling protocol and enclosure system was establish based on health and veterinarian conditions (Caillouet Jr 2000). This project stablished the bases to hold sea turtles under controlled condition.

By early 80s some of the Kemp's ridleys were sent to several places in order to diversify efforts and reduce the risk if something happened in Galveston laboratories. In 1980, Cayman turtle center (CTF Ltd.) received sea turtles from Galveston laboratories, reducing the mariculture activities almost to zero (sea turtle products can only be sold to islanders), in favor of conservation sea turtle rearing, starting also a head starting program with green turtles. Cayman turtle center were the first to breed Kemp's ridleys under controlled conditions in 1986.

Kemp's ridley head starting project finished in 1988 with a total of 23000 kemp's ridleys and hundreds of loggerheads released into the Gulf of Mexico and the last kemp's ridleys adults from CTF Ltd, were released in 1992 (Caillouet Jr 2000).

In the Cayman turtle center hatchlings were held in concrete tanks of 25 cm deep with a seawater volume around 600 L, and between 300 and 30 hatchlings were disposed per tank depending on the size of the animals. Hatchlings were placed in those tanks around one year until they reached an average weight of 2.75 kg. Then the animals were transferred to bigger tanks according to the size of the animals, with tank capacity ranged between 18 to 130 m^3. The protocols described within this facility were contributing to standardized protocols with specific feeding protocols related to the size and age of sea turtles, similar to the feeding protocols used in Galveston laboratories. The turtles were feed with processed and pelletized food (Fig 1.9), with either *ad libitum*, 3%, 2% 1% or 0.5% of their body weight, for animals until one year old, 1 to 2 y.o., 2 to 3 y.o., 3 to 4 y.o. and older than 4 y.o., respectively. This facility was based on a flow-through system, pumping seawater directly from the ocean with a total water renovation every 20 to 30 minutes (Márquez *et al.* 1992). After twelve years of the rearing program, from 1980 to 1992, more than 22 000 head started animals had been released (Stickney 2000).

Fig 1.9. A green sea turtle at the facilities of the CTF feeding on processed and pelletized food (World Animal Protection, Catherine mason, 2014)

Ten years before the first Kemp's ridley was successfully breed under controlled conditions, the Port of Nagoya Public Aquarium (PNPA), in 1996, accomplished the first successful breeding of loggerhead sea turtles held under controlled conditions (Uchida 1996). The

rearing facility of the PNPA consists on a doughnut shaped tank, 1.5 m depth and with a total water volume of 560 m^3. The protocols used in PNPA are not available in the literature, but additional information of light quality and feeding protocols were published. The tank receives natural light through glass windows in the ceiling; light supply is complemented with nine 400 w halide lamps. Water temperature ranged from 23 °C, in February, to 27 °C, in December. Turtles were fed three times per week on a mix of fresh fish and mollusks together with cabbage and seaweed. The mean amount of food per year was 0.5% of their body weight (Kakizoe *et al.* 2010, 2013).

1.2.3. Incubating sea turtle eggs for conservation programs

No one of the mentioned protocols gave information on the egg incubation procedures that is another important phase to consider on head starting and reintroduction programs. The incubation period in very important for sea turtles since hatchlings phenotype and performance are influenced by several environmental factors during incubation period, such as hydric properties of the substrate (Reece *et al.* 2002) and nest temperature (Booth *et al.* 2004), which also determine the sex of the hatchlings because sea turtles present temperature-dependent sex determination (TSD) (Mrosovsky 1994, Mrosovsky *et al.* 2002, Wibbels 2003).

In head starting programs the incubation of the eggs usually has followed the same standardized procedure, but two different procedures are used: a) eggs incubated on the beach and b) eggs incubated under laboratory conditions (Fig 1.10). For eggs incubated on beaches, the standardize protocol consisted on the collection of the eggs directly from the female from the donor population (i.e. Rancho Nuevo for the Kemp's ridley head start program), always during the oviposition before the eggs touch the sand, and transported to the beach of interest (i.e. PINS for the Kemp's ridley head start program), where eggs are incubated naturally (Simon 1975, Caillouet & Landry 1989, Caillouet Jr 2000). Other programs with the same characteristics did not need to collect eggs from the wild due to the own production of eggs in artificial beaches created for that purpose, as it has been done in CTF Ltd with Kemp's ridleys and greens or in the Nagoya Aquarium, Japan with loggerhead sea turtles (Owens & Blanvillain 2013).

For those programs with incubation under controlled conditions in the laboratory, including research programs to study sex determination or studies on how incubation

affects hatchling performance, the incubation of the eggs has been conducted in stoves, usually at stable temperatures (Booth *et al.* 2004, Mrosovsky *et al.* 2009, Fisher *et al.* 2014). After hatchling, sea turtles are placed in experimental tanks and follows the standardize protocols from each facility. However, constant incubation temperature is rare under natural conditions, which typically oscillate over the course of incubation (Packard & Packard 1988, Plummer *et al.* 1994, Shine *et al.* 1997).

Fig 1.10. Loggerhead eggs incubating in vermiculite under laboratory conditions at ECOAQUA facilities, Canary Islands, Spain (Dr. Ana Liria Loza)

Hold wild animals under controlled conditions, for long or short periods of time, is unavoidably associated to many challenges that an animal with a complex and partially unknown life cycle such as loggerhead sea turtle need to face. But keeping them under controlled conditions may provide insights about some life stages that are very difficult to observe and sample in the wild (Herbst & Jacobson 2002). The protocols to held the animals must be standardize to minimize deleterious effects on animals in terms of health and welfare, but, as mentioned above, most of the established parameters to rear sea turtle hatchlings under controlled conditions are based on experiences from each facility and based on results on health, measuring the survival or the prevalence of diseases or injures under certain temperatures and stocking densities (Fish and Wildlife Service, 2013) as well as with juveniles (Bluvias & Eckert 2010). Only few information is available on welfare aspects of sea turtles held under controlled conditions. Among the different species, green turtle has been the most studied species, information on loggerhead sea turtle being scarce in the literature.

1.3. Loggerhead sea turtles from the North Atlantic. The clue of a transatlantic voyage

The life cycle of sea turtles is characterized by include a diversity of ecosystems, terrestrial during oviposition and embryonic development, oceanic and neritic for growth and foraging (Bolten 2003). Post-hatchling and juvenile stages are the less understand phases on all species of sea turtles, traditionally known as "*The Lost years*" (Carr 1986, Bolten & Balazs 1995).

Among the different species of sea turtles, the juvenile stages of loggerhead sea turtles are the most studied (Frick *et al.* 2009) and withing the three different population that can be find in the Atlantic, two in the north Atlantic, the Northwest and Northeast Atlantic populations, and one in the south, the South Atlantic population (Wallace *et al.* 2010). The Northwest Atlantic population is the most studied and well known (Frick *et al.* 2009) and consequently the life cycle of the loggerhead sea turtle has been described using the Northwest Atlantic population as a model (Bolten 2003).

Northwest Atlantic population presents an oceanic-neritic development pattern. Hatchlings start with the frenzy stage, characterized by a hyperactive continuous swimming offshore after emergence and during the first 24h, followed by the post-frenzy stage, described as the active swimming during the flowing 5 days (Wyneken *et al.* 2008). After this, sea turtles drift and swim with currents for less than a year heading them to "nursery" oceanic zones (Mansfield *et al.* 2014), usually associated with oceanic algae concentrations, such as *Sargassum sp*. in the Sargasso sea, and then, they drift into the oceanic foraging areas, where they spend from 7 to 11.5 years reaching a size of 46 – 64 cm CCL (minimum curve carapace length). After this period in North Atlantic oceanic waters (Bjorndal *et al.* 2000), loggerhead juveniles leave the oceanic zone heading to neritic areas where they complete their development until the reach sexual maturity (Fig 1.11). During the adult stage, northwest Atlantic population moves between feeding grounds and reproduction areas, but always in the neritic zone (Bolten 2003, Braun-McNeill *et al.* 2008).

On latest nineties, the biologist Dr. Luis Felipe Lopez Jurado rediscovered the second most important loggerhead rookery in the Atlantic (Fig 1.12), and the third larger colony in the world after Florida and Oman colonies (Lopez-Jurado *et al.* 2007; Marco *et al.* 2011).

Fig 1.11. Loggerhead juvenile around Gran Canaria, Canary Islands, Spain (BuceoNorte Dive Center, 2019)

Fig 1.12. Female loggerhead sea turtle from the Northeast Atlantic population nesting during the day (Idaira Hernandez Rojas).

This new colony is located in the Archipelago of Cape Verde, a group of 10 volcanic and 5 islets situated in the Northeastern Atlantic, about 600km west of Senegal and 1.600 km south-southwest of Canary Islands. Main nesting activity of the archipelago occurs in Boa Vista Island (70%), and the remaining nesting activity is distributed within the rest of the archipelago, mainly on Sal and Maio Islands (Monzón-Argüello *et al.* 2010, Marco *et al.* 2011). This rookery presents a different life cycle from the one stablished for the Northwest Atlantic. For example, adults breeding on Boa Vista present a foraging behavior dichotomy, which seems to be related to the size of the individuals, where larger females occupying costal foraging grounds, while the smaller turtles usually use the oceanic habitats, been pelagic eaters (Hawkes *et al.* 2006) (Fig 1.13). In addition, this population is considered one of the eleven most endangered sea turtle population, considered as "endangered" by the IUCN due to a population decrease produced by a constant human pressure and habitat degradation, together with the reduced nesting area (<500km2), where larger amounts of nests are concentrated in less than 5 nesting locations (Casale & Marco 2015).

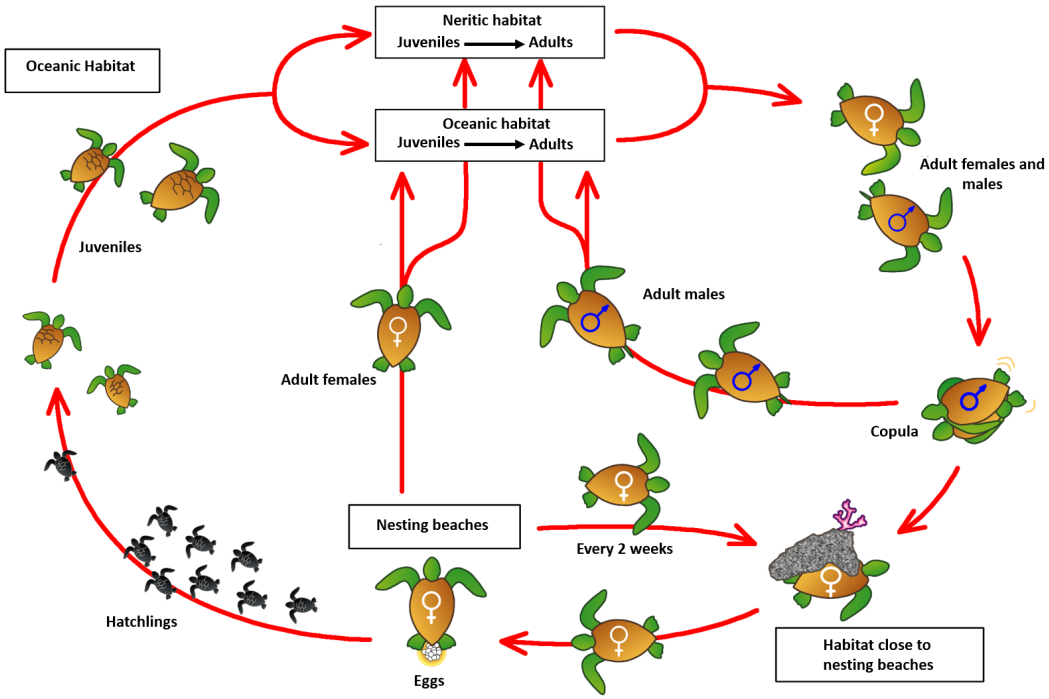


Fig 1.13. The life cycle of loggerhead sea turtles (Varo-Cruz 2010).

The situation of the Northeast Atlantic population demands important conservation plans. *"In situ"* plans have been conducted during the last 20 years, consisting on the protection of females on the beaches from human consumption, daily census to register the activity of females, and beach patrolling at night, for female tagging and data collection. Also, the implementation of hatcheries in order to increase the hatchling recruitment on the population and other research and conservation actions has been conducted. Another conservation tool, which is gaining in importance, is the ecotourism with sea turtles, offering alternatives to the local. *"Ex situ"* conservation plans for this particular population have been based the program "*Enlargement of the reproductive habitat of the loggerhead sea turtle in the Macaronesia region"* a collaborative project between the Governments of Cape Verde and the Government of the Canary Islands looking for the establishment of new breeding colonies in the Northeast Atlantic, through a reintroduction program of the loggerhead turtle in the Canary Island from eggs collected on the Cape Verde loggerhead colony.

1.4. Stress and welfare. Facing the controlled conditions in a proper way

Wild sea turtles during their life cycle are subjected to a very stochastic environment (Bjorndal *et al.* 2003) where they have to face a vast variety of natural and anthropogenic stressors (Milton & Lutz 2003), from the high predation pressure, mainly during their first life stages (Heithaus 2013) to great variations in food availability and temperature (Bjorndal *et al.* 2003) or human pollution (Milton & Lutz 2003). However, sea turtles held under controlled conditions do not have to deal with the same stressors, but they can be susceptible to other stressors like the ones derived from confinement, husbandry parameters, cleaning protocols, data collection or feeding strategies.

A stressor is described as an internal or external stimulus, which threatened the animal homeostasis (Moberg & Mench 2000, Conte 2004), setting off a physiological stress response to recover the allostatic equilibrium almost immediately (Denardo 2006, Schreck & Tort 2016). The classification of a stressor is wide, ranging from immediate or acute, as it could be the predator pressure or a direct handling, to a stressor prolongated on time

or chronic, as it could be a crowded tank while controlled conditions. The stress response starts just before the stressor perception with the activation of two neuroendocrine cascades: a) one driven by the autonomic nervous system with the synthesis and secretion of catecholamines by the chromaffin tissue, which affects most of the biological systems, inducing the animal into an alert mode, increasing the heart rate, the blood pressure and the fast mobilization of energy (Moberg & Mench 2000, Schreck & Tort 2016, Tort & Teles 2011), mainly to prepare the animal to escape or defense (*"fight or flight"*); and b) the neuroendocrine cascade activated during the stress response and driven by the hypothalamus pituitary adrenal (HPA) axis, which release corticosteroids in the blood stream to prepare the organism to resist by reallocation of energy reserves or changes in the immune defenses (Tort & Teles 2011). Both physiological mechanisms directly affect animal behavior, and the proper stress response is also mediated by the behavior of the animal itself, where the previous experiences and learning capacity of the species play an important role, even if they need to face husbandry practices.

It is widely accepted that the changes of the corticosteroids concentration on the bloodstream after HPA axis activation, indicate that the animal is experiencing a stressful situation, which would induce a hormonal cascade causing the release of corticosteroids from the adrenal cortex (Greenberg & Wingfield 1987, Gregory & Schmid 2001, Silvestre 2014). In reptiles, the main endocrine product released to bloodstream in the physiological regulation of stress response is the corticosterone (CORT) (Silvestre 2014). This steroid hormone would increase its levels in plasma within minutes to hours of exposure to a stimulus (Greenberg & Wingfield 1987) and changes in circulating CORT concentration can be triggered by internal or external factors (Romero & Wikelski 2002, Jessop & Hamann 2005, Flower *et al.* 2015).

Physiological and behavioral responses to stress would contribute to the animal adaption and subsequently animal welfare, mainly when the animal need to face controlled conditions (Wingfield *et al.* 1998, Sapolsky *et al.* 2000, Jessop & Hamann 2005). The concept of animal welfare usually is defined in terms of over time balance of affective states (Boissy *et al.* 2007). Affective states are variably described as feelings, emotions or moods, which might include, amongst others, happiness, fear, depression and pain, and comprise behavioral, physiological and cognitive components (Boissy *et al.* 2007). Measurement of affective state can be used to infer welfare state (Benn *et al.* 2019).

Resource-based measures are recorded in the environment surrounding the animals, whereas animal-based indicators are measured directly in animals, using a combination of physiological, behavioral and health variables (Whitham & Wielebnowski 2013), which include a wide variety of parameters such as spontaneous behavior or homeostatic equilibrium after a stressful situation (Benn *et al.* 2019). So, welfare can be assessed through behavior, levels of physiological indicators (i.e. stress indicators) or other indirect parameters like absence of illness or the proper appetite (Arena & Warwick 1995).

Most of the times, wild sea turtles can provide to themselves their own welfare, for example, swimming away from the stress source through a behavior response, or overcoming to it, from a physiological point of view. But, when sea turtles are under controlled conditions, they cannot freely face the stressors, because, for example, they cannot swim away from them, in consequence they cannot handled their own welfare as wild sea turtle can do. Besides, the proper controlled conditions have associated certain husbandry protocols that could induce changes in the welfare of the animals if they are not properly standardized. So, the welfare of animals held under controlled conditions needs to be controlled and managed by the people in charge of the facilities. Probably is even more important in an animal like the loggerhead sea turtle, which is listed as an endangered species, so monitoring their welfare when they are under these conditions should be a priority (Sherwen *et al.* 2018).

Changes of circulating CORT of wild sea turtles have been widely studied. For example, in adult females during the nesting season, CORT variations have been linked to reproductive success, high density nesting processes, season or to predation stimulus (Whittier *et al.* 1997, Jessop *et al.* 1999, Valverde *et al.* 1999, Rostal *et al.* 2001, Jessop 2001, Jessop *et al.* 2004a, Jessop & Hamann 2005, Flower *et al.* 2018). In wild juveniles have been related to handling or recovery/illness processes (Aguirre *et al.* 1995, Gregory *et al.* 1996, Jessop *et al.* 2004b, Jessop & Hamann 2005, Hunt *et al.* 2012) and in wild hatchlings during their dispersal behavior (Hamann *et al.* 2007). However, little is known about CORT variations when these reptiles are held under controlled conditions or during the human – sea turtle interaction in the wild for data collection or some particular research.

As said before, Northeast Atlantic loggerhead population is very fragile and different conservations plans are being developed. On *"in situ"* conservation actions interaction with nesting females is required for data collection, as well as for *"ex situ"* actions, where

rearing under controlled conditions (head starting programs), were carried out, so, animal welfare must be always considered on this fragile population. However, few information exists on the literature on how these situations could affect animal welfare on sea turtles, so the aim of this work was to establish standardized protocols based on the welfare of loggerhead sea turtles along their different life stages when sea turtles are held under controlled conditions, or during the human-sea turtle interaction on the wild.

1.5. Objectives

1. To determine the effect of incubation protocols, such as incubation temperature pattern (increasing or stable) and regime (low or high), on incubation time, hatching duration, hatching and emergence success, hatchling phenotype (carapace length and total mass) and hatchling self-righting time, in loggerhead hatchlings.
2. To improve head-starting protocols for loggerhead hatchlings, defining adequate rearing parameters (stocking density, seawater temperature or culture-related handling processes) based on welfare indicators (CORT concentration).
3. To define standardized husbandry protocols for loggerhead juveniles held under controlled conditions, based on CORT response and spontaneous behavior under different routine situations (isolation / multiple occupancy or dry-docking).
4. To evaluate standardized field procedures used in conservation programs with nesting female loggerhead sea turtles (data collection or recapture programs), based on animal welfare indicators (CORT variations).

2

Photo: Patricia Ostiategui Francia

General Material and Methods

2.1 Location of the study and origin of experimental animals

2.1.1. Location of the experiments

Studies included on this PhD were conducted with loggerhead sea turtles (*Caretta caretta*) from the Northeast Atlantic population. Animals come from some beaches located in the *'Reserva Natural das Tartarugas'*, southeastern Boa Vista Island, Cape Verde (Fig 2.1).

As was described in Chapter 1 the Northeast Atlantic loggerhead population is one of the eleven most endangered sea turtle population in the world (Casale & Marco 2015) where the 80% of the nests are concentrated in 30km of beaches located in the *Reserva Natural das Tartarugas (RNT)*, southeastern Boa Vista Island. This situation demands important conservation plans. "In situ" programs in Cape Verde began 20 years ago, back in 1998, where beach patrolling, daily census, female tagging, female data collection and the implementation of hatcheries started and have been conducted in Boa Vista Island by the NGO Cabo Verde Natura 2000. Experiments conducted in Boa Vista, with hatchlings (Chapter 4) and nesting females (Chapter 6), were conducted under the Official permits awarded annually by the *Direção Nacional do Ambiente (Cape Verde Government)* to the NGO Cabo Verde Natura 2000 for conservation and research activities with sea turtles in the RNT (Authorization n°2/2011, Authorization n° 2012, Authorization n°9/2013, Authorization n°12/2014).

In other hand, in 2006 an *"ex situ"* conservation plan was launched through the program *"Enlargement of the reproductive habitat of the loggerhead sea turtle in the Macaronesia region"*, supported by the Government of Cape Verde, the Government of Canary Islands, one regional council (Cabildo Insular de Fuerteventura), and two NGOs as scientific advisors to support the experimental research with sea turtles (NGO Cabo Verde Natura 2000 in

Boa Vista and NGO ADS Biodiversidad in Canary Islands), both presented official permits from the respective national authorities to conduct conservation and experimental activities with sea turtles. This program was included in several collaborative projects, such as Project PELAGOS (*"Un modelo para la Gestión Coordinada de los Recursos Naturales Marinos de la Macaronesia"*, Interreg MAC/3/178) and others. Experiments conducted in Canary Islands were conducted under the Official permits awarded by CITES authorities. The egg incubation under controlled conditions (Chapter 3) conducted in Gran Canaria, had been handled under CITES permits ES-DE-00008/08I and ES-DE-00005/09I. All the loggerhead juveniles included on the experimental trials described on Chapter

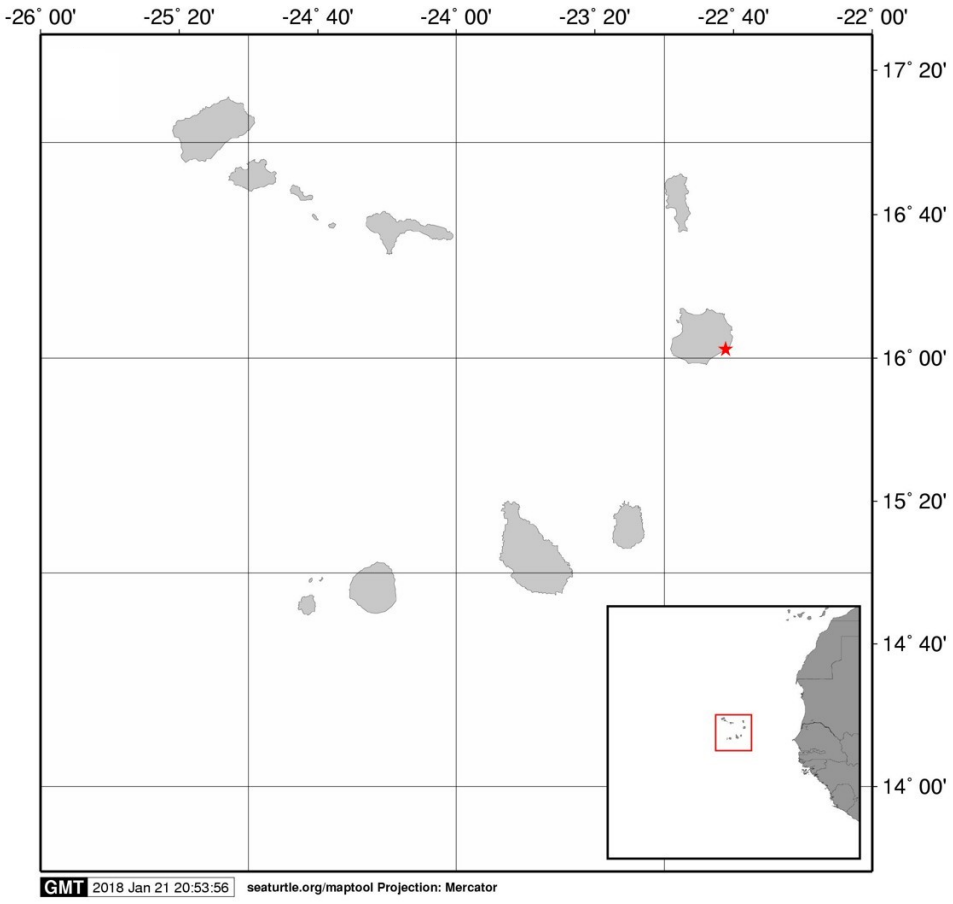


Fig 2.1. Map of Republic of Cape Verde, western Africa. The location of the archipelago (red square). Location of the "Reserva natural das Tartarugas" (red star), (SEATURTLE.ORG Maptool. 2002. SEATURTLE.ORG, Inc. (2018 Jun 21).

5 present their own individual CITES permits *(ES-DE-00042/19C, ES-DE-00043/19C, ES-DE-00044/19C, ES-DE-00045/19C, ES-DE-00046/19C, ES-DE-00047/19C, ES-DE-00048/19C, ES-DE-00049/19C, ES-DE-00050/19C, ES-DE-00051/19C).*

This program was divided in four phases, where each phase requires specific approaches, related with the particularities of each stage:

Egg collection from Cape Verde rookery and translocation to Canary Islands: eggs were collected on low productivity beaches from the "*Reserva natural das Tartarugas*", southeast Boa Vista Island (Cape Verde), directly from the cloaca, avoiding any contact with the sand to prevent pathogen (i.e. bacteria, fungi, etc.) introduction in the translocation destination (Canary Island). Eggs were placed into isothermal container (24x35x19 cm) (Fig.2.2a) to avoid temperature fluctuations during their transport, which could induce embryos mortality during the first hours after laying. Containers were filled with vermiculite at -150 KPa hydric potential, maintaining egg vertical position and oviposition order, arranged in columns and rows. The eggs from different nests were separated by plastic grids. The containers were transported by a 4x4 vehicle from the RNT beaches to Boa Vista airport and then taken to Gran Canaria Island (Spain) by plane (Fig.2.2b). The entire process required less than 24h and followed long distance nest translocation protocols established by Abella *et al* (2007) and López-Jurado (personal communication).

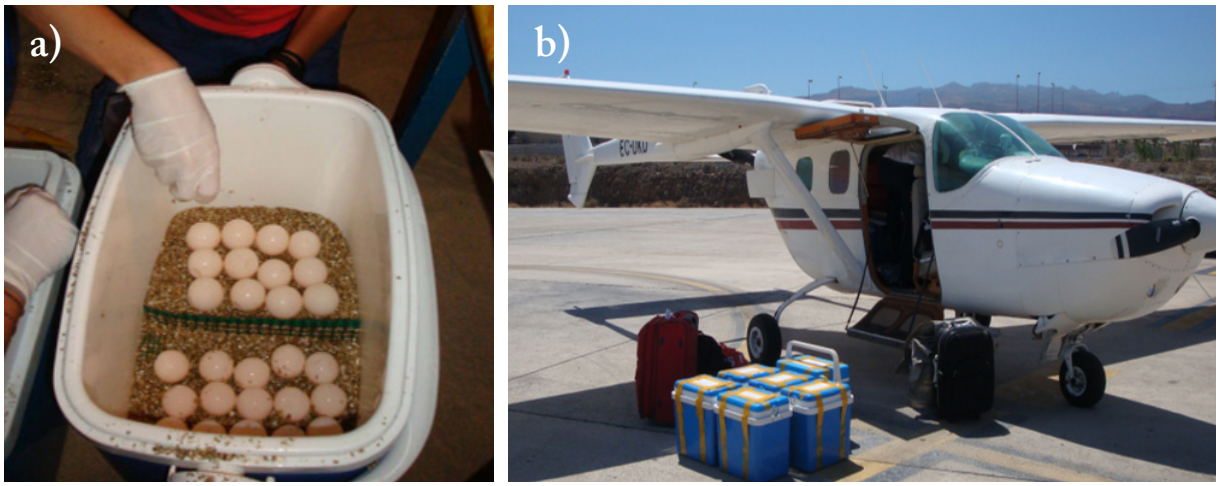


Fig 2.2. a) Eggs of loggerhead sea turtle placed in the plastic containers where are going to be transported to the Canary Islands; b) Plastic containers with the eggs inside ready to be transported by plane to the Canary Islands.

Eggs incubation on Canary Islands: two procedures were followed

a. Laboratory incubation (described in 2.2)

b. Beach incubation: After exhaustive analysis of beach characteristics (temperature, granulimetry, hidric conditions, beach slope, human alterations, etc.) conducted between 2003 and 2006 on Canary Islands, adequate beaches were identified and Cofete beach (Fuerteventura Island) were selected as the most adequate area. When eggs from Cape Verde arrived, an experimented researcher digged artificial nest holes in a previously determined are of the beach, by hand and with similar shape and deep to natural nests. Then, eggs from each original nest were picked up one by one, maintaining the original vertical position and minimizing any movement to avoid embryos death, and inserted in the artificial nest. Finally, each nest was covered with sand and the area were identified for monitoring. Direct incubation during all incubaion process has been conducted and monitored in Cofete beach (Fuerteventura). All neonates hatched on the beach were collected and brought to rearing facilities.

Head-starting programs: One year of head starting program with all hatchlings produced in Canary Islands has been conducted to increase survival rate in the wild after been released. After the first year, most of the animals were release into the wild, previously tagged with a passive integrated transponder (PIT) in the right front flipper. Husbandry procedures are described in 2.3.

Prolonged maintenance juveniles: maintenance under controlled conditions of a small group of yearlings were prolonged to increase knowledge on the biology of juvenile stages. Husbandry protocols described in 2.3.

2.1.2. Origin of experimental animals

In the first experiment (eggs incubation - Chapter 3), 200 loggerhead eggs were collected in 2008 and 2009 on Boa Vista beaches (Cape Verde), translocated to Canary Islands in less than 24 h, and incubated under controlled conditions on the ECOAQUA laboratories (Canary Islansd, Spain) (Fig 2.3). Incubation procedures are described in section 2.2. and experimental temperature ranges and patterns in Chapter 3.

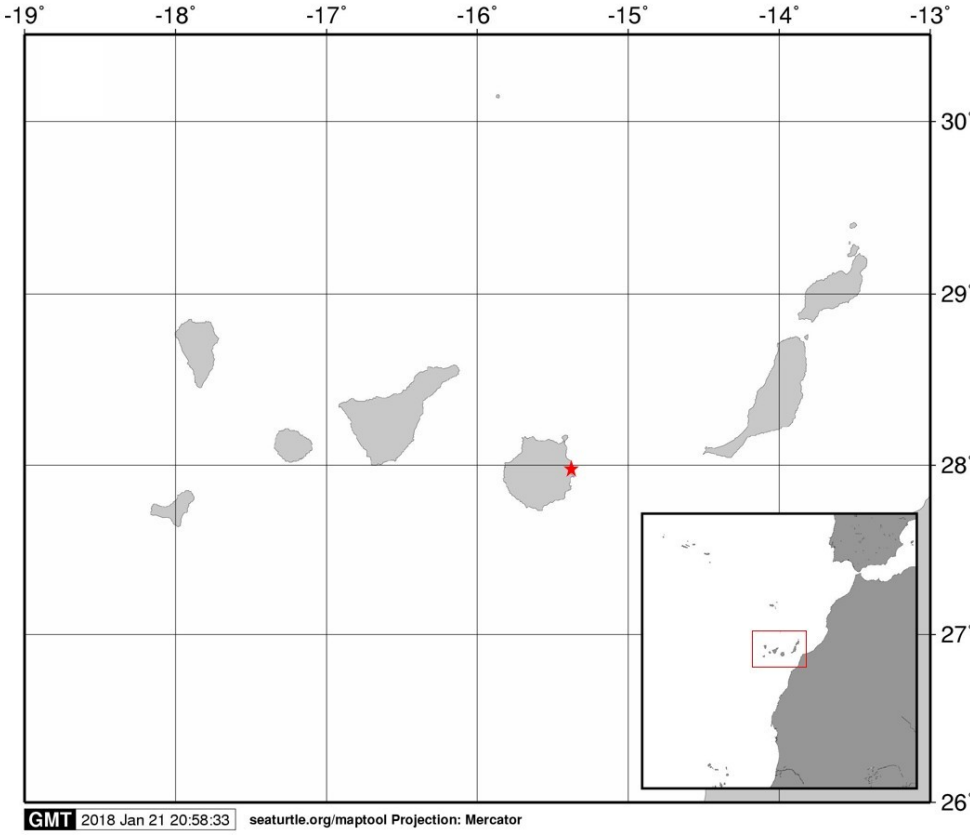


Fig 2.3. Map of Northeast Atlantic and western Africa. Location of Canary Islands (Spain) (red square) and location of ECOAQUA institute (red star) in Gran Canaria Island. Map elaborated with SEATURTLE.ORG Maptool. Inc. (2018 Jun 21).

For the second experiment (Chapter 4), 130 hatchlings were collected on 2013 and 2014 nesting season, from natural nests collected on low productivity beaches within the RNT, and translocated and incubated in the Ervatao hatchery, Boa Vista (Cape Verde). Hatchling were reared under controlled conditions in the facilities of the NGO Cabo Verde Natura 2000 under the License n°2/2015 from the *Direção Nacional do Ambiente (Cape Verde Government)*. Rearing procedures are described in section 2.3. and experimental trials in Chapter 4.

For the third experiment (Chapter 5), 9 juveniles derived from the program *"Enlargement of the reproductive habitat of the loggerhead sea turtle in the Macaronesia region"* described before, hatched in Cofete beach and ECOAQUA laboratories in 2009 and 2010, and reared under controlld conditions were used. Rearing procedures were described in section 2.3 and experimental trials in Chapter 5.

The last experiment (Chapter 6), were conducted with 99 loggerhead nesting females coming to nest in the RNT beaches. Standard protocols used by the NGO Cabo Verde Natura 2000 on the RNT beaches are described by Varo-Cruz *et al.* (2006), and the specific procedures analysed in the section 2.4. and Chapter 6.

2.2 Incubation procedures

In Boa Vista Island (Cape Verde), hatchlings reared under controlled conditions (Chapter 4) come from eggs collected from low productivity nesting beaches (*Ervatão* and *Ponta Cosme* beaches) within the "*Reserva Natural das Tartarugas*" (Fig 2.4), during the 2013 and 2014 nesting seasons. Whole nests were collected during the oviposition, placed

Fig 2.4. Ervatão beach, one of the most important nesting beaches for loggerhead sea turtles in the "Reserva natural das Tartarugas". In this particular beach, some of the eggs where collected and the nesting females sampled.

into plastic bags (one nest per bag) and translocated to an incubation-controlled area (*hatchery*) where they were incubated safely from tides and predation (Fig 2.5). Egg collection and translocation, as well as incubation process monitoring, were carried out by the experimented staff and trained volunteers from the NGO Cabo Verde Natura 2000.

Fig 2.5. The incubation-controlled area (hatchery) located in Ervatão beach (Boa Vista, Cape Verde), where sea turtle nests collected from low productivity beaches were incubated safely from tides and predation.

In the Canary Islands, experimental procedures on egg incubation were conducted on the ECOAQUA (ULPGC) laboratories, with eggs collected on Boa Vista (Cape Verde) beaches during the 2008 and 2009 nesting seasons. When the eggs arrived at the laboratory, there were divided and disposed in different plastic containers (3L) filled with vermiculite (at -150 KPa hydric potential). Eight eggs per container were disposed and only the 80% of the eggs were covered with vermiculite (-150KPa) to permit egg observation and monitoring during all the incubation process. Filled containers were placed in Medilow® incubators

Fig 2.6. Images of eggs incubation procedures under laboratory conditions. Left-up: eggs disposed into the containers filled with vermiculite; Left-down: Group of containers filled with eggs; Right: Medilow® stoves used for laboratory incubation.

at the temperature patterns and regimes described on Chapter 3 (Fig 2.6). Different parameters were monitored, such as the incubation time, defined as the period between the oviposition and the emergence, calculated in laboratory as 3 days after hatchlings were completely out of the egg (Godfrey & Mrosovsky 1997), hatch time, nest success, hatchling biometric parameters (SCLmin and weight), and hatchlings self-righting time.

2.3 Husbandry

2.3.1. Housing conditions

In Boa Vista Island (Cape Verde), rearing facilities consisted on 12 rectangular tanks (100 L) filled with seawater collected from the sea. Individual filters were disposed in each tank to maintain water quality, and a full cleaning were conducted by hand and with a total water renovation each 10-15 days (Fig 2.7a). Rearing seawater temperature and hatchling stocking density were established based on experimental trials described in Chapter 4.

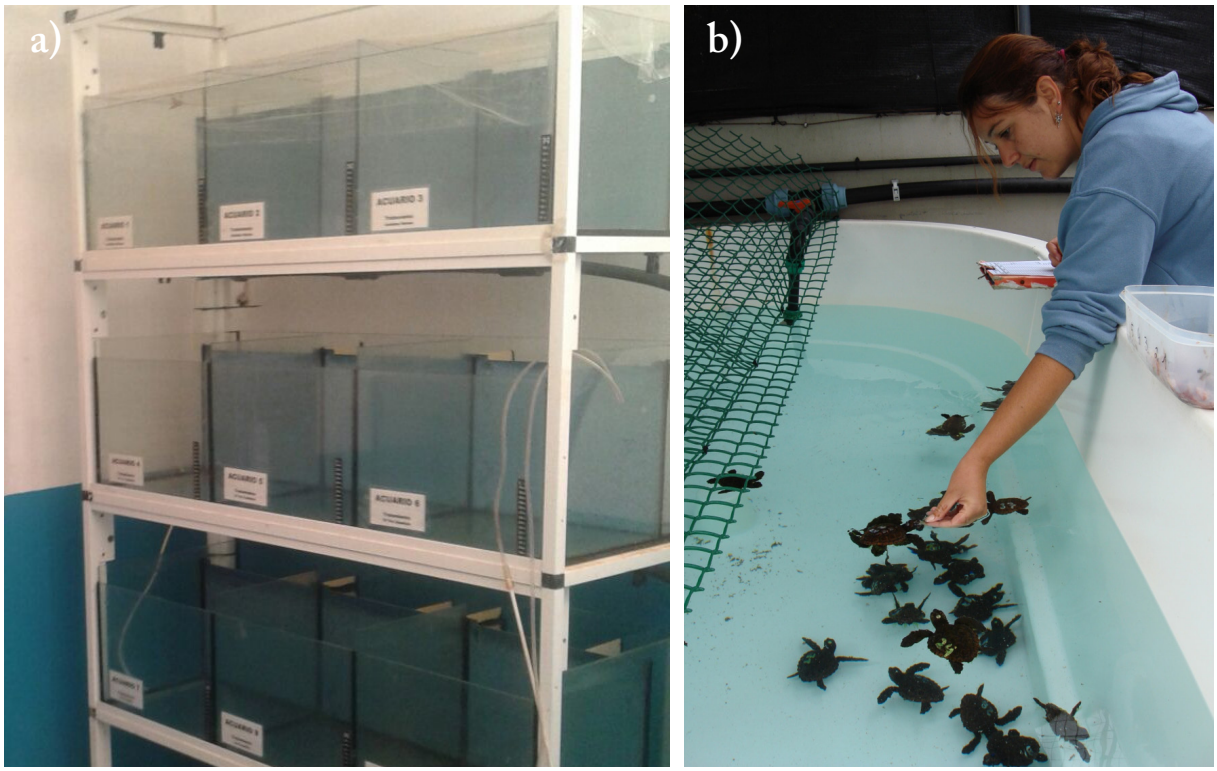


Fig 2.7. a) NGO Cabo Verde Natura 2000 rearing facilities for loggerhead hatchlings, b) rearing facilities for loggerhead hatchlings in the Canary Islands.

In the Canary Islands, during the first year of life, hatchlings were kept in 3000 L fiber tanks (oval shape), disposing between 30 to 40 hatchlings per tank (Fig 2.7b). As non-released animals were getting bigger the stock density per tank was reduced and the volume of the tanks increased, based on the availability of holding tanks within the facility. Finally, the optimal stock density for 8-9 years old animals (used in Chapter 5), was established in three animals per 5000 L tank, based on animal weight, to keep similar densities (kg/ L), the lack of aggressions between individuals, and the proper feeding behavior observed (Fig 2.8). The facilities were based on an open water circulation system, with flow-through system pumping seawater directly from the sea with any kind of filtration, treatment or temperature control. When injured or sick hatchlings were detected, the animal was removed and disposed into quarantine tanks, where water temperature was kept at 24°C.

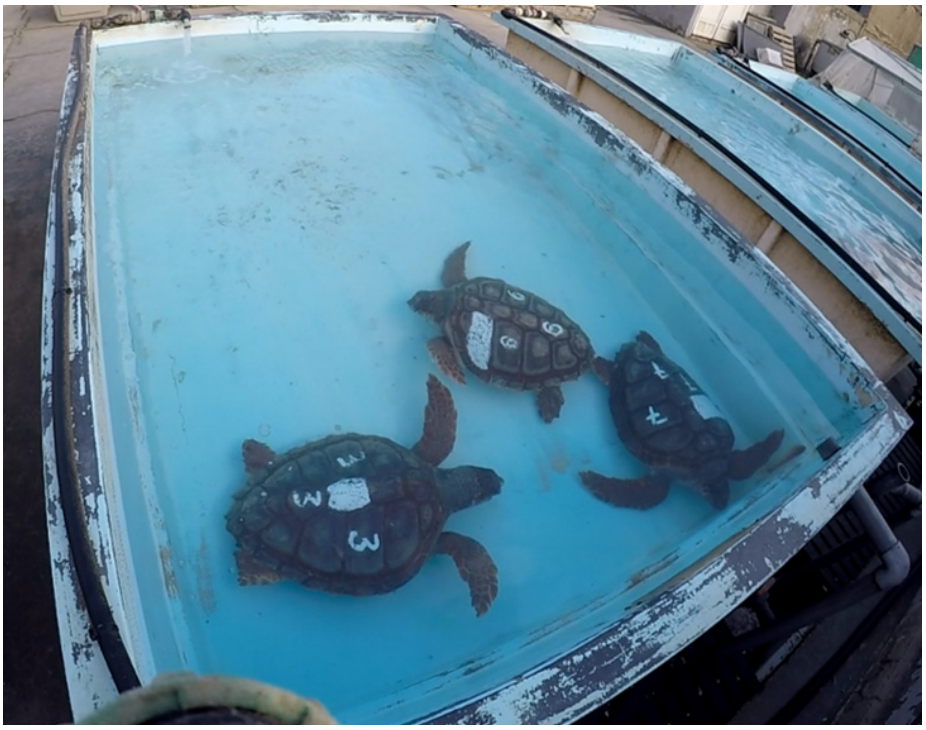


Fig 2.8. A tank of the rearing facilities viewed from above with the standardized stocking density for juvenile loggerhead sea turtles. Three animals per tank.

2.3.2. Feeding protocols

All feeding protocols were established following recommendations of Bluvias & Eckert (2010). In Boa Vista (Cape Verde), hatchlings were feed six times per week (from Monday to Saturday). Food amount per hatchling was adjusted to the 5% of their body weight per day. During all the experiment animals were feed on a mix of different fresh fishes, bought on the local market, and freeze mollusks.

In the Canary Islands, hatchlings were feed five times per week and food amount were adjusted to 5% of their body weight (quarantine animals were feed *ad libitum)*. After the first year (yearlings from 1 to 2 years old), periodicity decreased to four times per week and the amount of food was adjusted to body weight and seawater temperature, ranging from 3% to 5%. Finally, juveniles from 2 to 9 years old were feed three times per week (Monday, Wednesday and Friday) and food amount were also adjusted to body weight and seawater temperature, ranging from 2% to 3.5%. Seawater temperature in Canary Islands ranged from 18°C to 24°C. Since the beginning all animals were feed on a mix of different fresh fishes bought on the local markets and mollusks.

2.3.3. Handling procedures

During the rearing process sea turtles have to be handled for cleaning operation, both tanks and sea turtles, for data collection and for veterinarian treatments if needed. Handling standardized protocols were established according to their life stage.

In Cape Verde, standard handling procedures with hatchlings were established to define husbandry protocols, where the handling frequency and the number of hatchlings per tank were defined in the experimental trials described in Chapter 4. For data collection, each animal was taken out of the water gently, biometric data (carapace length and weight) was collected, and the animal was directly replaced in the tank. The whole process took no more than 2 minutes, and when blood samples were required, similar procedure was followed. Handling protocols were applied the same day of the week, at the same time and in the same order. Total cleaning of tanks were conducted each 10-15 days, where hatchlings were kept in provisional containers filled with water during around 60min. The same day was used to individual hatchling cleaning, with a soft brush, to avoid algae proliferation on the carapace and skin; and retouching the identification number (painted with non-toxic nail polish on their carapaces) to avoid identification lost.

In Canary Islands, hatchlings tanks were cleaned 5 times per week (from Monday to Friday), to maintain hygienic conditions to avoid infections. Cleaning process consisted on draining the tanks, removing food remains and hatchlings feces, rinsing with freshwater and filling again with clean seawater. All hatchlings were gently extracted from the tank before cleaning process and allocated in dry plastic containers for 10-20 minutes during the cleaning process. Once per week (every Friday) hatchlings were weighted and measured to monitor their growth and were exhaustively observed to monitor their health status. The same day was used to individual hatchling cleaning, with a soft brush, to avoid algae proliferation on the carapace and skin; and retouching the identification number (painted with non-toxic nail polish on their carapaces) to avoid identification lost. Cleaning operation, data collection and feeding procedures were always done in the morning between 9:00 am to 14:00 pm.

Standardized handling protocol for rearing juveniles consisted on cleaning the tanks twice a week (Monday and Friday), similar to hatching cleaning process. During the cleaning process sea turtles were dry-docked in plastic boxes (Fig 2.9) for no more than 15 minutes. Every four weeks, or more frequent if needed, during dry-docking time the epibiota on the carapace was cleaned with a scourer and rinse with freshwater. Also, every four weeks, always the same day (Friday) turtles were weighted and measured, to monitor their growth during all the on-growing period. Cleaning operation, data collection and feeding were always done in the morning between 9:00 am to 12:00 pm. Number of animals per tank and dry-docking time changed according to the requirements of the studies made with them (Chapter 5).

Fig 2.9. A juvenile loggerhead sea turtle in a plastic container in the dry-docking time during the cleaning process of the rearing tank.

2.4 Standard protocols with loggerhead nesting females

Studies with nesting females were conducted on Boa Vista Island, in three beaches of the '*Reserva Natural das Tartarugas*' (*Ervatão*, *Ponta Cosme* and *Calheta* beaches) under the standard protocols established by the NGO Cabo Verde Natura 2000 and detailed in

Varo-Cruz *et al* (2006). Nesting loggerhead females were intercepted during nigh patrols conducted by the NGO staff, where interaction with females is only allowed just after egg laying or when female come back to the sea after a failure nest. At this moment females were examined for external (metal tags) and internal (passive integrated transponder, PIT) identification tags, and if no tags were found a PIT was placed on the right flipper. Then, biometric data were collected as described in section 2.5.1. In addition any possible injury or anomaly was registered. All data collection process did not take more than 15 minutes. Blood samples were collected only when females were coming back to the sea, before and after the interaction with NGO staff for data collection. Different parameters were tested, as described in Chapter 6.

2.5 Sampling methodologies

2.5.1. Biometric parameters

In the present work the main biometric data to describe turtle size was the minimum carapace length, which is the distance from the anterior point at midline nuchal scale to the posterior notch between the supracaudal scales (Bolten 1999).

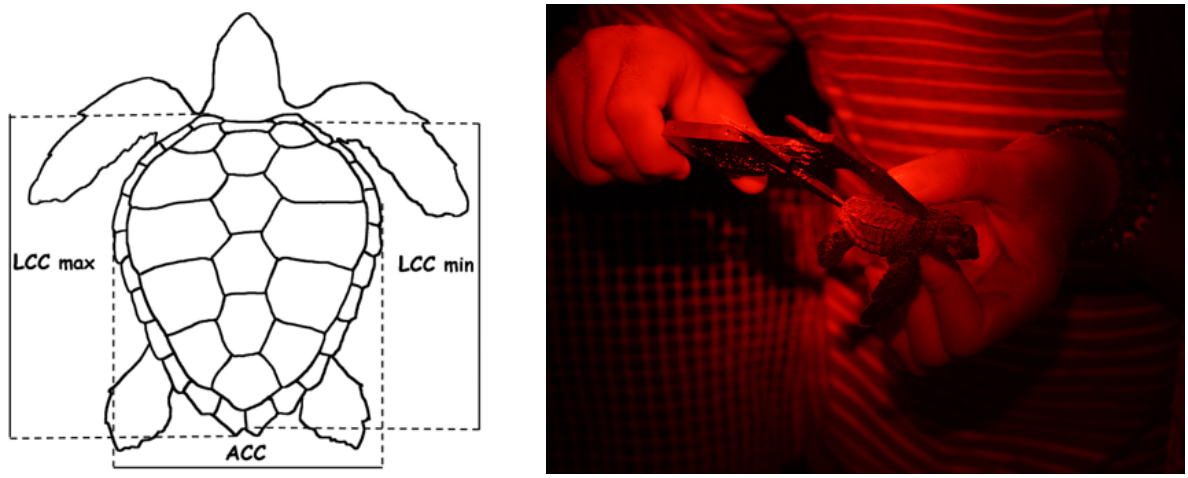


Fig 2.10. Left: sea turtle scheme representing the different measurements taken during data collection. Right: Loggerhead hatchling been measured with a caliper right before emerging in the hatchery, Ervatão.

On hatchlings, there were used the Minimum Straight Carapace Length (SCLmin), collected with calliper (Fig 2.10), and in juveniles and nesting females there were used the

Minimum Curved Carapace Length (CCLmin), collected using flexible tapes (Fig. 2.11). Other data were collected on hatchlings and juveniles, such as the weight, in grams and kilograms respectively.

Fig 2.11. Loggerhead nesting female measured by a researcher from the NGO Cabo Verde Natura 2000 during the night patrolling of Ervatão beach.

2.5.2. Blood extraction

In all the studies presented, blood samples were always collected following the same standardized protocol. Blood was collected from the dorsal cervical sinus with 1 ml and 0.5 ml syringes with a 29G/12.7 mm needle in hatchlings (Fig.2.12) and with a 5 ml or 10 ml syringe with a 21G/38 mm needle in juveniles and adult females (Fig.2.12). The blood was dispensed into tubes containing lithium heparin (2 ml or 5 ml depending on the volume extracted). Tubes were kept in a cooler with ice packs until centrifugation at 3000rpm for 5 min to obtain plasma. Blood plasma was extracted and disposed into 2ml eppendorf tubes and kept frozen (-30 °C).

Fig 2.12. Blood extraction of loggerhead hatchling (up), juvenile (left down) and nesting female (right down).

2.5.3. Corticosterone analysis

Frozen plasma samples from hatchlings and adult females were sent to the Department of Animal Health and Anatomy (Veterinarian Faculty) from the Universitat Autònoma de Barcelona (UAB) to analyze CORT levels. CORT concentration and all the validation tests were done using competitive EIA kits (Neogen® Corporation Europe, Ayr, UK). Each assay needs an exhaustive biochemical validation for the species and sample of interest (Buchanan and Goldsmith 2004). Therefore, assay validation was conducted following the criteria for an immunological validation: precision, specificity, accuracy and sensitivity (Reimers and Lamb, 1991) using extracts from several samples.

Intra-assay 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 pools 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 can distinguish and measure.

Frozen plasma samples from juveniles were sent to the Department of Cell Biology, Physiology and Immunology from the UAB to analyze CORT levels using double antibody in equilibrium Radioimmunoassay (RIA). In brief, corticosterone radioimmunoassay used [125I] corticosterone - carboxymethyloxime - tyrosine - methylester (ICN - Laboratorios Leti, Barcelona, Spain) and synthetic corticosterone (Sigma, Barcelona, Spain) as the standard and an antibody raised in rabbits against corticosterone - carboximethyloxime - bovine serum albumin. The range of the standard curve was between 6.25 and 1600 pg of corticosterone per tube. The antibody used has a cross-reactive 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 to avoid interassay variability (Scorrano *et al.* 2014).

2.6. Statistical analysis

All statistical analyses were conducted using R version 3.1.2 (R Department Core Team 2014). The main statistical test performed for this works were different Analysis of the Variance (ANOVA) together with Generalized Linear Models, as well as different correlations and descriptive statistics to obtain means and standard deviations. The particular specifications of statistical tests used are described in greater detail in each chapter.

Photo: Dr Ana Liria Loza

Effects of incubation temperature on loggerhead hatchling performance and phenotype

Published in Usategui-Martín et al (2019). Endangered Species Research 38:45–53. https://doi.org/10.3354/esr00935

3.1 Abstract

On natural conditions sea turtle eggs are subjected to a changing thermal environment but little is known about the effect of these temperature fluctuations during incubation on the performance and phenotype of hatchlings. The aim of this study was to determine how incubation temperature pattern (increasing or stable) and incubation temperature regime (low or high) affect incubation and hatching duration, hatching and emergence success, hatchling phenotype (carapace length and weight) and self-righting interval at hatching. Loggerhead sea turtle (Caretta caretta) clutches were collected at different beaches of Cape Verde archipelago and divided among incubators with different temperature regimens and patterns. Minimum straight carapace length (SCLmin) and weight (g) of all individuals were measured at hatching. In addition, the hatching duration and the time interval required for each hatchling to self-right were recorded. Results showed that incubation temperature regimes influenced all parameters studied more than the increasing temperature patterns. Low incubation temperatures, both increasing and stable, increased incubation time, produced bigger hatchlings with slower righting response compared to the higher temperatures. An optimal range of incubation temperatures was determined by assessing the most favorable values for hatchlings, although some differences in this optimal range between rookeries were found in the upper temperature range.

KEY WORDS: Loggerhead sea turtle · *Caretta caretta* · incubation temperature regime · incubation temperature pattern · hatchlings performance · hatchlings phenotype · north Atlantic.

3.2 Introduction

The northeastern Atlantic subpopulation of loggerhead sea turtles, *Caretta caretta* (Linnaeus 1758), is considered as endangered based on its small area of occupancy and continuing decline in habitat area (Casale & Marco 2015). Anthropogenic threats at sea (bycatch, marine litter, pollution) and habitat destruction on nesting beaches and in feeding areas have added new pressures to their high levels of natural predation on nesting beaches and at sea in their early life stages (Heithaus 2013).

Hatchling phenotype and performance are influenced by a combination of maternal phenotype and fitness during egg formation (Hewavisenthi & Parmenter 2001, Andrews 2004, Glen *et al.* 2003). Furthermore, several environmental factors during incubation period, such as hydric properties of the substrate (Reece *et al.* 2002) and nest temperature (Booth *et al.* 2004) also contribute to hatchling performance, growth rate and size, and therefore the amount of residual yolk (Booth 2006, Booth *et al.* 2004, Burgess *et al.* 2006, Reece *et al.* 2002). Sea turtles exhibit temperature-dependent sex determination (TSD) (Mrosovsky 1994, Mrosovsky *et al.* 2002, Wibbels 2003). For the loggerhead turtle, equal amounts of males/females are produced around 29°C, known as the pivotal temperature (PT). Different proportions of both sexes are produced at 2 - 3°C around the PT, known as the transitional range of temperature (TRT) (Mrosovsky 1994). Incubation temperature outside the TRT results in 100% males at lower temperatures, and 100% females at higher temperatures (Godfrey & Mrosovsky 1997, Mrosovsky 1994). Higher temperatures are also known to accelerate embryonic development, decrease the incubation period and reduce the amount of yolk transformed into tissues (Booth & Astill 2001). In general eggs that incubated at temperatures lower than 23 °C greater than 33 °C for extended periods do not hatch (Miller 1997), with an optimal incubation temperature range between 31.5-32°C at which embryonic growth is maximum (Monsinjon *et al.* 2017). Consequently, it has been suggested that female hatchlings have shorter incubation times (Stokes *et al.* 2006), higher residual yolk (Booth *et al*. 2004, Burgess *et al*. 2006) and, at some extent, are smaller than males (Booth 2006, Booth & Astill 2001, Reece *et al.* 2002). While temperature is the main factor affecting embryonic development, moisture is also important in the way that it modulate the temperature of the nest environment, therefore, an increase in substrate moisture

will increase the incubation time and the length and weight of the hatchlings (Sifuentes-Romero *et al.* 2018).

Extreme low and high incubation temperatures decrease hatching success (Booth 2017, Fisher *et al.* 2014) and increase self-righting times (Fisher *et al.* 2014, Read *et al.* 2013, Wood *et al.* 2014). In addition, the incubation temperature affects hatchling locomotor performance and post-hatching growth (Booth 2006). For example, a hatchling from an egg incubated at a lower temperature would have a poorer swimming performance (Booth 2006, Booth *et al.* 2004) compared to a hatchling coming from warmer nest. In addition, the cool incubated hatchling would have a slower stroke rate that can continue the stroking rate longer (Burges 2006) and exhibit a better crawling performance (Ischer *et al.* 2009).

Studies on sex determination and hatchling performance have been done under controlled conditions at stable temperatures (Booth *et al.* 2004, Fisher *et al.* 2014, Mrosovsky *et al.* 2009). However, constant incubation temperature is rare under natural conditions, which typically oscillate over the course of incubation (Packard & Packard 1988, Plummer *et al.* 1994, Shine *et al.* 1997).

The aim of this study was to determine how temperature pattern (i.e., increasing or stable) and temperature regime (low or high) during the incubation, affect the incubation and hatching duration, the hatching and emergence success, the hatchling phenotype (i.e., carapace length and total mass) and the self-righting time in hatchlings of the loggerhead sea turtle.

3.3 Material and Methods

Eggs used in the experiment were collected from beaches from the "Reserva natural das Tartarugas", southeast Boa Vista Island (Cape Verde) and translocated to the laboratories of ECOAQUA Institute of Las Palmas de Gran Canaria University, Canary Islands (Spain).

Forty-eight eggs were collected from each of 4 different clutches on September, 2th 2008 and the same amount on August, 3rd 2009 by staff and volunteers from Cabo Verde Natura 2000 NGO, directly from the cloaca, avoiding any contact with the sand

to prevent the introduction of any kind of pathogen (i.e. bacteria, fungi, etc.) in the translocation location (Canary Island). Eggs not used in this study were incubated in the hatchery of NGO Cabo Verde Natura 2000 close to the nesting area. All eggs were placed into the same isothermal plastic container (24x35x19 cm) to maintain the temperature stable and avoid important temperature fluctuations during their transport, which could induce embryos mortality during the first hours after laying. Containers were filled with vermiculite at -150 KPa hydric potential, maintaining oviposition order arranged in columns and rows. Different nests were separated by plastic grids. The containers were transported by a 4x4 vehicle to Boa Vista airport and then taken to Gran Canaria Island (Spain) by plane. Finally, the containers were transported by car to ECOAQUA laboratories. The entire process required less than 24h and followed long distance nest translocation protocols established by Abella *et al.* (2007) and López-Jurado (personal communication) under the CITES permits (ES-DE-00008/08I; ES-DE-00005/09I).

Each group of 48 eggs was divided in six plastic containers (3L) filled with vermiculite (at -150 KPa hydric potential) such that 8 eggs from each group were placed in each container. Three containers from each group were placed in Medilow® incubators at different temperature patterns and regimes, with a total of 96 eggs per treatment. In the first year, incubators were set at stable (S) temperatures patterns: incubator one at 27.0 ± 0.5°C (stable low, SL) and incubator two at 31.0 ± 0.5°C (stable high, SH). The second year, incubators were set to gradually increase (I) 0.5°C every two weeks: incubator three increased from 26.5 to 28.5°C (increase low, IL) and incubator four increased from 30.5 to 32.0°C (increase high, IH). Twenty-four hours after the containers were placed into the incubators, viable eggs were identified by the development of a white area on the uppermost surface of each egg (Miller 1885). One group was excluded from the study because it did not show any sign of development.

The incubation time was defined as the period between the oviposition and 3 days after hatchlings were completely out of the egg (Godfrey & Mrosovsky 1997). Hatching duration was defined as the time between the pipping and when the hatchling was completely out the eggshell (Gutzke *et al.* 1984). Hatching success was the number of neonates that got completely out of their eggshell. Emergence success was the number of neonates that survived 3 days after hatching. To determine incubation and hatching duration, incubators were checked daily at 9:00, 12:00,15:30, 19:00 and 23:00 hrs.,

incubators revisions were done according to the standardized animal welfare protocols of the facilities, so the access to facilities were restricted at night. Hatching time was calculated in hours and then transformed into decimal days.

The minimum straight carapace length (SCLmin) was measured after emergence from the anterior point at midline (nuchal scute) to the posterior notch at midline between the supracaudals using calipers to the nearest 0.1 cm (Bolten 1999), and the weight was recorded using a precision balance (MOBBA bs3000a) to 0.1g.

Self-righting time was determined after emergence by placing the hatchling in supine position and recording the interval to right itself. The test was repeated three times per hatchling. Maximum time allowed was 60s, after which the hatchling was manually turned over. Hatchlings from the first year were used to compare self-righting times between high and low incubation temperature conditions; hatchlings from high temperatures were used to compare self-righting times between stable and increasing incubation patterns.

All hatchlings used in this study were released after a head-start program as part of the project *"Enlargement of the reproductive habitat of the loggerhead sea turtle in the Macaronesia region"*.

All statistical analyses were conducted using R v. 3.1.2 (R development Core Team 2014). Incubation time violated the assumption of homoscedasticity, so it was analyzed using a Welch's ANOVA with temperature pattern/regime as a factor and a Game-Howell post-hoc test. Hatching duration was analyzed using ANOVA test with temperature pattern/regime as a factor followed by Tukey's post hoc test. The percentages of hatching success were converted to binary data and analyzed using a logistic regression test, with temperature pattern/regime as a factor. Gamma GLM was fitted to analyze the effect of the temperature pattern/regime over hatchling size (SCLmin) and the weight, because of lack variance homoscedasticity of both variables. One-way ANOVA was used to analyze self-righting time with temperature regime and temperature pattern as separate factors. Critical p value was 0.05.

3.4 Results

3.4.1. Incubation and hatching duration

Incubation time was significantly affected by incubation temperature, both pattern and regime ($F = 9714$, $p < 0.05$). Game-Howell test determined that eggs incubated at high temperature presented shorter incubation times ($p < 0.05$) than eggs incubated at the lower temperature. Eggs incubated at stable temperature had shorter incubation times than eggs incubated at increasing temperatures ($p < 0.05$), within both temperature regimes (SH $\overline{X}$= 51.1 ± 0.22 days, IH $\overline{X}$= 51.7 ±0.44 days, SL $\overline{X}$= 67.5 ±1.18 days and IL $\overline{X}$= 71.2 ±1.30 days), (*Fig* 3.1).

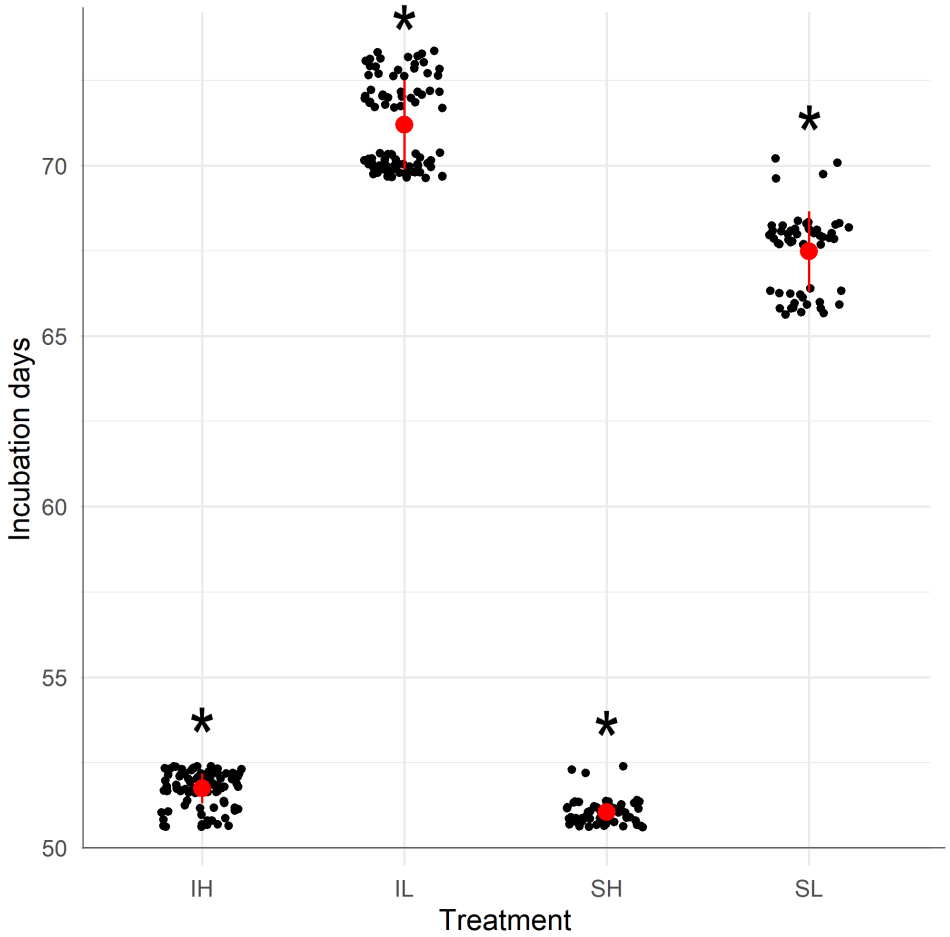


Fig 3.1. Incubation times distribution according to the incubation thermal treatments. Big red dots and lines represent the mean and the standard deviation respectively. Significance is marked with an asterisk (*).

Hatching duration was significantly modified by both the pattern and regime of the incubation temperature ($F = 12.36$, $p < 0.05$). Tukey's HSD test determined that hatchlings incubated at low temperatures spent significantly ($p < 0.05$) less time in the hatching process than the ones incubated at high temperatures, independent of the incubation pattern (SL $\overline{X}$= 1.2 ±0.52 days, IL $\overline{X}$= 1.0 ±0.61 days, SH $\overline{X}$= 1.5 ±0.57 days and IH $\overline{X}$= 1.3 ±0.52 days), (*Fig* 3.2).

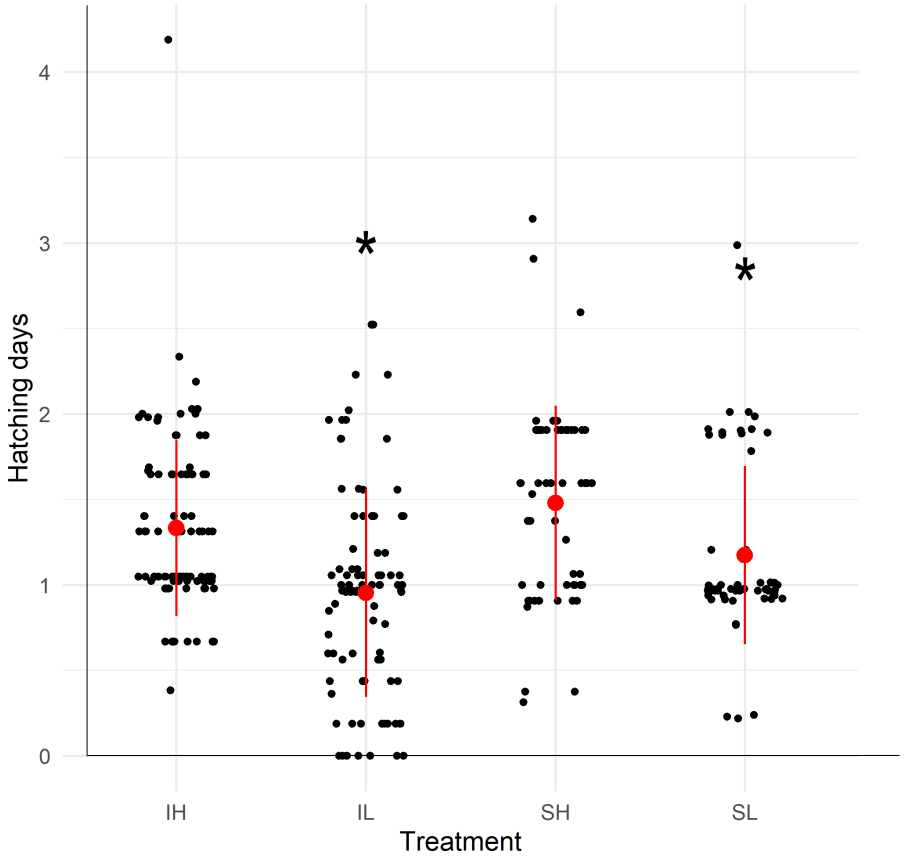


Fig 3.2. Hatching duration divided according to the four incubation treatments. Big red dots and lines represent the mean and the standard deviation respectively. Significance is mark with an asterisk (*).

3.4.2 Hatching and Emerging success

Hatching and emergence success were almost the same because only 2 hatchlings out of 336 died during emergence.

Hatching success was not affected by either the high regime or low regime temperature ($Z = -1.010$, $p > 0.05$) (90.40% *vs* 84.52%, respectively), but it was significantly improved by the increasing temperature pattern ($Z = 2.903$, $p > 0.05$) (INC *vs* STB pattern: 94.27% *5vs* 78.47%, respectively). When comparing the four experimental protocols, hatching success was higher for the IH treatment ($Z = 3.61$, $p < 0.05$) and for IL ($Z = 2.83$, $p < 0.05$), 96.88% and 91.66% respectively, compared to the other treatments.

3.4.3. Hatchling phenotype

Increasing temperatures produced significantly larger hatchlings than stable temperatures ($t = -4.13$, $p < 0.05$) (45.1 ± 1.0 mm and 44.2 ± 2.42 mm, respectively). Low temperature incubation resulted in larger individuals than high temperature incubation ($t = -3.65$, $p < 0,05$) (45.1 ± 1.60 mm and 44.4 ± 1.78 mm), (*Fig* 3.3).

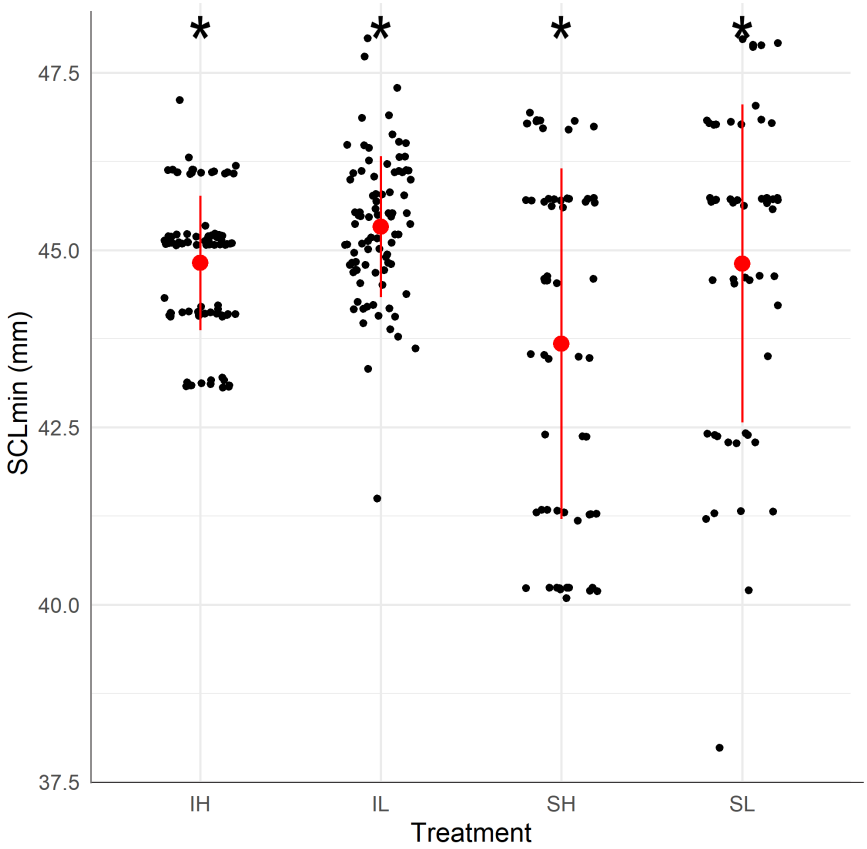


Fig 3.3. SCLmin distribution according to the four incubation thermal treatments. Big red dots and lines represent the mean and the standard deviation respectively. Significance is mark with an asterisk (*).

Same effect was found on hatchling weight, where increasing temperatures resulted in an increase in hatchlings weight ($t = -4.93$, $p < 0.05$) compared to hatchlings from eggs incubated at stable temperatures (19.4 ± 1.52g and 17.0 ± 2.92g, respectively). In addition, low temperatures produced heavier hatchlings than high temperatures ($t = -2.41$, $p < 0.05$) (18.9 ± 2.27g and 17.7 ± 2.23g, respectively), (*Fig* 3.4)

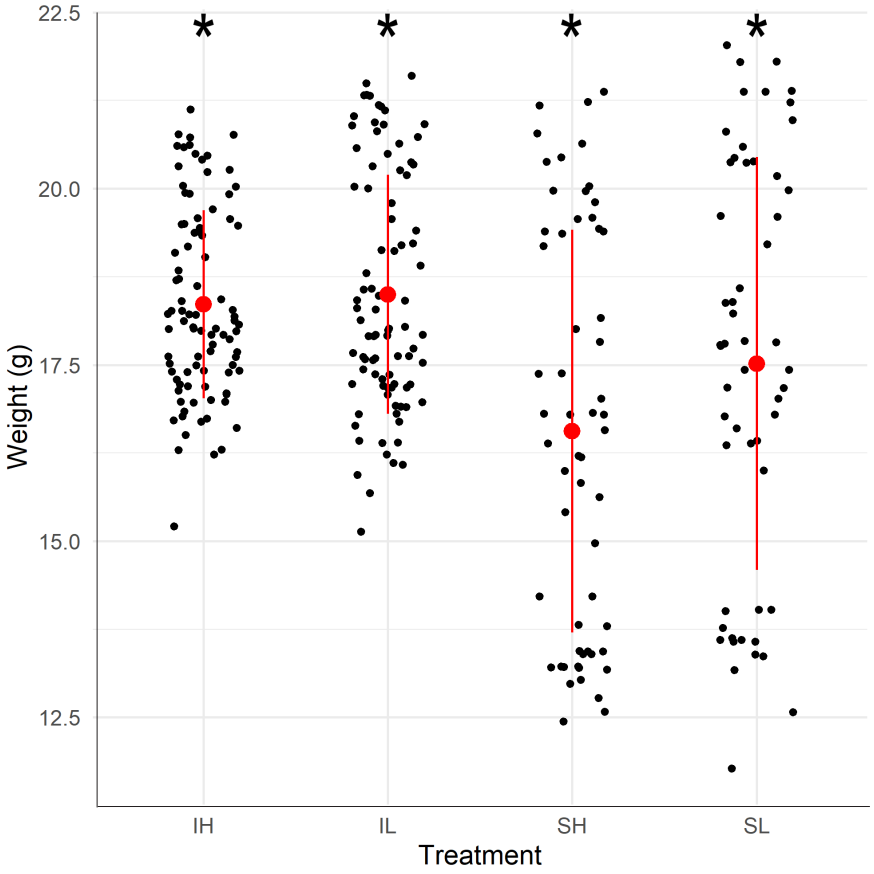


Fig 3.4. Weight distribution according to the four incubation thermal treatments. Big red dots and lines represent the mean and the standard deviation respectively. Significance is mark with an asterisk (*).

3.4.4. Self-righting response

The self-righting response of hatchlings was affected by temperature regime ($F = 104.9$, $p < 0.05$). Hatchlings incubated at low temperatures took longer times to turn over than

hatchlings incubated at high temperatures (37.5 ± 21.32s and 2.7 ± 1.72s, respectively), (*Fig* 3.5). Incubation temperature pattern had no effect on the interval of the self-righting response (F = 35, p > 0.05).

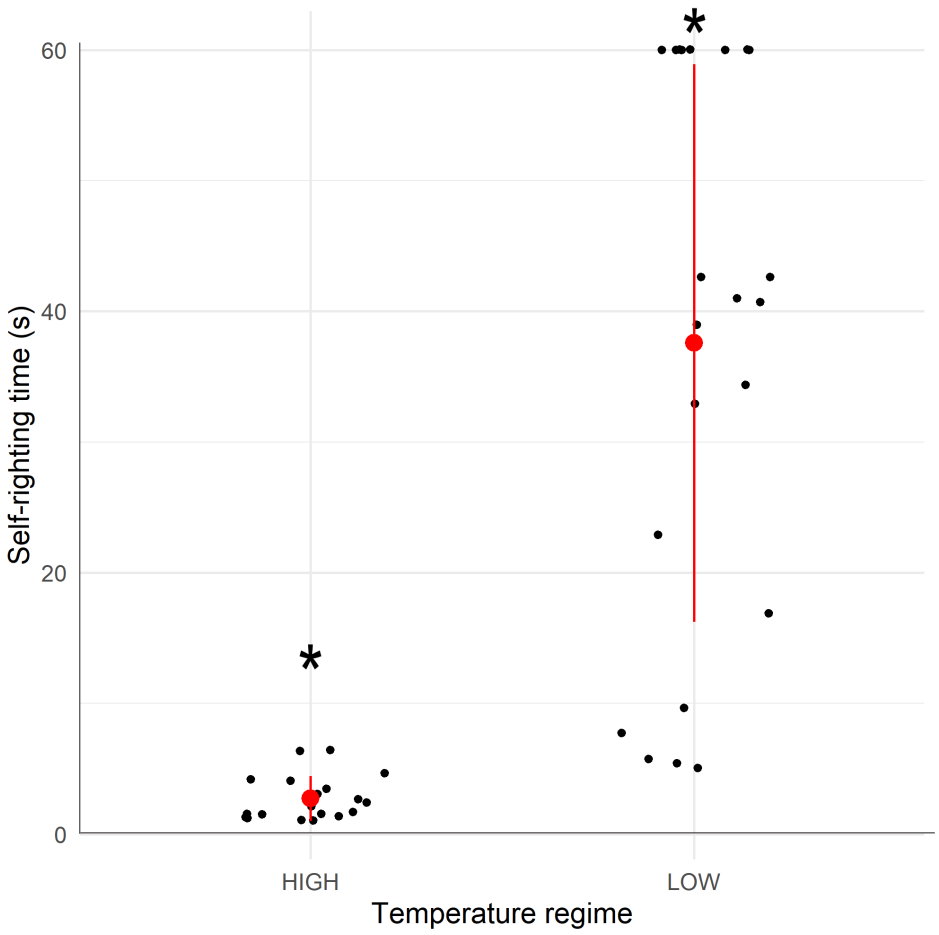


Fig 3.5. Self-righting time according to the both incubation thermal regimes. Big red dots and lines represent the mean and the standard deviation respectively. Significance is mark with an asterisk (*).

3.5. Discussion

Eggs of sea turtles have been incubated under controlled conditions in conservation programs (Plotkin 2007) and scientific studies, such as sex ratio (Booth *et al*. 2004,

Mrosovsky *et al.* 2009), hatchling fitness (Fisher *et al.* 2014) and embryonic development studies (Miller 1985). However, incubation temperature in wild nests typically oscillates over the course of incubation (Packard & Packard 1988, Plummer *et al*. 1994, Shine *et al*. 1997). Fisher *et al*. (2014) and Booth (2017) reported lower hatching success of loggerhead turtle eggs incubated at extreme low (50-60% at 27°C) and high incubation temperatures (less than 50% at 31°C), whereas maximum hatching success (69.2%), occurred at 29°C. Results obtained in the present study differ from Fisher *et al*. (2014), because greater hatching success (81.94%) occurred at 31°C than at 27°C (75%). This apparent discrepancy could result from latitudinal differences between the loggerhead population used by Fisher *et al*. (2014) and the population of the present study. Fisher *et al*. (2014) used eggs from the North Carolina rookery, which is the northern most breeding colony for loggerhead turtle in the north Atlantic (Bowen & Karl 2007), whereas in the present study we obtained eggs from Cape Verde colony which has a climate ranging from tropical dry to semi-desert (Duarte & Romeiras 2009). Laloë *et al*. (2017) studied the same Cape Verdean population, but under natural condition. The authors reported a higher emergence success at higher mean incubation temperatures (82.6% at 28.5°C and 81.6% at 32.2°C), which support our theory that the latitudinal plus genetic differences between Florida and Cape Verde populations (Shamblin *et al*. 2014) could provide the latter a higher or shifted thermal tolerance.

Translocation and handling issues can affect hatching success (Limpus *et al*. 1979), if not done carefully. Values obtained by Fisher *et al*. (2014) were lower (less than 70%) than the ones obtained in the present study (>75%), and translocation distance in their case was shorter. However, hatching success values above 75% has been reported by several authors (Marcovaldi & Laurent 1996, Mrosovsky *et al*. 2002, Wood **v** 2014), using similar long-distance egg translocation and hatchling handling protocol as used in the present study.

Significant differences in hatching success were found in relation to temperature pattern, where increasing temperatures produce almost 16% more hatchlings than stable temperatures in the present study. Strong maternal effects on eggs quality have been described by Booth *et al*. (2012). In the present study eggs incubated at different temperature patterns were collected in two different years (different females), therefore the maternal origin cannot be completely discarded. Further investigations are needed to

elucidate whether incubation temperature pattern or maternal origin (or both) produces the differences in hatching success.

Incubation temperature has an inverse relation with the incubation time (Booth 2017, Stokes *et al*. 2006, Booth & Astil 2001) but only within a certain range of temperature because very high incubation temperatures produced longer incubations (Monsinjon *et al*.2017). However, the effect of temperature pattern on incubation time under increasing temperature conditions has not been studied sufficiently. With the results obtained in the present study, we cannot affirm any conclusive hypothesis based on these results, and thus, we considered that suggestions on this topic could be speculative.

In our study, hatching duration, was longer for those animals incubated at high temperatures (with subsequent shorter incubation period). Ewert (1979, 1985) considered hatching duration, including the internalization of the residual yolk and the unfolding of the carapace and the plastron, to be controlled by the metabolic rate of hatchlings (i.e., affected by temperature). Ligon *et al*. (2009) reported that hatchlings incubated at higher temperatures had higher metabolic rates, thus suggested that hatchlings with faster metabolic rates would complete the hatching process sooner than hatchlings with slower metabolic rates. However, results from the present study found the opposite and this apparent discrepancy could be due to the size of the neonates. Larger hatchlings could go out of the egg faster breaking all the eggshell, while the small ones presented stages, first the pipping, followed by the head, then one flipper and at the end the other flipper and the rest of the body. Eggs incubated at lower temperatures (and hence longer incubation periods) produce hatchlings that are larger than those produced at higher temperatures (Booth 2006, Booth & Astill 2001, Reece *et al*. 2002) because embryos are able to transform more yolk into tissues (Booth *et al*. 2012, Read *et al*. 2013), this is also affected by nest moisture (Sifuentes-Romero *et al*. 2018), but it was not taken into account in the present study because the moisture levels were the same in all the eggs.

Metabolic heat produced by the embryos during development (Packard & Packard 1988, Plummer *et al*. 1994, Shine *et al*. 1997) reaches its highest values toward the end of the incubation period. The growth rate of sea turtle embryos follows a similar pattern, being slow at the beginning and increasing exponentially during the incubation period (Ackerman 1981, Booth & Astill 2001). In the present study, hatchlings produced at stable temperatures were smaller (44.2mm SCLmin) than the ones produced at increasing

temperatures (45.1mm SCLmin), probably because the increasing temperature may have influenced the exponential growth phase at the end of the incubation (Ackerman 1981, Booth & Astill, 2001). While incubation temperature has a strong effect on early stages of embryonic development, moisture conditions have a higher impact on later stages (Sifuentes-Romero *et al*. 2018). In our study, we maintained the same moisture levels in all the treatments, so the only factor affecting embryonic development was temperature, besides genetic or maternal effect (Andrews 2004, Glen *et al*. 2003). Hatchling size has been widely reported to be affected by incubation temperature, but few studies deal with the relationship between weight and temperature (Booth 2017, Fisher *et al*. 2014, Horne *et al*. 2014, Read *et al*. 2013). Hatchlings incubated at low temperatures have more body mass and less residual yolk, while the ones incubated at high temperatures present less body mass and more residual yolk (Booth 2017). Consistent with this, in the present study eggs incubated at low temperatures produced heavier hatchlings (18.93 ± 2.28g) than the ones incubated at high temperatures (17.67 ± 2.23g).

Although self-righting time is not an exact fitness estimator for sea turtles, the ability of a hatchling to turn itself over is essential to survival during their race to the sea after emerging from the nest (Fisher *et al*. 2014) and has been used several times as a proxy of hatchling fitness (Booth *et al*.. 2012, Read *et al*. 2013, Wood *et al*. 2014). Self-righting times increase at extreme low and high temperatures in both sea and freshwater turtles (Fisher *et al*. 2014, Read *et al*. 2013, Wood *et al*. 2014). Booth (2017) suggested an optimal range of incubation temperatures of 28°C to 32°C for shorter self-righting times. In the present study, the mean self-righting time of hatchlings incubated at 27°C was 37.5s while Fisher *et al*. (2014) got a mean self-righting time at that same temperature around 30s. However, at 31C hatchings took an average of 2.72s to turn themselves, which is faster than the values reported previously (approximately 60s, Fisher *et al*. 2014). The slight difference at low temperatures and the strong difference at high temperatures may result from the genetic differences between populations. The optimal temperature range for hatchlings from Cape Verde seems to be shifted to higher temperatures. More studies are needed to find the optimal range for each colony, rookery or location to be able to develop more efficient hatchery programs. Incubation temperature patterns did not induce any effect on this useful ability. This result is the same as described in smooth soft-shell turtles (*Apalone mutica*) (Ashmore & Janzen 2003).

In summary, low incubation temperatures produced larger hatchlings and extend the incubation time because according to previous studies, more yolk is transformed into tissues. Smaller hatchlings are produced at stable temperatures because stable pattern would not stimulate the embryonic growth during the exponential growth at later stages. Incubation temperature regimes induced more influence in hatchlings than temperature pattern, indicating that incubation temperature pattern of natural nests on laboratory studies has to be imitated, because even the temperature regime is the main hatchlings performance modulator, the temperature pattern also has an influence and its influence over hatching success is very important.

4

Photo: Dr Ana Liria Loza

Husbandry protocols for loggerhead hatchlings based on corticosterone response to temperature, handling frequency and stocking density

This manuscript has been submitted to Endangered Species Research.

4.1 Abstract

The head-starting programs are important conservation techniques for marine turtles to enhance recruitment of sea turtles in natural population. Within those programs, hatchlings must be collected from the wild and kept under controlled conditions for determined periods, ranging from few days to some months or even few years, before being released into the wild. During this rearing period, hatchlings need to be submitted to protocols such as handling for cleaning operations and health management or need to be disposed in different stocking densities depending on the housing tanks and available facilities. Standardization of handling and housing protocols are necessary to define the most adequate culture conditions to keep the best welfare of the hatchlings. The aim of this study is to define some parameters for head-starting protocols on loggerhead hatchling sea turtles (*Caretta caretta*), based on variations of circulating corticosterone concentration in blood under different stocking densities, temperatures and the culture-related handling processes, since corticosterone concentration in blood is a good indicator of welfare. To achieve it, two different trials were performed to analyze three specific parameters: i. Standardized handling; ii. Temperature housing conditions; iii. Stoking density. In both experiments corticosterone concentration in blood were analyzed and correlated with animal weight and carapace length increase. The corticosterone analyses demonstrated that loggerhead hatchlings held in controlled condition under handling standardized protocols applied regularly (once per week) are in better welfare conditions, ambient temperature must be maintained within the natural range of 26 ° to 28 °C.I Isolation of individual hatchling during the first 6 month of life must be refused, as this holding situation can induce a chronic elevation of plasma corticosterone.

4.2 Introduction

All species of sea turtle are considered endangered (Seminoff 2004, Abreu-Grobois and Plotkin 2008; Mortimer and Donnelly, 2008; Wallace *et al*. 2013; Casale and Tucker, 2017; Wibbels & Bevan 2019) due to the important decrease observed in their populations, mainly caused by anthropogenic threats at sea (bycatch, marine litter, pollution) and habitat alteration or destruction on nesting beaches. All those threats are increasing the pressure to the already high levels of natural predation on nesting beaches and at sea in their early life stages (Heithaus 2013). Therefore, conservation programs are very relevant, and the welfare of sea turtles should be considered.

The head-starting programs are important conservation techniques for marine turtles to enhance recruitment of sea turtles into the population (Bell *et al*. 2005). In these programs, hatchlings are collected from the wild (Heppell *et al*. 1996) and reared under controlled conditions during determined periods, ranging from a few days to some months or even years, before they are released into the wild (Shaver & Wibbels 2007). One-year period is considered the most appropriate, because they are larger enough to avoid most of predators associated with hatchlings and post hatchling stage (Caillouet Jr *et al*. 1997, Shaver & Wibbels 2007). Head-starting programs requires standardized protocols, where several factors must be considered, depending on the species characteristics and available facilities.

Animal welfare is an important factor to considered when holding sea turtle hatchlings under controlled conditions, including factors such as housing parameters, cleaning protocols, feeding strategies, among others. Welfare can be assessed through hatchling behavior, levels of physiological indicators (i.e. stress indicators) or other indirect parameters as the absence of illness or the appetite (Arena & Warwick 1995).

The homeostatic recovery of animals after changes in the environment or certain stressful culture-related situations is a key point to ensure animal welfare (Conte 2004). Traditionally, circulating corticosterone (CORT) has been used as an indicator of homeostasis (Gregory *et al*. 1996, Milton & Lutz 2003, Tokarz & Summers 2011), as this is the main glucocorticoid produced by reptiles to face stressful conditions (Carbajal *et al*. 2018, Cockrem 2013). Changes of 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, such as nesting density in beaches, season period or shark-attacks (Flower *et al.* 2018, Jessop *et al.* 2004, Jessop *et al.* 1999, Jessop 2001, Jessop & Hamann 2005, Rostal *et al.* 2001, Valverde *et al.* 1999, Whittier *et al.* 1997). Also, variations of CORT in wild juveniles have been related to handling or recovery/illness processes (Aguirre *et al.* 1995, Gregory *et al.* 1996, Hunt *et al.* 2012, Jessop *et al.* 2004, Jessop & Hamann 2005), and with the dispersal behavior in wild hatchlings (Hamann *et al.* 2007).

However, little is known about the variation of this hormone during the adaptation processes to culture conditions, a key point for head-starting programs. Most of established parameters to rear sea turtle hatchlings under controlled conditions are based on a health and behavioral point of view, measuring the survival rate or the prevalence of diseases or injures under certain temperatures and stocking densities (Fish and Wildlife Service 2013).

The aim of this study is the definition of specific parameters to improve protocols to keep loggerhead hatchlings (*Caretta caretta*) under controlled conditions, based on the CORT variations under different rearing conditions, such as stocking density, seawater temperature or culture-related handling processes. To achieve this goal, two different trials were performed. In the first trial, the effect of handling and temperature housing conditions were tested, and in the second one, the effect of rearing density was studied. In both experiments, the response to different variables were measured by CORT concentration in blood and correlated with weight gain and body size increase.

4.3 Material and methods

Loggerhead hatchings came from eggs collected from low productivity nesting beaches (*Ervatão* and *Ponta Cosme* beaches) located in the '*Reserva Natural das Tartarugas*', southeastern Boa Vista Island, Cape Verde. Whole nests were collected during the oviposition, placed into plastic bags (one nest per bag) and translocated to a controlled incubation area (*hatchery*), where they were incubated safely from tides and predation. Egg collection and translocation were carried out by the staff and trained volunteers from

the NGO Cabo Verde Natura 2000. The experiments were conducted under the License nº2/2015 from the *Direção Nacional do Ambiente (Cape Verde Government).*

4.3.1. Trial I: Handling protocols and housing temperature

In the first trial, 72 hatchlings were chosen from three different nests (24 from each nest), hatched between 31 October 2013 and 4 November 2013. Two hatchlings from each nest were placed on each of the 12 rectangular 100L tanks. The experimental tanks were located at the Cabo Verde Natura 2000 facilities, in Sal-Rei, Boa Vista Island (Cape Verde). Individual filters were disposed in each tank that were fully cleaned by hand and with a total water renovation each 10-15 days. Hatchlings were feed from Monday to Saturday with a combination of fresh fish, shrimp and squid. Food amount per hatchling was adjusted to the 5% of their weight per feeding day, based on feeding protocols established by Bluvias & Eckert (2010). All hatchlings ate normally during all the study period.

Four different treatments were tested with three replications (3 tanks per treatment). The first treatment (LowAmb) consisted on low frequent handling of the hatchlings (once every 2 weeks) and ambient seawater temperature (26°C). The second treatment (LowUp) consisted also on low frequent handling (once every 2 weeks) and seawater temperature increased in 2°C (28°C). The third treatment (HighAmb) was a high frequent handling (once per week) and with ambient seawater temperature (26°C) and the last treatment (HighUp) consisted on high frequent handling (once per week) and seawater temperature increased in 2°C (28°C). Handling protocols were standardized protocols performed the same day of the week, at the same time and in the same order, to obtain reliable biometrical parameters. Handling consisted on taking the animals out of the water gently, measured the carapace and weight them and replace them into the water. All the process took no more than 2 minutes. During handling days, also blood extraction was conducted, and hatchlings were cleaned from algae, rubbing them softly with a wet rag. Blood extractions were done along the whole experimental period (0.3ml per sample), at week 2 (w2), week 10 (w10), week 18 (w18) and week 26 (w26).

The biometrical parameters analyzed were the whole animal weight (W) and the minimum straight carapace length (SCLmin). A caliper to the nearest 0.1 cm (Bolten 1999) was used. Weight increase (WI) and carapace length increase (LI) were the increase of weight

and length for each hatchling during all the trials period, respectively. Mortality was also recorded during all the experimental period.

4.3.2. Trial II. Effect of stocking density

In the second trial, 57 loggerhead hatchlings were chosen from three different nests (19 from each nest) hatched on October 27th and 28th 2014, following the collection protocol described in the first experiment (see trial I). Tanks and feeding protocol were the same used in the first experiment (100 L tanks and 5% food amount), but the distribution of the hatchlings was stablished to evaluate the effect of rearing density. Seawater temperature was kept the same for all tanks (around 26°C).

Four different treatments were tested, with three replications for each treatment. The first treatment (D1) consisted on one hatchling per tank), where each hatchling came from a different nest. The second (D3) consisted on three hatchlings per tank (30 hatchlings/ m^3), one hatchling from each nest. In the third experiment (D6) there were six animals per tank (60 hatchlings/ m^3), two hatchlings from each nest. Finally, the fourth treatment (D9) consisted on nine hatchlings per tank (90 hatchlings/ m^3), three hatchlings from each nest.

All hatchlings followed the same handling protocol defined from the results obtained in the previous trial. Growth parameters were obtained following same procedures explained on trial I. Hatchlings were weighted once per week (every Friday) and SCLmin was measured once each four weeks.

Several blood samples from each hatchling were taken along the experiment, starting just after hatching (w0) and then at weeks 7 (w7), 15 (w15) and 23 (w23).

4.3.3. Blood collection and sample preparation

All blood samples were collected from the dorsal cervical sinus using 1 ml and 0.5 ml syringes with a 29G/12.7 mm needle and dispensed into 2ml lithium heparin tubes. Samples were kept refrigerated until centrifugation at 3000rpm for 5 min to obtain plasma. Blood plasma was pipetted into 2ml Eppendorf® tubes and kept frozen at -30 °C.

4.3.4. Analysis of circulating corticosterone in serum

Frozen plasma samples were sent to the Department of Animal Health and Anatomy (Veterinarian Faculty) from the Universitat Autònoma de Barcelona to analyze CORT

levels. CORT concentration and all the validation tests were done using competitive EIA kits (Neogen® Corporation Europe, Ayr, UK). Each assay needs an exhaustive biochemical validation for the species and sample of interest (Buchanan and Goldsmith 2004). Therefore, assay validation was conducted following the criteria for an immunological validation: precision, specificity, accuracy and sensitivity (Reimers and Lamb, 1991) using extracts from several samples.

Intra-assay 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 pools 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 can distinguish and measure.

4.3.5. Statistical analysis

All statistical analyses were conducted using R version 3.1.2 (R Department Core Team 2014). In both experiments, the effect of the different treatments over CORT concentration was analyzed using a mixed generalized lineal model (GLMM), with sampling time as random factor to compare the mean value per treatment on each sampling time. In both experiments, mortality in relation with treatments and time was analyzed using a binomial generalized lineal model with a lobby join function, where hatchlings were considered dead (0) or alive (1), in each treatment and time. Finally, Pearson's correlation was done between CORT levels and weight, weight increase and length increase, and also a one-way ANOVA was conducted to analyze any possible effect of the different treatments overweight and length increase. Results were considered significant at $p < 0.05$.

4.4 Results

4.4.1 Weigh and length increase

During the six months of the first trial, neither the temperature increment of 2°C nor the standardized handling protocols affected the WI and SCLmin increase of loggerhead hatchlings ($F = 0.02$, $p > 0.05$; $F = 0.52$, $p > 0.05$, respectively). The mean carapace LI

during all the trial ranged from 79.3 ± 9.0 mm to 85.6 ± 14.5 mm (mean ± SD) and the mean WI ranged from 285.1 ± 51.0 g to 296.2 ± 71.1 g (mean ± SD), (Table 4.1).

Table 4.1. CORT concentration in ng/ml, length increase (LI) in millimeters and weight increase (WI) in grams (mean ± SD) of the hatchlings from Trial I, according to the standardized handling protocols and housing temperature treatments.

Treatment	CORT	Lenght Increase (LI)	Weight Increase (WI)
High Up	5.93 ± 2.50	85.6 ± 14.5	293,0 ± 118.5
HighAmb	5.17 ± 3.76	79.3 ± 9.0	291.0 ± 54.4
LowUp	9.35 ± 4,17	82.5 ± 9.6	296.2 ± 71.1
LowAmb	8,87 ± 4.70	80,5 ± 7.9	285.1 ± 51.0

In the second trial, stocking density had no significant effect on the WI ($F = 1.90$, $p > 0.05$) neither on the SCLmin increase ($F = 2.45$, $p > 0.05$) of loggerhead hatchlings. The mean carapace LI ranged from 40.1 ± 5.4 mm to 50.2± 1.3 mm (mean ± SD) for turtles from D3 and D1 treatments respectively and the mean WI ranged from 86.5± 23.3 g to 117.1 ± 7.8 g (mean ± SD) for turtles from D6 and D1 treatments respectively (Table 4.2).

Table 4.2. CORT concentration in ng/ml, length increase (LI) in millimeters and weight increase (WI) in grams (means ±SD) of the hatchlings from Trial II, according to different stocking densities.

Treatment	CORT	Lenght Increase (LI)	Weight Increase (WI)
D1	5.86 ± 2.14	50.2 ± 1.3	117.1 ± 7.8
D3	3.72 ± 1.12	40.1 ± 5.4	89.3 ± 16.7
D6	3.18 ± 1.35	40.5 ± 6.3	86.5 ± 23.3
D10	3.01 ± 1.38	41.9 ± 6.2	88.0 ± 21.0

4.4.2. Biochemical validation of the EIA

The intra-assay coefficient of validation for CORT was 10.31%. In the dilution test, obtained and expected CORT concentrations were significantly correlated (r = 0.99, p < 0.05) with a mean percentage error of 6.42%. In the spike-and-recovery test, hormone standard spiked with the pool presented a mean recovery percentage of 103.06 ± 7.43 (mean ± SD). The sensitivity of the assay was 0.034 ng CORT/ml serum. These results demonstrate that the EIA kit used is precise, specific, accurate and sensitive measuring CORT level in plasma of the loggerhead sea turtle.

4.4.3. Trial I: Handling protocols and housing temperature

The different handling procedures had a significant effect (t = 7.12, p < 0.05) over the CORT concentration in blood serum. Hatchlings kept under a low frequent handling presented significantly more circulating CORT concentration (LowAmb: t = 3.12, p > 0.05; LowUp: t = 3.14, p > 0.05) than the ones subjected to high frequent handling procedures (Table 4.1, Fig 4.1). No significant effects of housing temperature or sampling time were found. Even though there was no effect of the housing temperature, animals with warmer water (28°C) presented higher, but not significantly (p > 0.05), circulating CORT in blood than ones kept at lower temperatures (Fig 4.1).

4.4.4. Trial II. Effect of stocking density

The number of hatchlings per tank induced a significant effect (t = 6.47, p < 0.05) on the circulating CORT concentration. Hatchlings from treatment D1 presented higher circulating CORT concentration in blood (D1 = 5.86 ± 2.14 ng/ml) compared to hatchlings from the other treatments (D3, D6 and D9), (Table 4.2, Fig 4.2). No differences were found between the different sampling times (p > 0.05).

No correlation between CORT levels and growth parameters was found in any of the different trials. There was no correlation between CORT levels in blood and weight (trial I: rho = -0.10, p > 0.05; trial II: rho = 0.05, p > 0.05), WI (trial I: rho = -0.08, p > 0.05; trial II: rho = 0.00, p > 0.05) nor with the carapace LI (trial I: rho = 0.19, p > 0.05; trial II: rho = -0.12, p > 0.05). The mortality of the animals during both experiments was not related with none of the parameters studied.

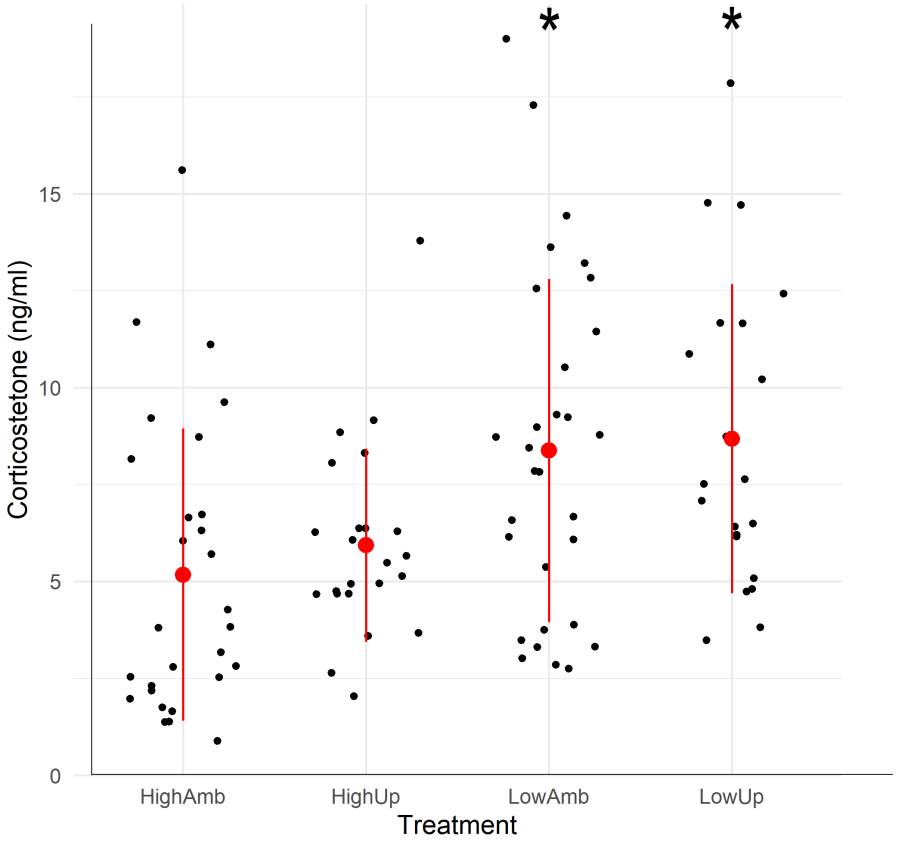


Fig 4.1. Circulating corticosterone concentration in serum according to the four handling treatments. Big red dots and lines represent the mean and the standard deviation respectively. Asterisk (*) denotes significant differences ($p < 0.05$) among handling protocols.

4.5 Discussion

When sea turtle hatchlings are reared under controlled conditions for short periods due to conservation or research purposes, or for longer periods on head-starting programs, different procedures, such as weighing, measuring or cleaning, need to be conducted (Hamann *et al.* 2007). Although different studies have determined how some of those situations can affect the behavior of the hatchlings, to our knowledge, none of them dealt on how controlled conditions, including handling procedures, could modify certain physiological parameters that could be used as welfare indicators at physiological level.

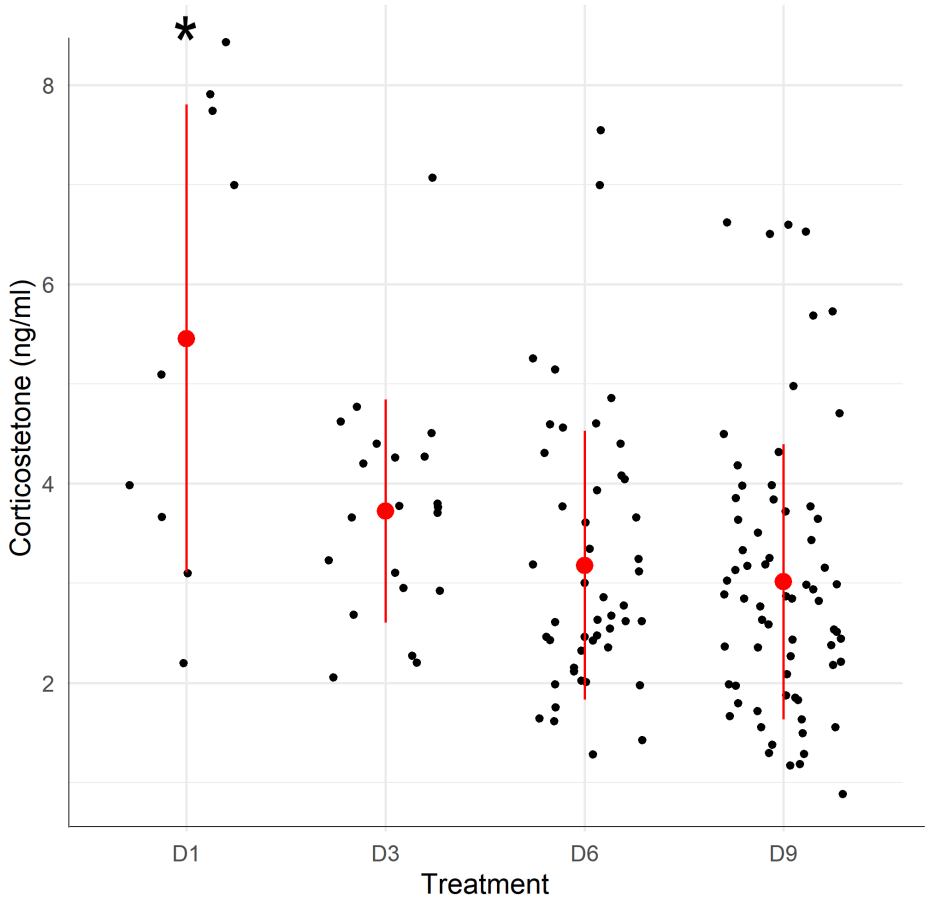


Fig 4.2. Circulating corticosterone concentration in serum according to the four density treatments. Asterisk (*) denotes significant differences ($p < 0.05$) among density treatments Big red dots and lines represent the mean and the standard deviation respectively.

Values of blood CORT concentrations obtained in the present study could be compared with values recorded for free-living loggerhead sea turtles obtained by Gregory *et al.* (1996) and also with other turtles such as red-eared slider turtle (*Trachemys scripta*) (Cash *et al.* 1997) and the Kemp's ridley sea turtle (*Lepidochelis kempii)* (Hunt *et al.* 2016).

In relation with handling protocols, the results of this study showed that high frequent handling protocols are beneficial for the hatchlings in short periods (at least 6 months) at any of the temperature assayed (26°C and 28°C). Animals with low frequent manipulation presented higher basal CORT concentration in blood, when compared to those animals subjected to high frequent manipulation protocols. Similar results have been found in

other reptiles, such as on rattle snakes *(Crotalus oreganus oreganus)*, where Holding *et al*. (2014) did not find any effect of repeated handling on baseline CORT concentrations. However, Fazio *et al*. (2014) found that corticosteroids levels increased in juveniles of Hermann's tortoise *(Testudo hermanni)* for more than 4 weeks after handling and a short transportation. These apparent discrepant results could be related with the ability of reptiles to adapt to continuous external stimuli. For example, French *et al*. (2008) described that the tree lizard *(Urosaurus ornatus)* presents less CORT levels when they inhabit on an urban environment (more exposure to external stimuli) than in a semi-natural or a natural environment. In other case, marine iguanas (*Amblyrhynchus cristatus)* exposed to continuous stimuli (tourists presence) showed less CORT when they face an acute stressor factor, such as capture, handling and restraint, when compared to naïve marine iguanas that had no interactions with tourists (Romero and Wikelski 2002). In our trial, the differences in CORT concentration were evident after the first blood sampling, when hatchlings were only two weeks old, suggesting that the adaptation to standardized handling was quite fast, similar to the rapid adaptation described for bearded dragons (*Pogona barbata*), where no variation in plasma CORT concentration were observed 3.5 h after capture and holding into cloth bags (Cree *et al.* 2000). In the present study, high frequent manipulation could permit the adaptation of hatchlings to the standardized protocols or continuous external stimuli.

Body temperature directly affects CORT levels in reptiles (Hunt *et al.* 2012, Telemeco & Addis 2014), where extreme temperatures pose a threat to performance and survival on ectothermic animals, so, glucocorticoid up-regulation might be an important component in response to extreme thermal conditions. On reptiles, low temperatures induce performance reduction, so, a decrease of CORT concentration could be expected (Cree *et al*. 2003, Tyrrell & Cree 1998). The results obtained in the present study agree, where loggerhead hatchlings reared at a lower temperature presented lower CORT levels (but not significant). This effect was not significant in the present study, perhaps because the experimental temperature (26 °C and 28 °C) ranged within the natural temperatures for loggerhead hatchlings in the North Atlantic (Mansfield *et al.* 2014). Significant alterations of CORT levels in reptiles subjected to temperature variations have been described in extreme temperatures, and specially related with suboptimal temperatures, where high CORT concentrations may help the organism to maintain an alert state, as suggested for

the Children's python (*Antaresia childreni)*, but not when snakes had access to preferred warmer body temperature (Dupoué *et al.* 2013). Indeed, at temperatures higher than 20 °C, a positive correlation of CORT and temperature was found for alligator lizards (*Elgaria sp.*) which was interpreted as a fully adaptive response and not an indicative of stress, because the temperature treatment assayed failed to induce a maximal CORT response in those lizards (Telemeco & Addis 2014).

Results of the first trial showed no correlation between CORT concentration and W, WI,L and LI, in agreement with those results found by Flower *et al.* (2018) on gravid loggerhead sea turtle and by Cash *et al.* (1997) on wild red-eared slider turtles (*Trachemys scripta elegans*).

From trial II in relation witheffect of stocking density, it could be assumed that hatchling social isolation was inducing an elevation of CORT levels in loggerhead hatchlings. Isolation in higher 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 (*Chamaleo calyptratus*) raised in isolation were less sociable and bold (Ballen *et al.* 2014); or the less social activity showed by water snakes (*Natrix maura*) when they were incubated isolated (Aubret *et al.* 2016). However, it is well known that sea turtles are solitary animals during their entire life, since they leave the nest on their natal beaches. Nevertheless, when sea turtle hatchings gain the sea, they swim strongly offshore going into the open ocean, until reach sargassum aggregations where they get refuge, food and thermal benefits (Mansfield *et al.* 2014). The differences in CORT levels found in this study between loggerhead hatchlings kept isolated and the ones sharing the tank with other hatchlings, could be the physiological response to face isolation, because they miss the protection given by natural enrichment structures such as seaweeds in the Sargassum sea. In the tanks with more than one hatchling, their tanks-mates could be playing a role of any kind of enrichment structure. Future studies including "environmental enrichment" on isolated hatchlings could be conducted to deep on the physiological and behavioral response to isolation on loggerhead hatchlings, suggested by Case *et al.* in (2005).

In conclusion, according to our results, when loggerhead hatchlings need to be held under controlled condition, handling standardized protocols could be applied regularly (once per week), as growth is not affected, and CORT levels suggested better welfare conditions

than those subjected to low frequent handling protocols. The ambient temperature must be maintained within the natural range of 26 ° to 28 °C, as this range did not induce any effect on growth and welfare conditions (CORT levels). The stocking density of loggerhead hatchlings could be as higher as 90 hatchlings per m^3 during the first 6 months of rearing under controlled conditions to keep appropriate welfare parameters. Isolation of individual hatchling must be refused, as induced a chronic elevation of plasma CORT and experiments with environmental enrichment need to be conducted.

Photo: Lisa Anna Matthiesen

Preliminary husbandry protocols for juvenile loggerhead sea turtles based on stress response to stocking density variation and dry-docking time

This manuscript has been submitted to Endangered Species Research.

5.1 Abstract

Juvenile loggerhead sea turtles *(Caretta caretta)* are mainly hold under controlled conditions for veterinarian reasons in rescue or rehabilitation centers and, to a lesser degree, for conservation breeding programs. Accordingly, protocols and basic guidelines have been described for husbandry of sea turtles with veterinarians needs, which stablish recommendations on holding conditions and on how animals have to be handled for cleaning operations, health management and data collection. Although those considerations are not based on physiological indicators of welfare. As loggerhead sea turtle is listed as endangered species, monitoring the welfare when kept under controlled conditions should be a priority. The aim of this study was to define standardized husbandry protocols for loggerhead juveniles under controlled conditions, based on the corticosterone response and spontaneous behavior, which have been described as good indicators of welfare. To achieve it, two different trials were performed. In the first one, eventual changes in standardized stock density were assayed, while in the second one dry-docking time-course was studied. Corticosterone analyses stablished that the number of juvenile loggerheads per tank held in controlled condition can be modify punctually without affecting their welfare. However, time of dry-docking must be no higher than 15 min, as indicated by elevation of circulating corticosterone. Results also showed that standardized protocols tested, did not affect behavior parameters measured.

5.2 Introduction

Loggerhead sea turtle *(Caretta caretta)* juveniles can be held under controlled conditions if basic guidelines of action with animals affected by injuries or traumas recommend their

transport to a rescue/ rehabilitation center or willing veterinarian for observation and/ or treatment (Phelan & Eckert 2006), from days to months on recoverable animals and years if they are considered unrecoverable. There are also animals held under controlled conditions for conservation breeding programs (Owens & Blanvillain 2013, Kawazu *et al.*2018). As loggerhead sea turtle is listed as threatened species, monitoring the welfare when kept under controlled conditions should be a priority (Sherwen *et al.* 2018). The concept of animal welfare usually is defined in terms of over time balance of affective states (Boissy *et al.* 2007). Affective states are variably described as feelings, emotions or moods, which might include, amongst others, happiness, fear, depression and pain, and comprise behavioral, physiological and cognitive components (Boissy *et al.*2007). Measurement of affective state can be used to infer welfare state and reliable indicators of welfare for reptiles need to be identified, including a transition from resource to animal-based indicators, and from group to individual assessment (Benn *et al.* 2019). Resource-based measures are recorded in the animals' environment, whereas animal-based indicators are measured directly in animals, using a combination of physiological, behavioral and health variables (Whitham & Wielebnowski 2013), which include a wide variety of parameters such as spontaneous behavior or homeostatic equilibrium after a stressful situation (Benn *et al.* 2019).

As a response to stress, the Hypothalamic Pituitary Adrenal (HPA) axis induces a hormonal cascade causing the release of corticosteroids from the adrenal cortex (Greenberg & Wingfield 1987, Gregory & Schmid 2001, Silvestre 2014). It is widely accepted that measurement of products of the HPA axis will indicate that the animal is experiencing a stressful situation. In reptiles, the main endocrine product released to bloodstream in the physiological regulation of stress response is the corticosterone (CORT) (Silvestre 2014). This steroid hormone would increase its levels in plasma within minutes to hours of exposure to a stimulus (Greenberg & Wingfield 1987). Stress response not only consists on CORT increase, but also it is associated to different behaviors, and both, the physiological and behavioral response would contribute to the animal adaption and subsequently animal welfare (Wingfield *et al.* 1998, Sapolsky *et al.* 2000, Jessop & Hamann 2005).

Changes in circulating CORT concentration can be triggered by internal or external factors (Romero & Wikelski 2002, Jessop & Hamann 2005, Flower *et al.* 2015). For

loggerhead sea turtles, the changes on circulating CORT have been studied under different field conditions and during different life stages. For example, in breading and no-breading adults, CORT variations have been linked to reproductive success, seasonality, shark-attacks or human handling (Whittier *et al.* 1997, Jessop*et al.* 2004, Valente *et al.* 2011, Flower *et al.* 2015, 2018). Variations of CORT in wild juvenile loggerheads have been related to handling or recovery/illness processes (Gregory *et al.* 1996, Flower *et al*. 2015) and in wild loggerhead hatchlings during their dispersal behavior (Pereira *et al.* 2013). While CORT response has been widely studied in wild loggerheads, few data exist on CORT or stress response in juvenile sea turtles subjected to different controlled conditions.

Detailed protocols and basic guidelines have been described for husbandry of marine turtles subjected to trauma (Bluvias & Eckert 2010). Recommendations on holding conditions and on how animals have to be handled for cleaning operations, health management, sampling procedures and data collection, although those considerations are not based on physiological indicators of welfare (Bluvias & Eckert 2010).

Among the different protocols, housing is considered one of the most important parameters to be defined (Arena *et al.* 2014) to avoid deleterious effects of multiple-occupation pounds or social isolation, as it is sometimes unavoidable to accommodate multiple turtles together in a single tank. Dry-docking (maintenance of the animals out of the water for different purposes) for a period of time is also necessary for cleaning purposes, data collection or in order to give the turtle time to rest and absorb any fluids which may need to be administered (Bluvias & Eckert 2010).

Thus, the aim of this study was to preliminary define standardized husbandry protocols for loggerhead juveniles under controlled conditions, based on CORT response and spontaneous behavior under different routine situations such as separation/isolation situations or dry-docking. To achieve this goal, two different trials were performed. In the first one, eventual changes in standardized stock density were evaluated through CORT variations and behavior response whereas in the second one, dry-docking time-course study was conducted to evaluate elevations of plasma CORT subsequently the dry-docking time to avoid deleterious effects on welfare caused by this routine protocol.

5.3 Material and methods

Juveniles loggerhead sea turtles used in these experiments come from eggs collected on 2009 and 2010 nesting seasons from beaches sited on the *"Reserva natural das Tartarugas"*, southeast Boa Vista Island (Cape Verde) and translocated to the laboratories of ECOAQUA Institute of Las Palmas de Gran Canaria University, Canary Islands (Spain) for controlled incubation. Transport and incubation protocols were previously described by Usategui-Martín and co-authors (2019) and conducted under CITES permits (ES-DE-00008/08I; ES-DE-00005/09I).

All loggerheads were raised under similar controlled conditions during 7 and 8 years, before performed the two trials. They were kept in 5000l rectangular tanks fill with a continuous seawater flux directly from the sea. There were three animals per tank allocated according to their weight trying to have the same density and based on the availability of holding tanks within the facility. All of them were feed similarly with a mix of different fishes and cephalopods. The amount of food per animal was establish according to their weigh, age and seawater temperature, based on feeding protocols established by Bluvias & Eckert (2010). They were feed three times per week (Monday, Wednesday and Friday). The feed intake of all the animals was adequate to the protocols established (Bluvias & Eckert 2010) and no aggressions between them were observed, that is why three animals per tank were considered the optimal density tanking also into account the available space in the installations.

Sea turtle juveniles were handled following the same standardized handling protocol. Tanks were cleaned twice a week (Monday and Friday), where tanks were drained, the turtle feces and algae were cleaned with a scrub brush and rinse with fresh water and fill again with clean seawater. Sea turtles were dry-docked and the epibiota on the carapace was cleaned with a scourer and rinse with freshwater every four weeks or more frequent if needed. Sea turtles were weighted (kg) and measured (cm) every four weeks to monitor their growth during all the on-growing period. Then growth in centimeters per year (cm/y) was calculated to compare with growth rates calculated by other authors on wild juvenile loggerheads.

5.3.1. Trial I. Effect of stocking density variation

In order to assess the effects of punctual isolation or multiple in-tank-allocation of sea turtles on plasma CORT concentration, nine animals (four females and five males) were used in the trial, following the standardized procedures used during their growth at the facilities. All the three tanks used in this trial were identical, rectangular with 5000 L capacity, same orientation (270° W) and same ratio sun/shade. Two different treatments were assayed. The first one consisted on decrease the original density of three turtles per tank to one (D1), and the second consisted on increased the density from three to five animals per tank (D5). The remaining tank was kept with three juveniles as control group. This experience lasted 9 days and was replicated three times. Between replicates turtles were relocated, being again three animals per tank for seven days to let them rest. The process to distribute the animals between the different treatment and the resting periods was random and different at each replicate, trying to avoid the possible effect produced at an individual level.

Three blood samples per turtle were taken along each triplicate, one just before starting the trial (day0), then at day 4 (day4) and the last just before finishing each replicate at day 9 (day9). Besides, in order to study the spontaneous behavior, each tank was separately studied by recording one hour in the morning (from 9:00 to 10:00 am) and one hour in the afternoon (from 16:00 to 17:00 pm) during the experimental period. GoPro® cameras were allocated in an elevated position (Fig 5.1a) to film the tanks from above to have a clear image of the whole tank (Fig 5.1b). Cameras were activated remotely to not interfere in the animal behavior. Then, videos were analyzed to measure different sea turtle behavior parameters: a) number of breaths per our (breaths/h), defined as each time a sea turtle take its head out the water in one hour; b) number of movements per hour (moves/h), defined as the number of times that the juvenile sea turtles started to move through the tank and stops on the bottom of the tank in one hour; c) Duration of the movements (min/move), define as mean duration, in minutes, of all the moves in one hour; d) Number of breaths per movement (breaths/move); e) the total time the juvenile stayed moving expressed in %.

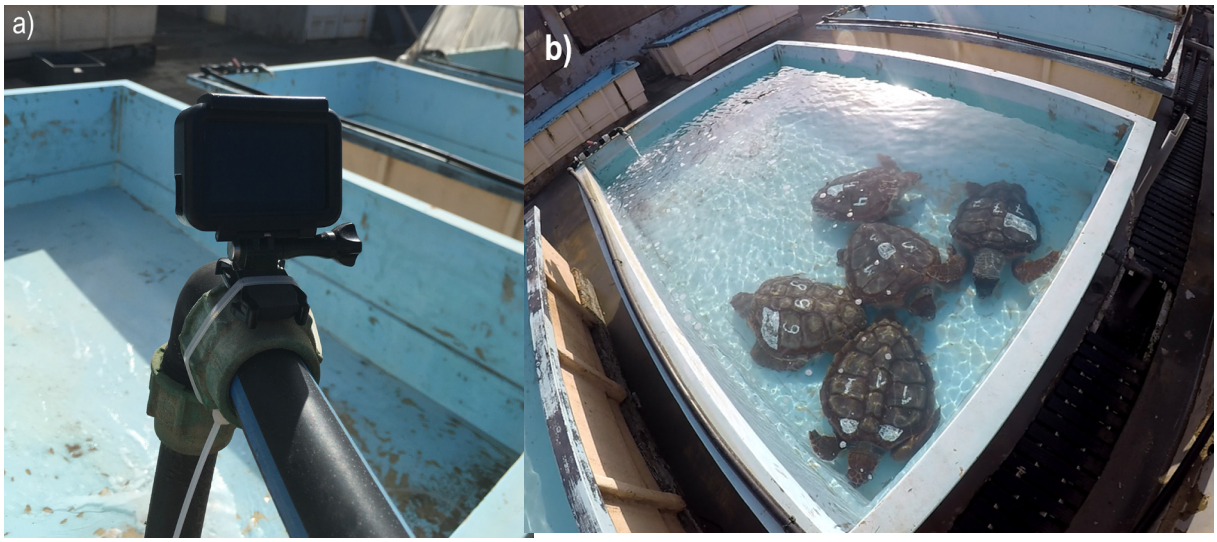


Fig 5.1. a) Set up of the video camera to film the behavior of sea turtles, b) One of the experiment tanks view from above

5.3.2. Trial II. Effect of dry-docking time

To study the evolution of plasma CORT during dry-docking time protocol, eight animals were used (six females and two males). Four rectangular tanks with similar water capacity (5000l) were used in this trial with two turtles per tank. Four different treatments were assayed. The beginning of all four treatments was the same, following the steps of the cleaning and data collection routine protocols from the facilities, that consist on: i. takes the animals out of the water, ii. measure and weight them (less than 5 minutes) and iii. put them back into the water. The four treatments differed in the time that sea turtles were exposed to the dry-docking protocol for the standardized manipulation. In the first treatment (T0) animals were sampled immediately after the 5 min standardized handling protocol and did not spend any minute in dry-docking period. In the second (T15), the third (T30) and the fourth (T60) treatments, animals were dry-docked by 15, 30 and 60 min, respectively. Every Friday during a month, the experiment was repeated in order to avoid possible effects of individual response, where the eight animals experiencing all the treatments.

Two blood samples per turtle were taken, the first just after the animal was take out from the water, previous to 5 minutes handling protocol, and the second just before

putting it back into the water. The first one was considered as the control group. Both mean CORT concentration after dry-docked protocol and individual increment of CORT (IncCORT) (defined as CORT after dry-docked - CORT control) were obtained. To analyze spontaneous behavior after dry-docking protocols, juvenile sea turtles were recorded for one hour after putting them back to water, using same cameras set up and video analysis as described in trial I.

5.3.3. Blood collection and sample preparation

All blood samples were collected from the dorsal cervical sinus using a 5 ml syringe with a 21G/38 mm needle and dispensed into 2 ml tubes containing lithium heparin. Tubes were kept in a cooler with ice packs until centrifugation at 3000rpm for 5 min to obtain plasma. Blood plasma was pipetted into 2ml eppendorf tubes and kept frozen (-30 °C). Frozen plasma samples of both trials were analyzed at the Dpt. Cell Biology, Physiology and Immunology from the Universitat Autònoma de Barcelona to analyze CORT levels using double antibody in equilibrium Radioimmunoassay (RIA). In brief, corticosterone radioimmunoassay used [125I] corticosterone - carboxymethyloxime - tyrosine - methylester (ICN - Laboratorios Leti, Barcelona, Spain) and synthetic corticosterone (Sigma, Barcelona, Spain) as the standard and an antibody raised in rabbits against corticosterone - carboximethyloxime - bovine serum albumin. The range of the standard curve was between 6.25 and 1600 pg of corticosterone per tube. The antibody used has a cross-reactive 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 to avoid interassay variability (Scorrano *et al.* 2014).

5.3.4. Statistical analysis

All statistical analyses were conducted using R version 3.1.2 (R Department Core Team 2014). CORT concentrations from both trials were analyzed using Two-way Analysis of the Variance (ANOVA). Data from the first trial were analyzed using treatment and sampling day together as one factor and sex as the second factor. Data of the second trial were analyzed with dry-docking time and sex as factors. Besides, the IncCORT was also analyzed with a two-way ANOVA. Tukey's post hoc analysis was conducted when needed.

Behavior parameters were correlated with CORT concentration in both trials. In addition, correlations between the difference in CORT variation pre and post dry-docking and all behavior parameters were also conducted. In trial I, Three-way ANOVAs were conducted

using behavior parameters as response variable and treatment/sample, sex and daytime as factors. In trial II, behavior parameters were subjected to Two-way ANOVAs with treatment and sex as factors. All ANOVAs were followed by Tukey's post hoc tests in order to explore any possible significant differences between study groups. Results were considered significant at $p < 0.05$.

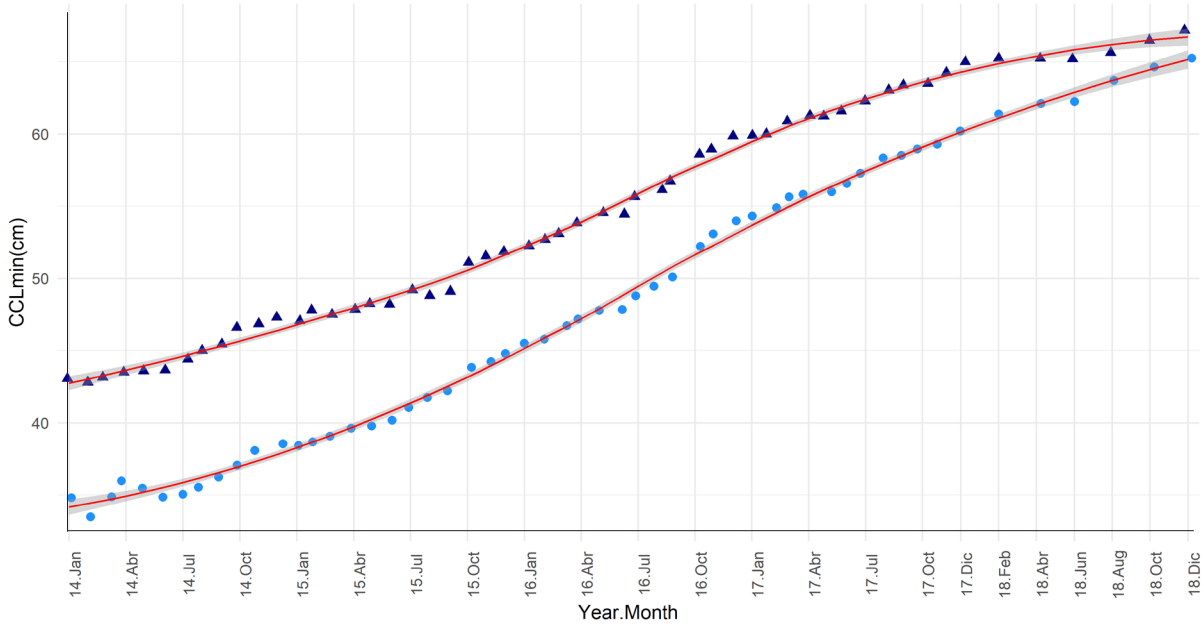


Fig 5.2. Juvenile loggerheads carapace growth for five years (2014 -2018), with the mean SCLmin increase in cm by month separated in the two cohorts, dark blue triangles represent juveniles hatched in 2009 and light blue dots animals hatched in 2010.

5.4 Results

Along the last five years the animals showed a mean total Growth of 31.4 ± 4.92 cm in carapace length increase (Fig 5.2) and annual growth rates ranged from 4.95 ± 1.43 to 8.31 ± 1.35 cm/y (Table 5.1).

5.4.1. Trial I. Effect of stocking density variation

CORT concentration was significantly different ($F = 6.309$, $p < 0.05$) among densities along the sampling period but, there were no significant differences ($F = 0.666$, $p > 0.05$) according to the sex of the animals neither of the interaction of both ($F = 0.218$, $p > 0.05$).

Table 5.1. Mean CCL min and growth rates with its SD in cm/y, of the last five years in juvenile loggerhead sea turtles held under controlled conditions.

Year	CCLmin	Growth
2014	40.2 ± 4.7	5.00 ± 0.86
2015	45.1 ± 4.5	6.07 ± 0.92
2016	52.3 ± 4.2	8.31 ± 1.35
2017	59.7 ± 3.1	7.73 ± 0.96
2018	64.5 ± 1.8	4.95 ± 1.43

Table 5.2. Mean corticosterone concentration and SD in ng/ml, according to stocking density and sampling day. Asterisk (*) denotes significant differences ($p < 0.05$) among groups.

Day/Treatment	Mean	SD
Control	0.59	0.21
d4.D1	0.21*	0.03
d4.D3	0.65	0.32
d4.D5	0.35*	0.03
d9.D1	0.42	0.10
d9.D3	0.57	0.17
d9.D5	0.42	0.15

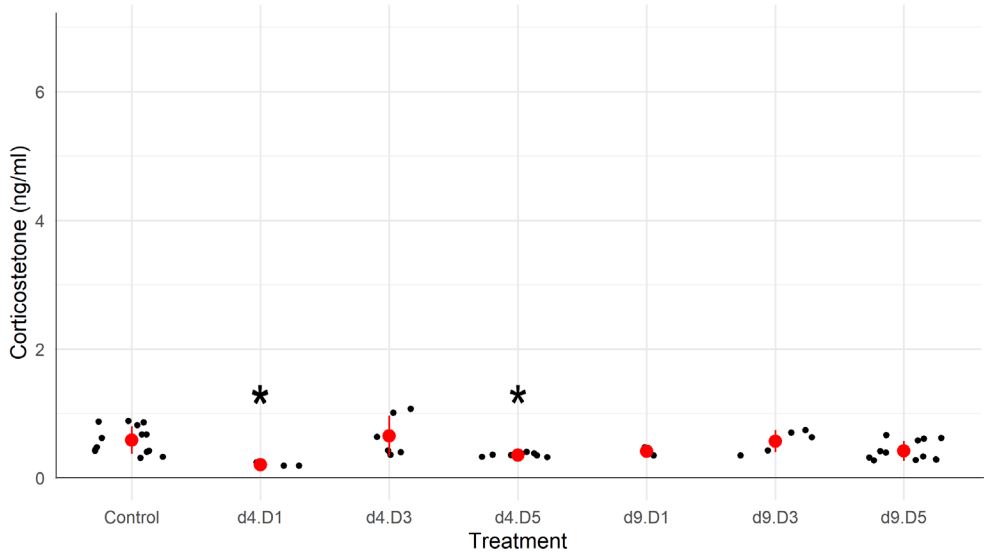


Fig 5.3. Circulating corticosterone concentration in serum according to control group and different stocking density (D1, D3 and D5) by sampling days (day 4 -d4- and day 9 -d9-). Asterisk (*) denotes significant differences (p < 0.05) among groups. Big red dots and lines represent the mean and the standard deviation respectively.

Tukey´s post hoc test showed that plasma CORT levels were significantly lower ($p < 0.05$) at day four in treatment D1 and D5 respect to Control group, rising up both at day nine and recovering values from control group (Fig 5.3, Table 5.2).

No significant differences ($p > 0.05$) were found in most of the behavior variables measured according to the different density treatments. However, there were significant differences ($F = 25.206$, $p < 0.05$) in the breaths/h according to the daytime, and also there were significant differences according to the sex of the juveniles in the moves/h ($F = 11.954$, $p < 0.05$) and in the average duration of each move ($F = 4.656$, $p < 0.05$). Finally, no correlation was found between CORT concentration and behavior parameters studied.

4.2 Trial II: Effect of dry-docking time

CORT concentration was significantly affected by the dry-docking time ($F= 37.96$, $P < 0.05$). No effect was found in relation with individual sex ($F = 3.13$, $p > 0.05$), neither on the interaction of dry-docking time and sex ($F = 0.54$, $p > 0.05$). CORT levels after 60 minutes out the water were significantly ($p < 0.05$) higher than after 15 or 30 minutes, as well as, after 30 minutes out the water, sea turtles presented significantly ($p < 0.05$)

Table 5.3. Mean corticosterone concentration (CORT) and corticosterone increase (IncCORT) ± SD in ng/ml, as well as the mean ± SD of the different behavior parameters by dry-docking times. Asterisk (*) denotes significant differences ($p < 0.05$) among groups.

Treatment	CORT	incCORT	Breaths/h	Moves/h	Breaths/move	Min/move	Move %
Control	0.51 ± 0.24	-	-	-	-	-	-
T0	0.62 ± 0.34	0.09 ± 0.35	12.9 ± 5.9	2.4 ± 1.7	9.1 ± 7.7	33.7 ± 24.8	92.3 ± 13.8
T15	0.75 ± 0.44	0.32 ± 0.53	14.5 ± 9.4	1.6 ± 0.6	10.9 ± 10.5	30.3 ± 24.8	69.6 ± 31.2
T30	1.51 ± 0.67*	1.05 ± 0.72	16.6 ± 6.5	2.0 ± 1.3	10.2 ± 9.7	32.4 ± 27.3	70.2 ± 34.3
T60	5.28 ± 1.49*	4.32 ± 1.32*	23.4 ± 11.2	1.4 ± 0.5	21.6 ±12.8	42.1 ± 20.7	91.3 ± 17.42

higher circulating plasma CORT than the control (T0) (Fig 5.4, Table 5.3). The dry-docking time induced significant differences ($F = 25.97$, $p < 0.05$) in the variation pre – post CORT response, being the increase of T60 significantly higher ($p < 0.05$) than the other treatments (Fig 5.5, Table.5.3). There was no effect of the sex ($F = 1.50$, $p > 0.05$) neither of the interaction between the different treatments and sex ($F = 0.07$, $p > 0.05$).

The dry-docking time did not affect significantly ($p > 0.05$) the behavior parameters, neither the sex of the animals ($p > 0.05$). Meanwhile, there has been found correlation between breaths/h and circulating CORT ($R = 0.45$, $P < 0.05$) and with time out of the water ($R = 0.39$, $p < 0.05$), as well as correlation between breaths/move and CORT ($R= 0.55$, $p < 0.05$), (Table 5.3) and incCORT ($R = 0.49$, $p < 0.05$).

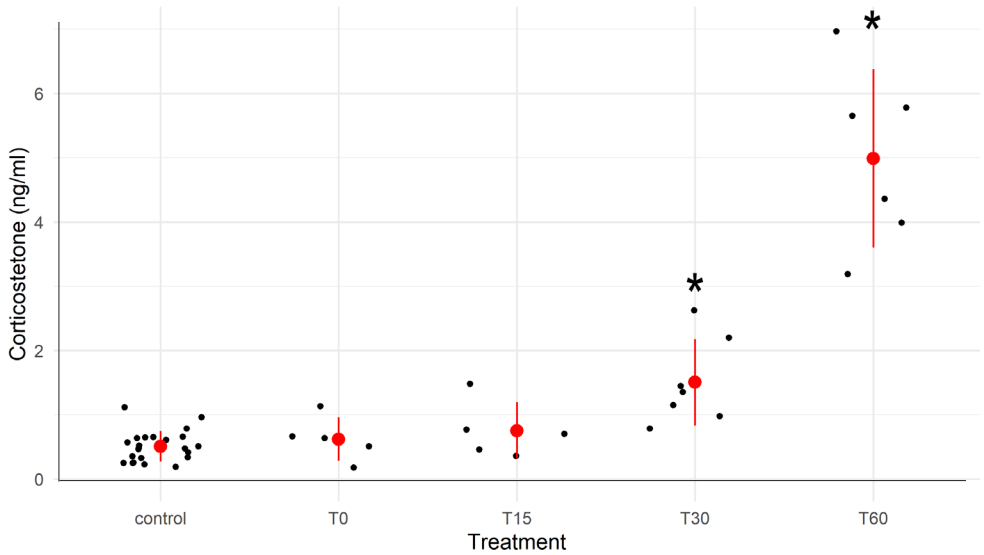


Fig 5.4. Circulating corticosterone concentration in serum according to control group and different dry-docking times. Asterisk (*) denotes significant differences ($p < 0.05$) among groups. Big red dots and lines represent the mean and the standard deviation respectively.

5.5 Discussion

There are few studies of loggerhead juveniles held under controlled conditions, so the discussion must compare the results obtained with growth models and CORT values obtained from wild animals.

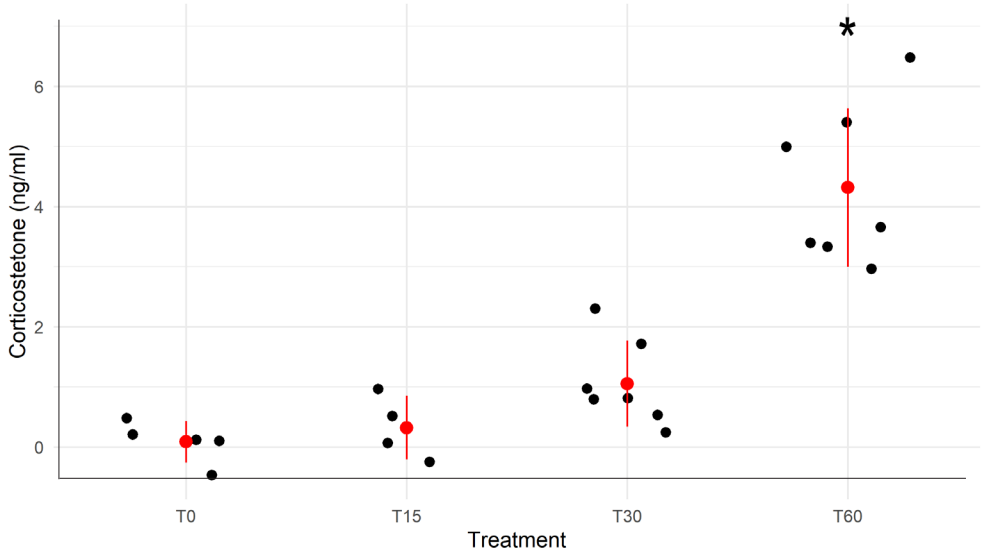


Fig 5.5. increment of circulating corticosterone concentration in serum after different dry-docking times. Asterisk (*) denotes significant differences (p < 0.05) among groups. Big red dots and lines represent the mean and the standard deviation respectively

The loggerhead sea turtle juveniles used in the present study ranged from 4/5 years old (in 2014) to 8/9 (in 2018), that correspond to the oceanic stage, as described by Bjorndal *et al.* (2000) where in this species ranges from 6.5 yr (46 cm CCL) to 11.5 yr (64 cm CCL). CCL at maturation has been estimated ranging from 83 to 102 cm on the Northwest Atlantic population (summarized in Avens *et al.* 2015) and from 69 to 82 cm in the Northeastern Atlantic population (Cape Verde) (Varo-Cruz 2010). Bjorndal *et al.* (2003) estimated that loggerhead sea turtles of 7.0 yr had around 46 cm of CCL. Lenz and co-authors (2016) estimated an average length of around 71 cm for loggerhead sea turtles of 15 years. Our results have shown that juvenile loggerheads grew under controlled conditions reached 65 cm CCL at 8/9 yr, denoting a faster growth than the estimated growth in different wild populations. The data obtained for CCL in the present study would correspond to animals of 10 yr following the Von Bertalanffy growth curves based on adjusted age estimates calculated by Klinger & Musick (1995) for this species. The faster growth of juvenile loggerheads held under controlled conditions has been previously described by Swingle and co-authors (1993), although those authors based their observations on total weight of individuals and not on CCL. This faster growth could be due to the availability of food, as wild juveniles of this species in the oceanic-stage have a stochastic lifestyle with great variation in food availability and temperature,

which in turns induce higher variable growth rates (Bjorndal *et al.* 2003, Lenz *et al.* 2016). Indeed, loggerhead sea turtles under controlled conditions showed growth rates ranging between 4.95 to 8.31 cm per year, which are slightly higher than the natural growth ranges estimated for wild loggerheads from the Northwest Atlantic population, estimated to be up to 7.4 cm per year (Braun-McNeill *et al.* 2008) and higher to those estimated for neritic individuals of the Mediterranean population, ranging between 0.57 to 6.5 Cm/y^{-} (Casale *et al.* 2011), the Southwestern Atlantic population (Brazil) with 2.04 cm/y (Petitet *et al.* 2012), or 2.1 cm/y (Lenz *et al.* 2016).

Even when the growth of the animals within the present study was higher than the estimated ones for wild juveniles, basal CORT level obtained in this study around 0.50 ng/ml (by mean of all data coming from undisturbed juveniles under controlled conditions) were similar to the ones recorded from wild loggerheads (Gregory *et al.* 1996, Flower *et al.* 2015), denoting that our population is growing properly and have no chronic stress under the standardized husbandry protocols conducted in our facilities.

During the holding period within our facilities, the animals were held in a multiple occupational regime, being established the adequate number of animals per tank in three, based on the growth rate, taking into account weight and size of the animals, combined with absence of aggression between them. Based on husbandry protocols established for rehabilitation centers, the recommended number of animals per tank is one, in order to avoid possible infections between animals or aggressions (Higgins 2003, Bluvias & Eckert 2010). Although, these recommendations are based on health aspects derived from housing conditions of animals coming from the wild to be rehabilitated or recovered. As far as we know, no information has been reported on the number of animals per tank for healthy individuals held under controlled conditions for long periods, being an important parameter to consider from the welfare point of view (Arena *et al.* 2014). CORT analysis of this study confirmed that under the stock density established empirically, juvenile loggerheads were in a proper welfare conditions, CORT levels did not exceed 1.07 ng/ml and aggression were not presented. CORT blood concentrations have been studied in different sea turtle species (Gregory *et al.* 1996, Valverde *et al.* 1999, Jessop 2001, Hamann *et al.* 2002, Al-Habsi *et al.* 2006) and the CORT values recorded in the present study (between 5.45 and 8.48 ng/mL) were similar than those found for the same species in the wild (Gregory *et al.* 1996).

Husbandry protocols for loggerhead juveniles not only need to determine the number of animals to be held by tank depending on the facilities, but also the effect of punctual variations in the housing conditions such as isolation or multiple occupation derived from husbandry necessities such as cleaning or health treatments (Bluvias & Eckert 2010). Results from the present study determined that punctual changes of tank occupation (isolation practices or multiple occupancy) for 9 days did not altered the circulating CORT of the animals, together with no aggressions recorded, indicating that welfare of loggerhead juveniles were not affected. No studies of changes in multiple occupancy of tank have been done with sea turtles, but our results are similar to those reported for harvest-size saltwater crocodiles (*Crocodylus pososus)* housed in a farm in communal or individual pens (Isberg & Shilton 2013, Isberg *et al*. 2018). Other reptiles showed different CORT response to changes in the occupancy of tank, such as the Eastern box turtle (*Terrapene carolina carolina)* subjected to one hour of isolation and confinement, that had 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 effects of the different tank occupancies assayed could be related to the availability of this species to adapt to the controlled conditions and to the standardized husbandry protocols. Flower and co-authors (2015) described a delay of CORT response in rehabilitating turtles after stress, compared to wild ones, that may be associated with the daily contact (visual or direct) they have with their human caretakers.

There are other handling protocols that need to be standardized from the welfare point of view. Handling for cleaning protocols are necessary although they must be as faster as possible, and dry-docking periods are also necessary for transport, cleaning routines or other husbandry practices related with health and sea turtle care (Bluvias & Eckert 2010). Flowers *et al*. (2015) described a significant CORT increase after 6 min of handling in juvenile loggerheads from Jekyll Island (Georgia, USA), reaching the highest pick of circulating CORT after 30 minutes, being those values 8-fold higher that the resting values. Gregory *et al*. 1996) found a similar increase of 7.2-fold 30 minutes after handling loggerhead juveniles from Florida population. From the present study, the handling protocol assayed (five minutes of handling for biometric data collection) did not induced significant changes in circulating CORT concentration, as seen when compared T0 (5 min) values with control ones. Dry-docking protocols induced a significant increase of

circulating CORT after 30 min, with 3 and 10-fold increase after 30 and 60 min of dry-docking respectively. Those results showed a delay of CORT response when compared with other loggerhead sea turtle studies described before. Differences in the time and intensity of CORT response after stress could be due to the different handling protocols, the size of the individuals, the acclimation to controlled conditions or even the geographic origin of the animals (Gregory *et al.* 1996, Gregory & Schmid 2001, Flower *et al.* 2015).

The results from the present study suggested that the maximum time that a juvenile loggerhead can be out the water without compromising its welfare must be 15 minutes, because at 30 minutes corticosterone level were already significantly higher. This suggested husbandry protocol is more restrictive that the previously proposed by Higgins (2003), who determined a dry-docking time of 30 min to prevent carapace desiccation and peeling, that could provoke incidences of opportunistic pathogen.

The standardized protocols proposed within the present study did not induced changes in the behavior of the loggerhead juveniles. No correlations were found among the dry-docking time and the different behavior parameters studied. However, a significant correlation ($p<0.05$) between circulating CORT levels and breaths per hour was found. Changes in circulating CORT have been associated with changes in reptile behavior (Denardo 2006, Silvestre 2014), including enhancement of antipredator responses (Thaker *et al.* 2009), reduced aggressive behavior (Tokarz 1987), increased defensive behavior (Stepanek *et al.* 2019); Cash & Holberton (1999) showed a significant increase in locomotor activity within 48 hr after CORT implant in Red-Eared Slider Turtle, *Trachemys scripta elegans*. Those authors suggested that the effects of CORT on behavior may be context-dependent (i.e., whether the turtles can find food) and concentration-dependent (Cash & Holberton 1999). However, within the present study, no food deprivation occurs, and standardized handling protocols did not affect behavior parameters measured.

In conclusion, changes during 9 days in the multiple - occupancy of the tank did not induce changes in the circulating CORT or behavior of loggerhead turtle juveniles. Husbandry protocols of isolation or increasing the multiple - occupancy of the tank can be applied punctually without affecting loggerhead turtle welfare. However, more than 30 min of dry-docking protocols must be refused, time of dry-docking must be no higher than 15 min, as indicated by elevation of circulating CORT.

6

Photo: María Medina Suárez

Corticosterone response of loggerhead females during the last phases of the nesting process on Boa Vista (Cape Verde) beaches

6.1 Abstract

Loggerheads sea turtles spend almost their entire life on marine ecosystems, where many difficulties are found to study their biology and ecology. Only females spend short time at terrestrial environment to accomplish the nesting process, when most of the information about their populations is been collected. The acquisition of baseline data on endangered species, such as loggerhead turtle, is crucial for conservationists to measure successes and failures of the conservation programs as well as for the implementation of new ones. The interaction with nesting females during data collection is unavoidable, however animal welfare should be considered to prevent stressful situations that could impact on sea turtle behavior or even reproductive success. The aim of this study was to evaluate how different data collection protocols used on nesting females could affect their welfare, in order to establish a more adequate standardized field protocol. To achieve this goal several nesting females were sampled under different situations and subjected to different data collection procedures to assess welfare indicators. Broadly nesting females showed low corticosterone levels, inversely related with their size, and low hormonal response when different protocols were applied. However, even if standardized protocols had no effect on CORT concentration an especial care must be taken while interacting with nesting females on the beach because their behavior can be modified by human presence and subsequently their welfare.

6.2 Introduction

Loggerhead sea turtle (*Caretta caretta)* is actually considered as "vulnerable" by the UICN (Casale & Tucker 2017) because their rookeries had slightly improve their conservation status in the very recent years, after the important decrease suffered in precedent

decades that positioned them as "*endangered*", mainly due to fisheries bycatch, meat and eggs consumption and habitat destruction (Wallace *et al.* 2010). Only the Northeastern Atlantic rookeries, focus on the Cape Verde breeding colony, is still been classified as *"endangered*", due to the strong concentration of nests in a very restricted area, where few kilometers of beaches, sited on Boa Vista Island and included in the "*Reserva Natural das Tartarugas" (RTN)*, concentrates the 60-70% of the reproductive effort of this colony.

New menaces such as extreme weather events and sea level rise brought by climate change have a direct impact on sandy beaches that are crucial habitats for the survival of sea turtle colonies. Important fluctuations on the reproductive success of sea turtles are being observed in response to habitat alterations, population dynamics, or natural patterns in nesting behavior, challenging conservationists to gain knowledge on how to protect these important breeding areas and engage new stakeholders (Hamann *et al.* 2010). Massive tourism development of pristine areas is rising sharply in recent decades, which is strongly threatening the natural habitats used by sea turtles to breed. Uncontrolled *"turtle-watching tours*" are also affecting nesting females on the beaches. Besides, sustainable tourism must be seen as an important part of the turtle conservation 'toolbox' that can be used to raise awareness, to provide funds for conservation and management plans, and to create 'alternative livelihoods' and revenues for local communities (Meletis & Harrison, 2010).

The acquisition of baseline data is crucial for conservationists to measure successes and failures of the conservation and management programs and enable them to assess their impacts (Meyer *et al.* 2014). For example, baseline data are required to establish adequate standardized protocols for data collection on nesting females, as well as for *turtle-watching tours*, considering the welfare of the animals to prevent stressful situations that could impact on sea turtle behavior or reproductive success.

Loggerheads sea turtles spend almost their entire life on marine ecosystems, where many difficulties are found to collect data to study their biology and ecology. Only females spend short time at terrestrial habitats to accomplish the nesting process (Flower *et al.* 2015, 2018), when most of the information about their populations is been collected. During the nesting process females come ashore to the sandy beaches, look for the adequate place, dig the egg chamber with the rear flippers, lay the eggs, cover them with sand and camouflage the nesting place before return to the ocean. Not always that a female crawls

up the beach succeed on the nesting process, many times they come back to the sea without laying the eggs (Eckert *et al.* 1999). All conservation programs conducted on the nesting beaches collect data on the females or during the oviposition or just after, but never before eggs laying, to avoid altering the success of the nesting event.

Animal welfare can be assessed through behavior, levels of physiological indicators (i.e. stress indicators) or other indirect parameters like absence of illness or appetite (Arena & Warwick 1995). In wild nesting sea turtle females, welfare can only be studied via physiological indicators, due to the short time available for their observation. Traditionally, circulating corticosterone (CORT) has been used as welfare physiological indicator (Gregory *et al.* 1996, Milton & Lutz 2003, Tokarz & Summers 2011), because is the main glucocorticoid produced by reptiles to face and adapt to stressful conditions (Cockrem 2013, Carbajal *et al.* 2018). Corticosterone response in adult sea turtles has been studied in different species and under different conditions (Whittier *et al.* 1997, Jessop *et al.*1999b a, 2002, 2004, Valverde *et al.* 1999, Rostal *et al.* 2001, Jessop 2001, Jessop & Hamann 2005, Valente *et al.* 2011, Flower *et al.* 2015, 2018, Hunt *et al.* 2016). For example, in sea turtles CORT concentration in plasma has been related with progesterone and testosterone levels by Valente *et al.* (2011). In adult loggerheads corticosterone levels have been studied during the reproductive period, observing their decrease along the reproductive season (Whittier *et al.* 1997), even if very low levels have been observed during the reproductive season (Whittier *et al.* 1997, Flower *et al.* 2018) even in response to external stimuli (Jessop *et al.* 2004, Flower *et al.* 2015). The CORT response in sea turtles has been related with age, size and sex, been more sensitive in juveniles than in adults and being the same between sexes during the non-breading season (Jessop & Hamann 2005). Green turtles in high density nesting areas produce slightly higher CORT levels than sea turtles in low-density nesting areas (Jessop *et al.* 1999b). The different stages of the reproductive behavior induce changes in CORT concentration, where females present higher CORT levels during the swimming activity than during the copulatory activity (Jessop *et al.* 1999a). CORT concentration has been also related with daily cycles in juveniles, whereas nesting females had no differences between day and night (Jessop *et al.* 2002). The plasma CORT concentration in adult leatherbacks (*Dermochelys coriacea*) during the nesting season has been observed to present stable values during the course of the nesting season (Rostal *et al.* 2001).

Even CORT response has been widely studied in mature sea turtles, little information is available on how data collection is affecting the welfare of the nesting females. Some protocols conducted during night patrols, such as handling nesting females for tagging or to obtain biometric parameters, could induce stressful situations for the animal that trigger any CORT increase. Welfare indicators could be used to determine which are the best procedures to collect data, in terms of number of people working on the female or distance to the tide line, based on animal welfare.

The aim of our study was to evaluate how different protocols used on data collection on nesting loggerhead females could affect their welfare and consequently compromise their reproductive success, in order to establish more adequate standardized field protocols, if needed. To achieve this goal several nesting females were sampled under certain situations and subjected to different data collection procedures to assess welfare indicators.

6.3 Material and methods

Between July and September of 2011 and 2012, 99 nesting loggerhead females were sampled at three beaches of the *RNT*, southeastern Boa Vista Island (Cape Verde), *Ervatão*, *Ponta Cosme* and *Calheta* beaches. Sampling was conducted during the night patrols included in the loggerhead conservation program managed by the NGO Cabo Verde Natura 2000 (Varo-Cruz *et al.* 2006) in the RNT

Biometric and tagging data from each female were obtained, as well as blood samples to measure corticosterone concentration. All samples were collected when females were coming back to the ocean, in order to not interfere in the nesting process, as was indicated by the standardized protocols of the NGO (Varo Cruz *et al.* 2006). Sampling was done even if the female succeeds in the nesting process or not. Once the female was found on the beach, the phase of the nesting process was noted, considering two types of contact depending on the distance of the people from the female: i) *no contact*, when female was encountered coming out of the sea, rising beach slop, or looking for the nesting place, where the team stay far away from the female; and ii) *direct contact* when the female was digging, laying eggs, covering, camouflaging or returning to the sea, where the team approach the female and stay behind the turtle to not disturb the nesting

process or directly approach the females for data collection.. When the female begins their return to the sea, first blood sample (preCORT) was taken. Once blood sampling was finished, researchers proceed to collect standardized biometric data: minimum curve carapace length (CCLmin) and curve carapace width (CCW) (Bolten 1999), and tagging information: females were examined for external (metal flipper tags) and internal (passive integrated transponder -PIT-) identification tags, and if the turtle presented no tags, there was identified as "*first observation*" and a PIT tag was placed intramuscular on the right flipper. If the turtle presented any tag was identified as "*recaptured*". Just after finish data collection, which takes between 13 and 17 minutes, a second blood sample was taken from the female (postCORT). Increment of CORT (incrCORT) was also evaluated by the difference between postCORT and preCORT.

Other parameters collected were: distance from the place where the female was first sampled to the tide line; if the female succeed or not in the nesting process (successful nest / failed nest); and how many people was around the animal during data collection (2, 3, 4, 6 or 8 people), where two people is the minimum number of people required.

A complete history of all individual sampled was obtained from Cabo Verde Natura 2000 databases, where the following parameters were extracted: number of encounters registered on the same season (*season recaptures)*; total number of recaptures registered since the animals was observed/tagged for the first time (*total recaptures*); and number of nesting seasons where each female was observed from the first interception (*total seasons*).

All those studied parameters were classified in two groups: i) natural variables, which include nesting process success, female size and distance to the tideline; and ii) human-turtle interaction factors, which include nesting phase on first contact, precedent interactions (recaptures), number of people and response to manipulation (Fig 6.1).

Holding the female during blood sampling was not required. Blood samples were collected from the dorsal cervical sinus using 5 ml syringes with 20G/38 mm needles, and sampling did not exceed 2 minutes. Blood was first conserved on 4ml lithium heparin tubes, then centrifuged for 5 minutes at 10000 rpm in the first 2 hours after sampling, and finally storage at -20 °C in the basecamp and at -80°C in the laboratory. Frozen plasma samples were sent to the Department of Animal Health and Anatomy (Veterinarian Faculty) from

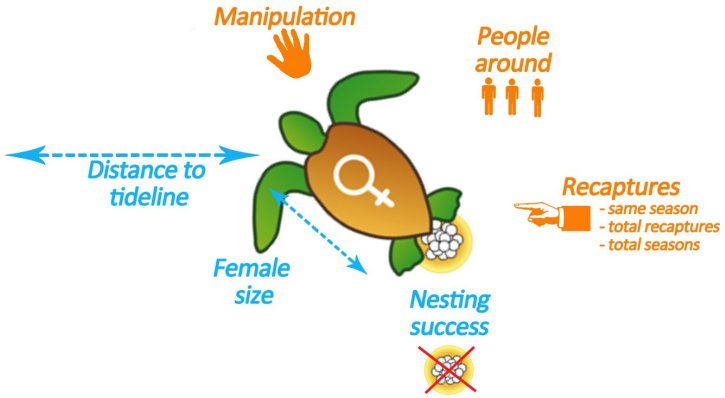


Fig 6.1 Graphic representation of the studied factors on loggerhead females. Factors in orange are natural, and factors in blue correspond to human-sea turtle interactions.

the Universitat Autònoma de Barcelona to analyze plasma CORT concentration levels using competitive EIA kits (Chapter 2 and 4)

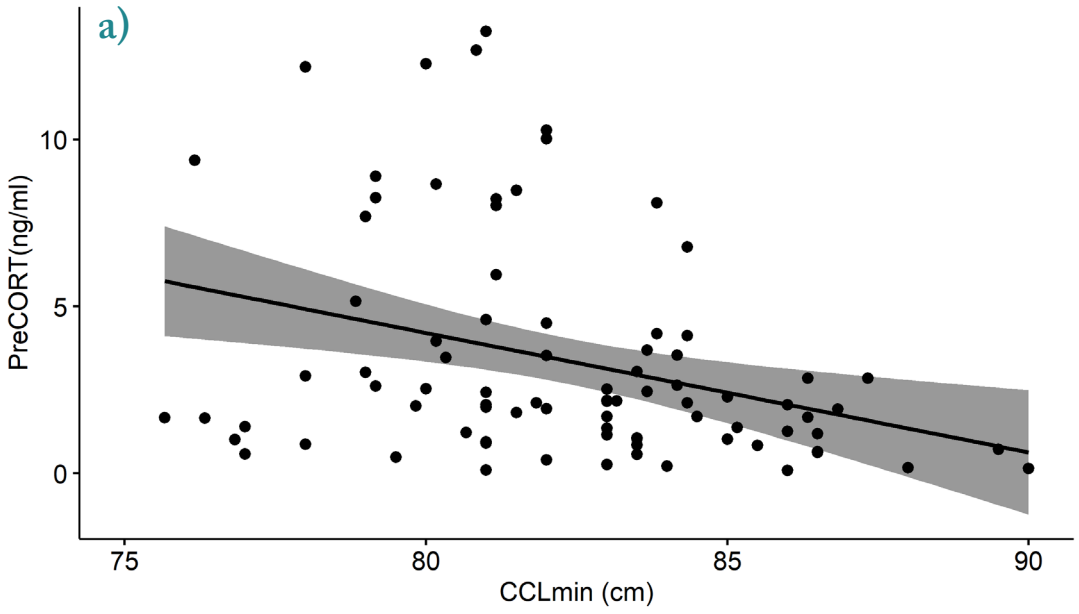
All statistical analyses were conducted using R version 3.1.2 (R Department Core Team 2014). First, natural factors were analyzed to identify its effects on CORT levels, CCLmin and distance to the tideline was correlated with preCORT, postCORT and incrCORT, in order to explore the dependence of CORT with female size; and the second one to analyze the increase of "anxiety" suffered by females when closer to the tide line as has been observed by field staff of Cabo Verde Natura 2000. On anthropogenic factors, preCORT levels were correlated with *season recaptures*; *total recaptures*; and *total seasons* to identify the effect of precedent interaction with humans on females. In other hand, the incCORT concentrations were correlated with the number of people around the female during data collection.

Gamma GLM was fitted for preCORT with nesting process success (*successful nest/failure nest)*, type of interaction (*no contact/direct contact)*, tagging situation (*recapture/first observation*) and *total seasons* as factors and for postCORT and incCORT the factor *people around* (two/more than two) was added to the GLM, to explore if one factor by itself, or combined, produce any change on CORT levels. PreCORT and postCORT together were also analyzed with an one way ANOVA to explore if the manipulation of the females

meant a CORT response, and also their relation with nesting succeed, recapture, people group size or the type of interaction approach could modulate the response of the females facing human manipulation. Results were considered significant at $p < 0.05$.

6.4 Results

A significant correlation (rho = -0.31, $p < 0.05$) between the female size (CCLmin) and their preCORT and postCORT levels was found, where bigger females showing lower plasma CORT concentrations (Fig 6.2a, b). No correlation was found between the female size and the difference of CORT before and after manipulation (rho = 0.05, $p > 0.05$), (Fig 6.2 c). No correlation has been observed between the distance to the tide line and basal CORT (rho = -0.28, $p > 0.05$) nor with the difference of CORT (rho = 0.10, $p > 0.05$).



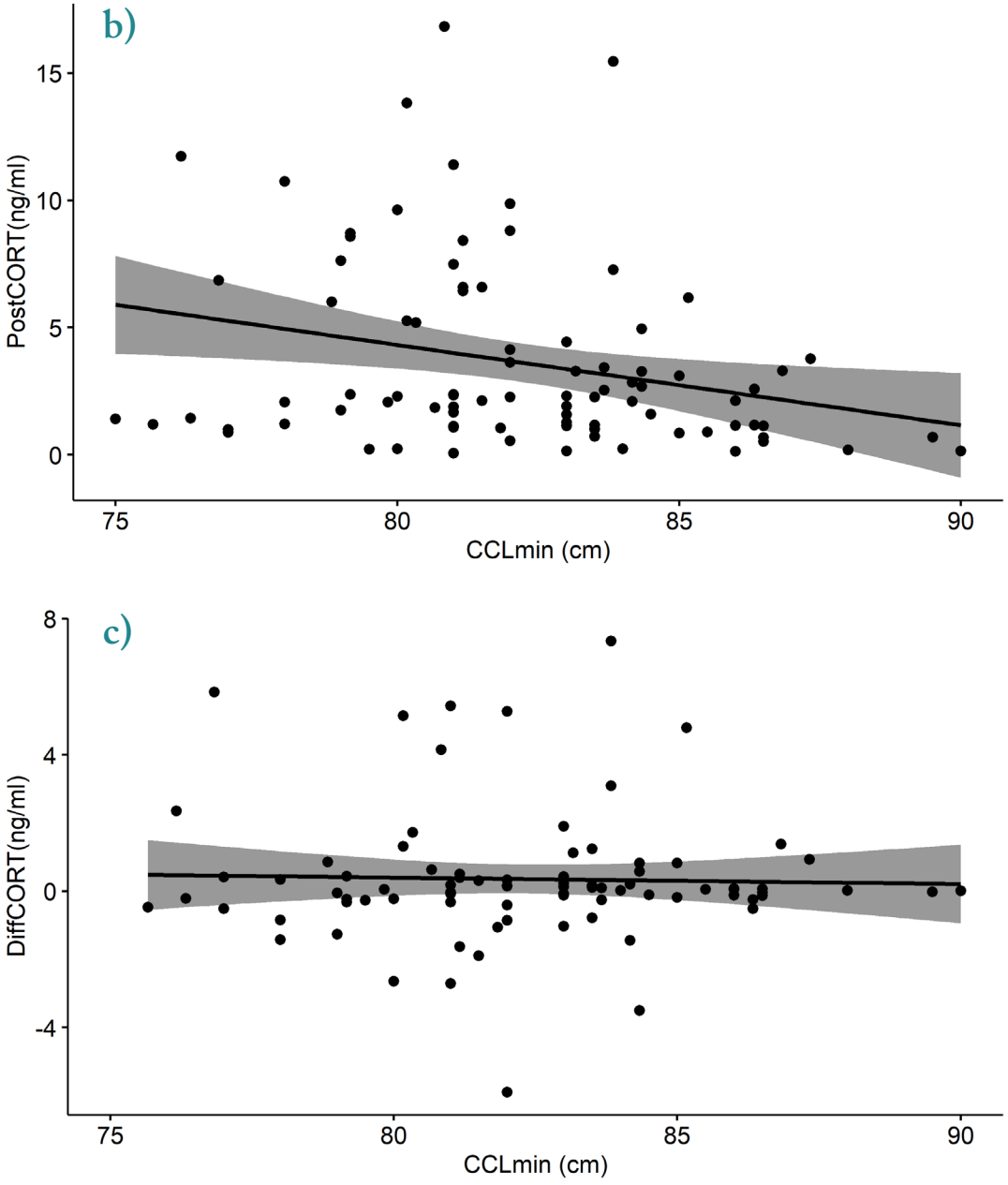


Fig 6.2 a) Negative significant correlation between corticosterone concentration in nesting loggerhead females and their CCLmin before manipulation. b) Negative significant correlation between corticosterone concentration in nesting loggerhead females and their CCLmin after manipulation. c) Correlation between the different of corticosterone concentration before and after manipulation in nesting loggerhead females and their CCLmin.

CORT levels were not correlated with any of the three different types of precedent interactions (*season recaptures:* rho = 0.04, p > 0.05; *total recaptures*: rho = 0.03, p > 0.05; and *total seasons***:** rho = - 0.07, p > 0.05), neither with the number of people around the female (rho= 0.14, p > 0.05).

Nesting success, recapture, interaction type and the number of seasons recaptured did not produce any significant effect on the preCORT levels, neither individually (t = 0.42, p > 0.05; t = -0.26, p > 0.05; t = 0.05, p > 0.05; t = -0.18, p > 0.05, respectively), nor combined (p > 0.05). These parameters together with people group size neither affected postCORT levels (t = 0.54, p > 0.05; t = -0.04, p > 0.05; t = -0.26, p > 0.05; t = -0.84, p > 0.05; t = -0.17, p > 0.05, respectively). Finally, the studied parameters, nesting success (Fig 6.3), recapture (Fig 6.4), interaction (Fig 6.5), people group size (Fig 6.6) or manipulation (Fig 6.7), did not trigger a CORT response to produce a significant difference between pre and post CORT levels (F = 0.94, p > 0.05; F = 0.58, p > 0.05; F = 0.67, p > 0.05; F = 0.97, p > 0.05) (Table 6.1).

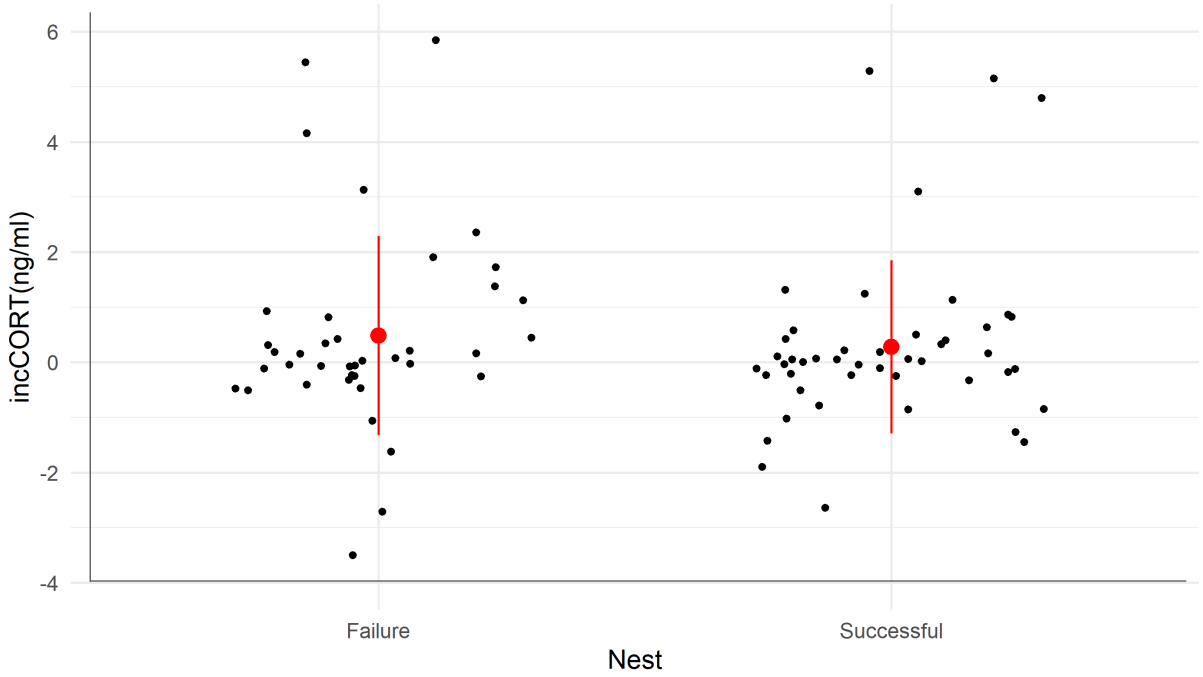


Fig 6.3 Increment of circulating corticosterone concentration divided by nesting process outcome. Big red dots and lines represent the mean and the standard deviation respectively.

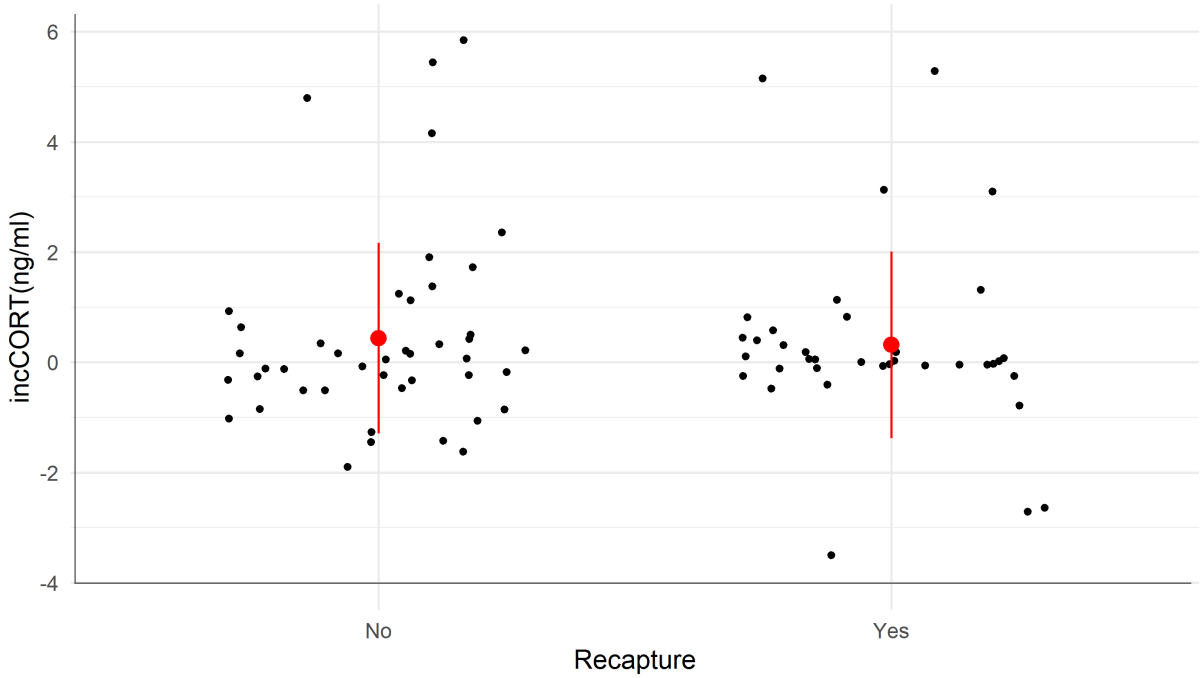


Fig 6.4 Increment of circulating corticosterone concentration divided by if the female has been recaptured or not. Big red dots and lines represent the mean and the standard deviation respectively.

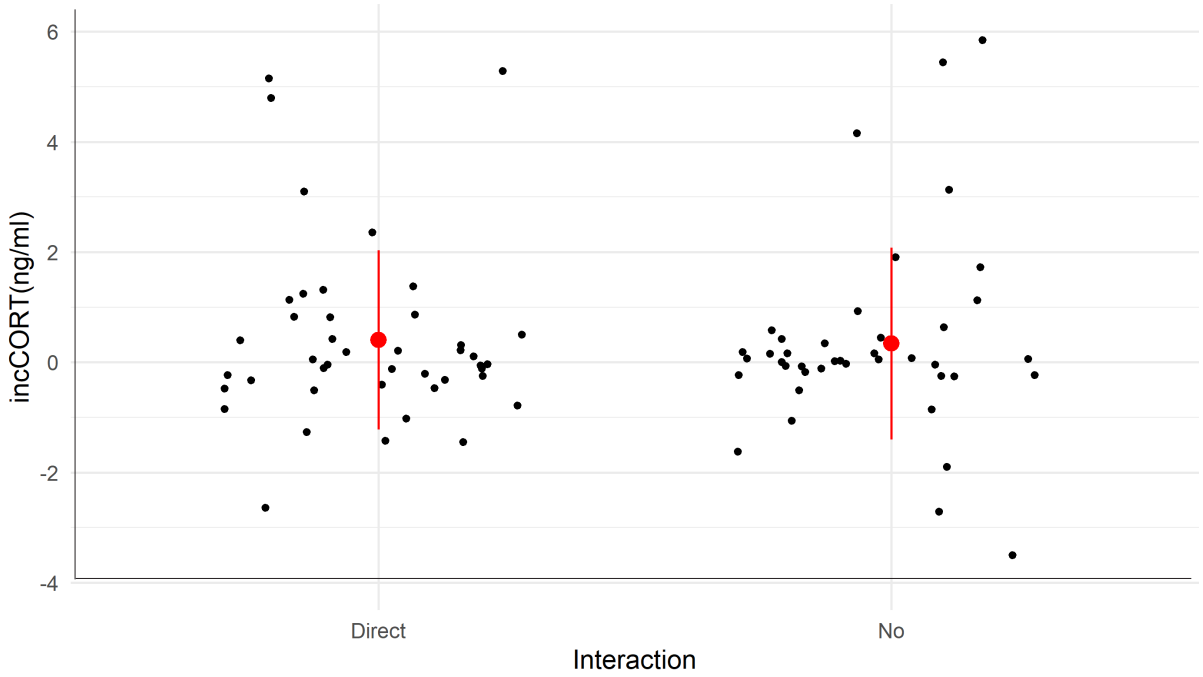


Fig 6.5. Increment of circulating corticosterone concentration divided by the first interaction type. Big red dots and lines represent the mean and the standard deviation respectively.

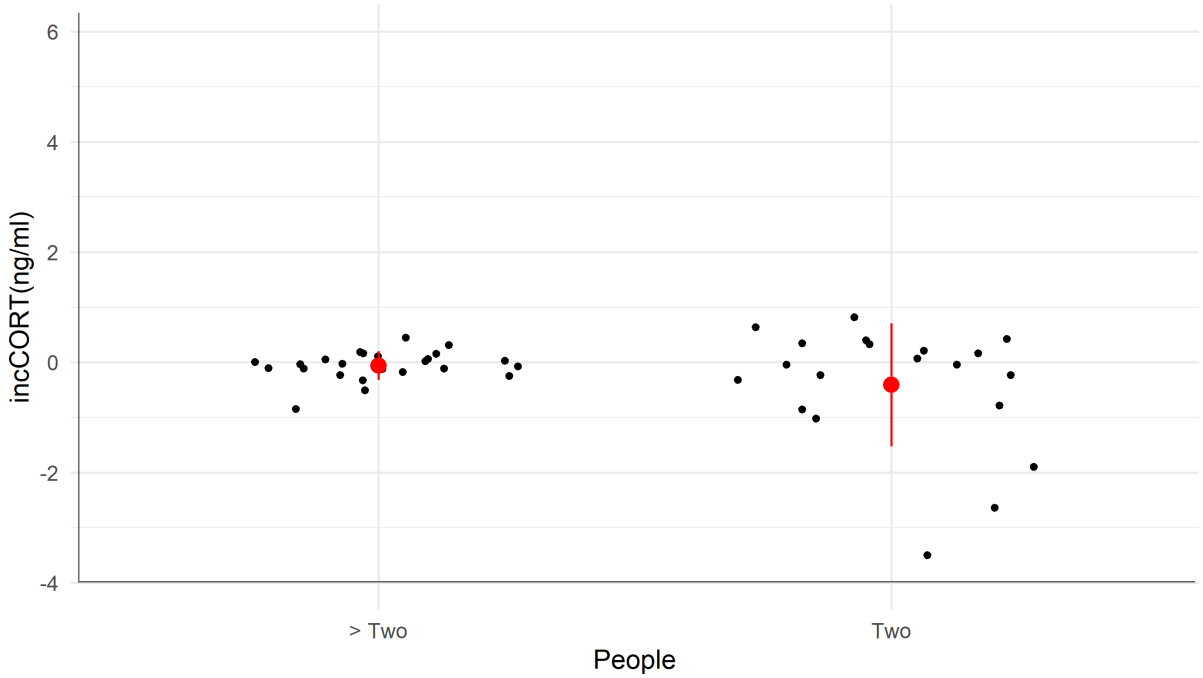


Fig 6.6 Increment of circulating corticosterone concentration divided by the number of people around the female. Big red dots and lines represent the mean and the standard deviation respectively.

Fig 6.7 Corticosterone concentration after (postCORT) and before (preCORT) manipulation. Big red dots and lines represent the mean and the standard deviation respectively.

Table 6.1. Mean corticosterone concentration and SD in ng/ml, before and after manipulation (preCORT and postCORT) of the nesting females as well as the difference between them (IncCort), according to each factor studied.

Factor	Output	preCORT	postCORT	incCORT
Nest	Successful	3.33 ± 3.38	3.48 ± 3.49	0.15 ± 1.80
	Failure	3.75 ± 3.42	4.58 ± 4.45	0.48 ± 1.80
Recapture	Yes	3.97 ± 3.79	3.49 ± 4.32	0.15 ± 2.00
	No	3.17 ± 3.08	4.45 ± 3.76	0.44 ± 1.73
Contact	Direct	4.18 ± 3.63	4.76 ± 4.25	0.26 ± 1.87
	No	2.81 ± 2.96	3.15 ± 3.52	0.34 ± 1.74
People	Two	3.30 ± 3.12	3.29 ± 3.19	-0.41 ± 1.12
	>Two	1.68 ± 2.27	1.62 ± 2.27	-0.05 ± 0.26
Manipulation	-	3.86 ± 3.97	4.15 ± 4.15	0.30 ± 1.80

6.5 Discussion

According to several searchers nesting females reduce the CORT concentration during reproductive season to invest all their available energy in the breeding process (Jessop 2001, Jessop & Hamann 2004, Flower *et al.* 2015). The results obtained in the present study agree with precedent works, where nesting females sampled on Boa Vista Island showed very low CORT levels (average 3.53 ± 3.30 ng/ml).

The present study also found an inverse correlation of CORT levels with female size, where larger females showed lower CORT concentration in their bloodstream. These differences could be explained by the direct link of body-size with fecundity and reproduction effort described Van Buskirk & Crowder (1994) on sea turtles, where larger females invest more energy resources in reproduction process. Even more when the loggerhead population breeding on Boa Vista Island (Cape Verde) present an interesting dichotomy on their foraging behavior linked to their size, where larger females occupying costal foraging

grounds, with more available resources, while the small ones usually are pelagic eaters using the scarcest oceanic habitat (Hawkes *et al.* 2006). Therefore, larger females nesting on Boa Vista Island, feeding on richest neritic habitats, obtain more energy that could be invested on the reproduction process with the subsequent reduction of the plasma CORT concentration (Jessop 2001, Jessop & Hamann 2004, Flower *et al.* 2015, Jessop *et al.* 2004).

In other hand, Avens *et al.* (2015) found a direct relation between the carapace length and the turtle age, where generally larger animals would be older. Moreover, Whittier *et al.* (1997) related the number of nesting episodes of an adult female with the CORT response, suggesting that on sea turtles the previous experience of more nesting episodes is correlated with less CORT levels in blood, where females with more than two breading seasons presented lower CORT levels. The present study did not find this relation in the Northeast Atlantic population, where the number of recaptures, intra and inter seasons, nor the registered number of breeding seasons, showed any significant relation with CORT concentration on blood. The discrepancy between both populations could be caused because Whittier *et al.* (1997) determined the number of breeding seasons by laparoscopy, getting the real number of breeding seasons, and the present study obtained the number of nesting seasons from the number of encounters with researchers on the beach, that could not been the real amount of breeding seasons. So, the results obtained determine that females with more interaction with humans on the beach, during the same year or accumulated along the time, showed no significant differences on CORT response neither on CORT levels.

Other natural factor analyzed was the nesting outcome, founding no significant differences on PreCORT between females that successfully laid the eggs or the ones that fail in the attempt, matching the results obtained by Whittier *et al.* (1997) on loggerhead females nesting in Mon Repos, Australia.

The inhibition of the CORT response in nesting females was also evident in the Cape Verde loggerhead population, when the effect of anthropogenic variables, such as female handling and the number of people around the turtle, were studied. None of the two factors showed any effect on the CORT levels and its response. However, the inexistence of a hormonal response to human presence or manipulation does not mean that their welfare is maintained. Welfare can be assessed using a combination of physiological,

behavioral and health variables (Whitham & Wielebnowski 2013), which include a wide variety of parameters such as spontaneous behavior (Benn *et al.* 2019). Alterations of behavior have been reported when different number of people is present on the beach at night, since human presence interferes in the nesting process of the females, inducing their return to the sea before they complete the nesting process, or scaring them in their rise up the beach, or even inducing a decrease on the nesting activities those days with higher numbers of people on the beach (Jacobson & Lopez 1994, Wilson & Tisdell 2001, Meletis & Harrison 2010).

Even though there were a correlation between the size of the females and their CORT levels, in the present study was not observed a reaction of the Hypothalamic Pituitary Adrenal (HPA) axis, which cause the release of corticosteroids from the adrenal cortex (Greenberg & Wingfield 1987, Gregory & Schmid 2001, Silvestre 2014), as no significant changes were observed in incrCORT or postCORT after any of the different parameters measured, denoting a low responsiveness of the nesting females to potentially stressful events. In olive ridley (*Lepidochelys olivacea*) CORT response has been studied during massive nesting events (*arribada*), establishing that nesting females showed a slower CORT response than non-nesting females, and also, solitary nesters presented a faster CORT response than massive (*arribada*) nesters (Valverde *et al.* 1999). A suppression of the adrenocortical response during the nesting period has been suggested in other sea turtle species, such as hawksbills (*Eretmochelys imbricata*) and green turtles (*Chelonia mydas*) (Jessop 2001). A decreased CORT response has been also observed in other oviparous reptiles during the gravid life-history stage, including female tree lizards (Woodley and Moore, 2002) or tuatara (*Sphenodon punctatus*) (Anderson *et al.*, 2014). This low HPA axis responsiveness during nesting season could be related with an adaptive interruption of certain stress response reactions (e.g. flight response in birds) to achieve a successful nesting process. Also, could be associated with the pre-breeding CORT-flexibility hypothesis, suggesting that the CORT levels during periods preceding breeding delays the onset of breeding in cases where supplemental cues, such as low food availability and inclement weather, indicating no suitable environment for breeding (Lattin *et al.* 2016). The results of the present study do not permit to elucidate possible physiological mechanisms, but changes in binding proteins (CBGs) and/or CORT receptors at target tissues has been suggested to play a role in the CORT response to stress in gravid individuals (Romero, 2002).

An attenuated CORT response could also be a product of adaptive maternal effects such as protecting eggs/embryos from potential deleterious effects of hormones or allowing maternal programming of offspring to occur (Anderson *et al*. 2014). Maternal hormones have been discovered in yolk of hatchlings on certain reptiles (Rhen *et al*. 2006) and increased levels of maternal CORT can influence hatchlings phenotype, behavior and fitness performance. For example, in garter snakes (*Thamnophis elegans*) Robert *et al*. (2009) found that females with increased plasma CORT concentrations produced offspring with decreased anti-predator behavior, and Belliure *et al*. (2004) found decreased activity, sprint speed, and motivation to run in common lizard hatchlings (*Lacerta vivipara*). Also, experimental treatment of females with CORT implants induces increased offspring philopatry to the natal area on common lizard (De Fraipont *et al*. 2000) and gravid lizards in poorest conditions produce offspring that have increased natal philopatry compared to offspring from mothers in good condition. No evidences have been recorded on those mechanisms for sea turtles, thus, further studies are necessary to elucidate the different mechanisms associated to the low responsiveness of HPA axis during sea turtle nesting period.

In conclusion, loggerhead sea turtle nesting females from Cape Verdean rookery present low basal CORT concentration during the nesting season, as described for other loggerhead rookeries and other species. CORT concentration and female size are negatively correlated, where larger females showing reduced CORT concentration, which could suggest that older females had low CORT concentration probably mediated by previous nesting experiences. A low CORT concentration in response to stressful situations has been also recorded that could be indicating a low HPA responsiveness during nesting season, that could be related to a CORT-flexibility during reproduction. In general, the standardize protocols for data collection used by the NGO Cabo Verde Natura 2000 on nesting loggerhead females in Boa Vista Island, had no effect on circulating CORT concentration, suggesting no alteration of welfare on the animals. However, although the standardized protocols show no effect of on CORT concentration, especial care must be taken as behavior can be also modified by human presence and subsequently welfare can be affected.

7

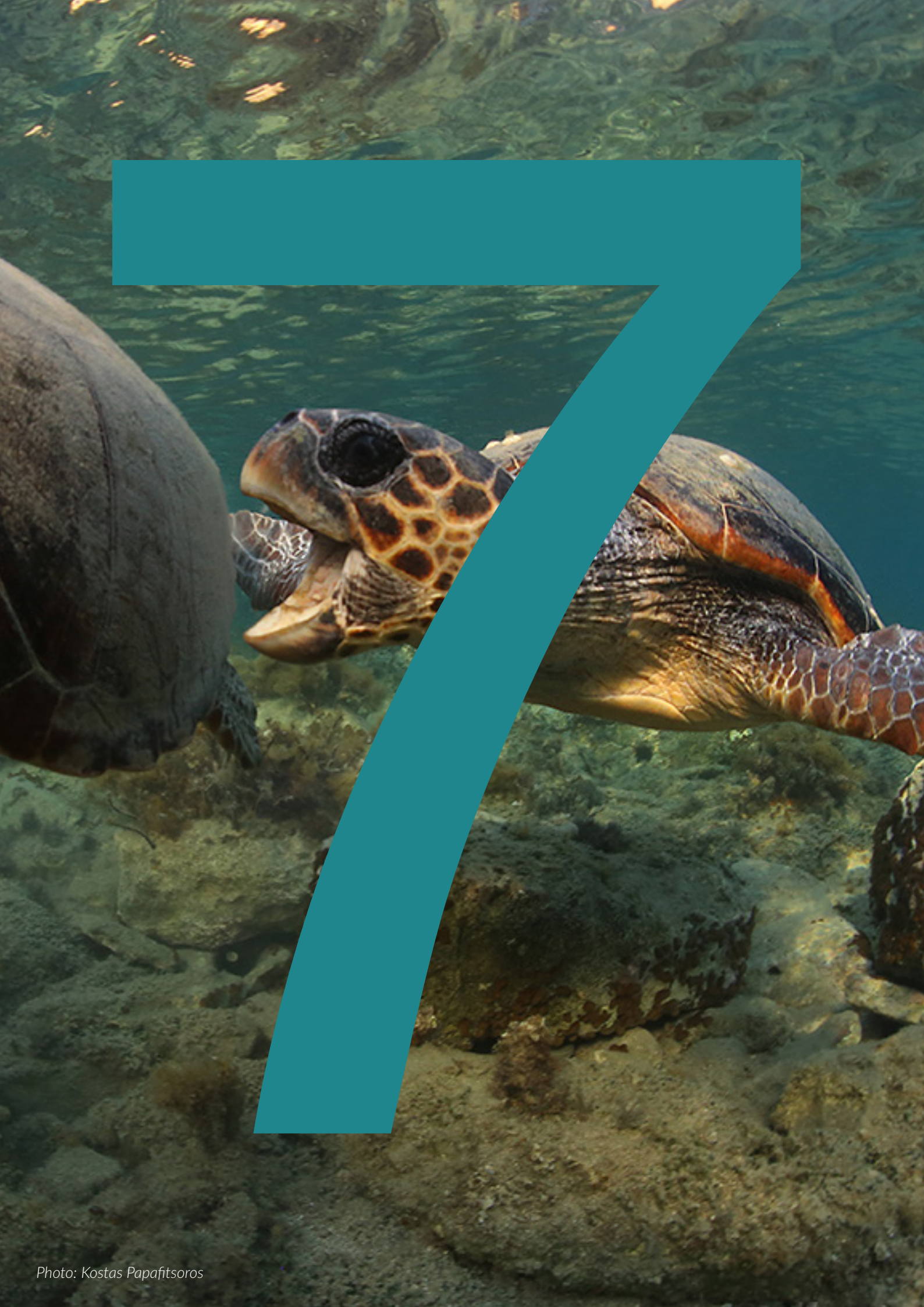


Photo: Kostas Papafitsoros

General Discussion

7.1 Why humans have to interact with sea turtles in the wild or even kept them under controlled conditions?

Humans had interacted with sea turtles since long time ago, the first evidence of this relationship dating from 5000 BCE (Mendonca *et al.* 2016). From that time until today, the perception, the use and the interaction with them has changed. An important transition had occurred in how sea turtles are seen by humans, from being considered as a protein and raw material sources to be a target species for conservation.

This change on the perception was necessary after the indiscriminate fishing and consumption of sea turtles since ancient times, together with other threats that sea turtles have to face in the recent years, such as marine litter, pollution and habitat destruction. These threats have produced a general decline of all its populations and consequently the necessity to include all the seven sea turtle species in the consideration as endangered species (Seminoff 2004, Abreu-Grobois and Plotkin 2008, Mortimer and Donnelly 2008, Wallace *et al.* 2013, Casale and Tucker 2017, Wibbels & Bevan 2019).

The serious situation of sea turtles makes necessary to launch important conservation plans worldwide, but even when sea turtles have been widely studied during the last decades, there are still important gaps of knowledge on sea turtle biology and ecology, as well as human-turtle interactions and threats, which need to be filled in order to improve their situation (Hamann *et al.* 2010).

To increment the knowledge about sea turtles and to apply the adequate conservation and management programs, the interaction human – sea turtles is unavoidable. For example, researchers and conservationist have to interact with adult sea turtles while working on nesting beaches to collect data; and veterinarian and biologists interact with them during the recovery process of injured animals, having to be held under controlled conditions

during certain periods of time. There is also interactions with sea turtle hatchlings during head - starting programs and during some particular research programs, in which hatchlings must be held under controlled condition, to provide insights about some life stages that are very difficult to observe and sample in the wild (Herbst & Jacobson 2002), as well as with the eggs during the incubation process under laboratory conditions. Also, ecotourism related with sea turtles is an important part of the turtle conservation 'toolbox' which also implies a direct interaction human–sea turtles.

7.2 Are there any established protocols to regulate the human-sea turtle interactions?

Depending on the type of interaction, there are different protocols that must be standardized to minimize any kind of deleterious effect on sea turtles. Since conservation of sea turtle started some decades ago, the applied protocols during human – sea turtle interactions had been evolving and improving at the same time as the knowledge of sea turtles has improved. Hence, most of the established protocols to hold sea turtles under controlled conditions are based on experiences from each facility or project and supported by results based on health status, measuring the survival rate or the prevalence of diseases under certain holding conditions, such as temperature or stocking densities. Other indirect health parameters have also been used such as an adequate growth, proper feeding and no aggressive behavior. In the particular case of recovery of injured wild sea turtles, the protocol applied should be determine by the animal recovery process itself and by the treatments required. (Fish and Wildlife Service 2013, Bluvias & Eckert 2010).

While interacting with sea turtles in the wild, the followed protocol is mainly based on the minimum interference with biological processes. For example, during the conservation programs conducted on the nesting beaches, the data obtained from females must be collected or during or just after the oviposition, but never before the eggs laying, to avoid altering the success of the nesting event (Varo Cruz *et al.* 2006).

During the last years, animal welfare has been gaining in importance and it is widely accepted that welfare can be measured through behavior, levels of physiological

indicators (i.e. stress indicators) or other indirect parameters such as the absence of illness or the proper appetite (Arena & Warwick 1995). So, it could be said that protocols followed during human - sea turtle interaction for the last decades have been partially based on welfare parameters even if hardly ever the word "welfare" can be read in the literature about sea turtles. Even, the welfare in sea turtles have never been assessed from the physiological point of view as it has been done in other reptiles by measuring neuroendocrine products released to blood stream after the activation of the HPA axis as a consequence of a stressful event. In sea turtles, the main endocrine product released to bloodstream in the physiological regulation of stress response is the corticosterone, which also has an indirect role as modulator of the behavior and the indirect parameters used to assed welfare. The present studies aimed to verify if standard protocols used on the interaction human-sea turtle in different life-stages, are adequate based on welfare parameters.

7.3 For an adequate and successful incubation of sea turtle eggs, which parameters should be considered?

The incubation of sea turtle eggs is modulated by different factors, like hydric properties of the substrate and the temperature (Reece *et al.* 2002, Booth *et al.* 2004). These factors, together with a combination of maternal phenotype and fitness during egg formation would affect the posterior hatchling phenotype and performance (Hewavisenthi & Parmenter 2001, Andrews 2004, Glen *et al.* 2003, Booth 2006, Booth *et al.* 2004, Burgess *et al.* 2006, Reece *et al.* 2002). The temperature is one of the most important parameters determining the success of the incubation, as temperatures out of the range between 23 °C and 33 °C for large periods of time, would kill the embryos (Miller 1997).

During most of the incubations of sea turtle eggs performed under laboratory conditions, temperature has been kept stable without considering its effect over posterior hatchlings phenotype and performance, while under natural conditions nest temperature oscillate over the course of incubation (Packard & Packard 1988, Plumme *et al.* 1994, Shine *et al.* 1997). Results from Chapter 3 showed that, during the eggs incubation under laboratory conditions, temperature regime (high or low temperature) is an important

modulator of hatchlings performance, but the temperature pattern (constant or variable) have to be also considered, since incubation at unchanging temperatures produce smaller hatchlings and decrease hatching success.

7.4 If conservation programs or research projects require holding hatchlings under controlled conditions, what are the main welfare parameters to be aware of?

As it has been explained before, the protocols used while holding hatchlings under controlled conditions are partially based on welfare parameters, mainly based on indirect parameters, such as absence of illness, proper appetite and proper growth rates, or some direct parameters like the behavior of the animals with their conspecifics. Those parameters have been used to stablish the adequate seawater temperature on the facilities, between 23 - 31 °C, based on mortality observed at temperatures below 20 °C. The adequate stocking densities depend on the species and the facilities. For example, the loggerhead and Kemp's Ridley hatchlings have been held isolated to avoid aggressive behavior (Caillouet Jr. 2000) whereas in Cayman islands green turtle hatchlings have been held at high densities, ranging from 300 to 30 hatchlings per tank based on their body weight (Márquez *et al.* 1992). The cleaning procedures or direct handling of the hatchlings vary between the requirements of the different facilities depending on particular necessities, but most of the time, the periodicity of those procedures is stablished to avoid deleterious effects on the hatchlings.

Results obtained in Chapter 4 showed that there was no negative increase of circulating CORT when hatchlings were held at a sea water temperature ranged between 26 ° and 28 °C. On the other hand, isolation of loggerhead hatchlings for a period of 6 months induced higher CORT levels than their conspecifics held together in the same tank. Thus, isolation of the hatchlings must be refused in order to maintain their welfare, as isolation induced a chronic elevation of plasma CORT.

Even when hatchlings have been reared in some facilities, there is not a clear protocol on how periodical handling and cleaning process must be done. After studies with loggerhead hatchlings performed in Chapter 4, a periodicity and handling duration for

hatchlings can be stablished. The use of a frequent handling protocols (once per week) in shorter periods did not compromised hatchlings welfare, where no circulating CORT response was observed, while animals with low frequent manipulation (once every 2 weeks) presented higher basal CORT concentration in blood. The manipulation during this experiment was not higher than 2 min and no CORT response was observed so in consequence, handling can last at least 2 minutes.

7.5 When loggerhead juveniles must be hold under controlled conditions, do they have similar husbandry requirements as hatchlings?

Considering the comparison among results from Chapter 4 and 5, it seems that under controlled conditions loggerhead juveniles are less restrictive in terms of welfare parameters than hatchlings. While isolated hatchlings from Chapter 4 presented high CORT levels, juveniles on Chapter 5 did not showed CORT response when they were isolated from their conspecifics for 9 days. In addition, on multiple - occupancy of the tank for short periods no aggressive behavior was observed between individuals, and CORT levels found during all experimental stocking densities were similar to levels found in wild loggerhead juveniles (Gregory *et al.* 1996). It can be established that protocols of isolation or increase of the multiple - occupancy of the tank can be applied for short-periods (9 days) without affecting loggerhead turtle CORT, suggesting no variations in welfare.

The previously established protocols for sea turtle juveniles suggested that sea turtle handling and its dry-docking time must not exceed 30 min to prevent carapace desiccation and peeling, that could provoke incidences of opportunistic pathogen (Higgins 2003). However, as demonstrated in Chapter 5, dry-docked time higher than 15 min induced a rise of circulating CORT, suggesting consequently a deleterious effect on welfare. Besides, handling frequency (twice per week) did not induced changes in circulating CORT of juveniles from this study (Chapter 5), being their CORT levels similar to those reported for wild loggerhead juveniles (Gregory *et al.* 1996). Besides, no variations of juvenile behavior have been found under the different standardized protocols assayed, suggesting all together no effect or compromise of their welfare.

7.6 Could the new insights found on welfare under controlled conditions be extrapolated to adults nesting females?

Taking an overview of the results from the different chapters together, it could be suggested the fact that as loggerhead turtles get bigger, the influence of external factors over the circulating CORT decreased. Hatchling incubation (Chapter 3) is influenced by temperature regime and temperature pattern, as well as by moisture and by maternal phenotype and fitness; the welfare of hatchlings held under controlled conditions (Chapter 4) is affected by handling frequency and stocking densities but not by the same range of temperature that affects embryo performance during incubation (Chapter 3). Juveniles (Chapter 5), are less sensitive to stocking density and isolation compared to hatchlings, where circulating CORT is only affected by the dry-docking time, and no alterations of behavior occurs after standardized protocols assayed, suggesting low or no variation of their welfare. Finally, the welfare at a physiological level on loggerhead females during the nesting season in Boa Vista (Cape Verde) (Chapter 6) presented low CORT levels if compared with hatchlings and juveniles, suggesting that adult females did not invest energy in the CORT response destinating most of it to reproduction, as described by (Jessop 2001). In general, loggerhead nesting females were not affected by the interaction human – sea turtle, no CORT response were observed, even when they spend long time out the water during the nesting process (around 1 hour), while welfare of loggerhead juveniles was compromised after been 15 min out of the water (Chapter 4).

So, the new insights found about welfare for hatchlings and juveniles (Chapter 4 – 5) cannot be extrapolated to adults nesting females on the beach, since adult females present a very low or even no response of the HPA axis during the nesting season. However, other parameters like different aspects of behavior or other hormones have to be studied to be able to assed other aspects of their welfare.

7.7. Could some recommendations be givento improve the standardization of the protocols, based on the welfare results obtained?

As preliminary results conducted within this species under controlled conditions, and taking into account the variations of circulating CORT, consider as indicator of welfare,

different recommendations can be suggested to improve the standardized protocols used during human – sea turtle interaction at different levels of the loggerhead sea turtle life cycle, that are summarized within the Table 7.1.

*Table 7.1. Summary of previous protocols together with different recommendations suggested to improve the standardized protocols during human – sea turtle interaction at different levels of the loggerhead sea turtle life cycle. *according to sea turtles body weight **depending on seawater temperature.*

	Incubation	Hatchlings	Juveniles	Source
Temperature	Between 23-33 °C Variable pattern (27-32°C)	 Between 23-31 °C Between 26-28 °C	 Between 23-27 °C Between 18-24°C	Miller (1997) Chapter 3 Caillouet Jr (2000) Chapter 4 Kakizoe *et al.* (2010, 2013) Chapter 2
Handling		3 times per week Every day Once per week	 Twice per week	Caillouet Jr (2000) Márquez *et al.* (1992) Chapter 4 Chapter 5
Density		30-300ind /600 L 3-9 ind / 100 L	 Three ind/5.000L Punctual variation (1 and 5 ind/tank, 9 days)	Márquez *et al.* (1992) Chapter 4 Chapter 5 Chapter 5
Dry-docking		2 min	 30 min 15 min maximun	Chapter 4 Higgins (2003) Chapter 5
Food amount*		2% 5% ad libitum	 0.5% 0.0 - 0.8 %** 2 - 2.5 %**	Caillouet Jr (2000) Chapter 2 Márquez *et al.* (1992) Márquez *et al.* (1992) Kakizoe *et al.* (2010, 2013) Chapter 2
Feeding regime		Every day Monday to Saturday	 3 times/week	Caillouet Jr (2000) Chapter 2 Kakizoe et al. (2010), Chapter 2

Photo: Alejandro Usategui Martín

Conclusions

1. Under laboratory conditions, the temperature range of 30.5 - 32.0°C is the most adequate for the incubation of loggerhead eggs from the Northeast Atlantic population, because this temperature regime induces shorter incubations and smaller and better fit hatchlings, when compared to lower temperatures.
2. Under laboratory conditions, a variable temperature regime along the incubation period, mimicking temperature oscillations in natural nests, induces the increase of the weight and length on loggerhead hatchlings, without altering their fitness or the incubation time.
3. Handling protocol conducted each week does not affect basal circulating corticosterone concentration, but larger interval (2 weeks) induces an increment, suggesting an adaptation of the loggerhead hatchlings to frequent standardized handling protocols.
4. The isolation of loggerhead hatchlings under controlled conditions increased circulating CORT levels, denoting a poor welfare.
5. Dry-docking time for loggerhead juveniles must be no longer than 15 min.
6. On juvenile loggerheads, punctual change in the number of turtles per tank, subjected to husbandry necessities and for short periods (until 9 days), does not induce changes of the circulating CORT levels or the behavior parameters, suggesting no deleterious effect on their welfare.
7. The standardized protocols for data collection publish by the NGO Cabo Verde Natura 2000 (Varo-Cruz *et al*, 2006) for nesting loggerhead females from the Northeast Atlantic Population, do not modify the circulating CORT concentration, suggesting no alterations of welfare under those standardized protocols.

8. There is an inverse correlation between the CCLmin and the circulating CORT levels in nesting loggerhead females from the Northeast Atlantic population.
9. Based on circulation CORT levels, loggerhead sea turtles are more susceptible to environmental or human induced alteration factors during the early stages of their life cycle. In consequence particular attention must be paid to standardize the protocols during that stages.

Photo: Ege Yacindag

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Photo: Kostas Papafitsoros

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