Ecology and conservation of the leatherback sea turtle (*Dermochelys coriacea*) nesting in Brazil

Submitted by

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I certify that all material in this thesis that is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

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Female leatherback turtle returns to the sea after nesting in Espírito Santo, Brazil

Abstract

Knowledge of life-history parameters of marine turtles is essential for understanding their ecology and for effective conservation. The leatherback turtle (*Dermochelys coriacea*) is globally classified as Vulnerable by the International Union of Conservation of Nature (IUCN), however, distinct subpopulations are recognised and require specific data for the assessment of their conservation status. The Southwest Atlantic Ocean subpopulation is known to regularly nest only in eastern Brazil, on the coast of the state of Espírito Santo. This thesis presents a collection of chapters that investigate the ecology and conservation of this taxon. Multiple technologies and methodologies are employed to ask both ecological and management questions, assessing key population parameters to inform conservation. Here I use long-term data (30 years) to investigate the nesting ecology, population trends and conservation status of leatherback turtles in Espírito Santo. I explore their nesting environment and provide baseline data which will guide further research and management strategies under future scenarios of climate change. I investigate the marine habitat use through stable isotope approaches and satellite telemetry to infer spatial ecology, setting out the conservation situation and perspectives of this important population. Finally, I assess the potential impacts of artificial light on wildlife, using marine turtles in Brazil as a case study. The leatherback turtle rookery in Espírito Santo shows signs of population recovery, however, the existence of various threats means this population remains subject of conservation concern.

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Author's declaration

All chapters within this thesis were written by L. P. Colman under the supervision of B. J. Godley and A. C. Broderick at the University of Exeter and under mentorship of Joca Thomé and Cecília Baptistotte from Projeto TAMAR (the Brazilian Sea Turtle Conservation Programme) in Brazil. This work was funded by a CNPq (National Council for Scientific Development, Brazil) scholarship grant (207684/2014-6) with additional funding awarded by The Rufford Foundation and British Chelonia Group for the fieldwork activities to be conducted in Regência, Brazil, with the support of Projeto TAMAR. All stable isotope samples were prepared in the Daphne du Maurier research laboratories with samples analysed for Carbon and Nitrogen at Elemtex, Launceston. Platform Terminal Transmitters (PTTs) attached in 2017 were SPLASH10-295C (Wildlife Computers), funded by FUNBIO (Brazilian Biodiversity Fund) in collaboration with the Chico Mendes Institute for Biodiversity Conservation (ICMBio)/Ministry of Environment (MMA). Data processing was conducted in the satellite Tracking and Analysis Tool (STAT) facilitated by seaturtle.org. Fieldwork in Espírito Santo, Brazil was conducted as part of the Projeto TAMAR standard monitoring activities under the supervision of J. Thomé and C. Baptistotte, with further assistance by A. Marcondes, J. Borini, F. Ribeiro, and L. Vila-Verde in addition to the numerous volunteers who collected tissue and egg yolk samples, nesting and temperature data and helped during the deployment of satellite transmitters. Specific author contributions are detailed below:

Chapter I: Thirty years of leatherback turtle (*Dermochelys coriacea*) nesting in Espírito Santo, Brazil, 1988-2017: reproductive biology and conservation

Liliana P. Colman, João C. A. Thomé, Antônio de P. Almeida, Cecília Baptistotte, Paulo C. R. Barata, Annette C. Broderick, Flávia A. Ribeiro, Lucas Vila-Verde, Brendan J. Godley

LPC, JCAT, APA, CB, FAR and LV planned and conducted fieldwork. LPC, JCAT, APA and CB coordinated fieldwork logistics and research permits. LPC assembled and analysed data, produced all figures and tables, and was the lead author on the manuscript. JCAT, CB, PCRB, AB and BJG provided guidance on data analysis and writing and all co-authors provided useful comments on the manuscript.

Chapter II: Stable isotope analysis and satellite tracking of Southwestern Atlantic leatherback turtles (*Dermochelys coriacea*): habitat use and female-offspring relationships

Liliana P. Colman, João C. A. Thomé, Cecília Baptistotte, Annette C. Broderick, Julia C. Haywood, Ana C. Marcondes, Evandro A. de Martini, Thaís Pires, Flávia A. Ribeiro, Lucas Vila-Verde, Matthew J. Witt, Brendan J. Godley

LPC, JCAT, CB, ACM, TP, FAR and LV planned and conducted fieldwork. LPC, JCAT, CB, EAM, TP and ACM coordinated fieldwork logistics and research permits. LPC assembled and analysed data, produced all figures and tables, and was the lead author on the manuscript. MJW provided training on satellite tag configuration and deployment. EAM supported the logistics regarding satellite telemetry. JCAT, CB, AB, JCH, MJW and BJG provided guidance on data analysis and writing and all co-authors provided useful comments on the manuscript.

Chapter III: Estimating hatchling sex ratios of leatherback turtles (*Dermochelys coriacea*) in the Southwestern Atlantic Ocean

Liliana P. Colman, João C. A. Thomé, Cecília Baptistotte, Annette C. Broderick, Ana C. Marcondes, Ana Rita Patrício, Flávia A. Ribeiro, Dominic Tilley, Lucas Vila-Verde, Brendan J. Godley

LPC, JCAT, CB, ACM, FAR and LV planned and conducted fieldwork. LPC, JCAT, CB and ACM coordinated fieldwork logistics and research permits. LPC assembled and analysed data, produced all figures and tables, and was the lead author on the manuscript. JCAT, CB, AB, ARP, DT and BJG provided guidance on data analysis and writing and all co-authors provided useful comments on the manuscript.

Chapter IV: Assessing coastal artificial light and potential exposure of wildlife at a national scale: the case of marine turtles in Brazil

Liliana P. Colman, Paulo H. Lara, Jonathan Bennie, Annette C. Broderick, Juliana R. de Freitas, Ana C. Marcondes, Matthew J. Witt and Brendan J. Godley

LPC, PHL, ACM and BJG planned the study design. Data was collected by Projeto TAMAR in Brazil. JB, JRF and MJW provided help with the treatment and interpretation of remote sensing data. LPC assembled and analysed data, produced all figures and tables, and was the lead author on the manuscript. PHL, AB, ACM, MJW and BJG provided guidance on data analysis and writing and all co-authors provided useful comments on the manuscript.

List of notations and abbreviations

Notations:

δ – Gamma

Abbreviations:

BA – Bahia

CB – Comboios

CCL – Curved carapace length

CE – Ceará

CNPq – National Council for Scientific and Technological Development

DC – Leatherback turtle, *Dermochelys coriacea*

EI – Hawksbill turtle *Eretmochelys imbricata*

ES – Espírito Santo

FUNBIO – Brazilian Biodiversity Fund

GAM – Generalised additive model

GIS – Geographical Information System

GLMM – Generalized linear mixed model

GU – Guriri

I – *In situ*

ICMBio – Instituto Chico Mendes de Conservação da Biodiversidade

ID_{mid} – Middle third of the incubation duration

IUCN – International Union of Conservation of Nature

LO – Olive ridley turtle, *Lepidochelys olivacea*

MMA – Ministry of Environment (Brazil)

MND – median nesting date

MTRG – Marine Turtle Research Group

NS – Northern loggerhead turtle (*Caretta caretta*) stock

P – Relocated to other section of the beach

PG - Pontal do Ipiranga

PIT – Passive integrated transponder (tag)

PTT – Platform Terminal Transmitter

PV – Povoação

Rebio – Reserva Biológica de Comboios

RJ – Rio de Janeiro

SC – Santa Catarina

SE – Sergipe

SIA – Stable isotopes analysis

SS – Southern loggerhead turtle (*Caretta caretta*) stock

SSF – Small scale fisheries

STAT – Satellite Tracking and Analysis Tool

TAMAR – Brazilian Sea Turtle Conservation Programme

TRT – Transitional range of temperatures

TSD – Temperature-dependent sex determination

TSP – Thermosensitive period

T – Relocated to open-air hatcheries

General introduction

Marine turtles spend most of their life at sea. After emerging from clutches on nesting beaches, hatchlings crawl towards the sea and generally swim until they reach oceanic waters, where they are believed to follow favourable ocean currents and spend several years (Putman 2018). Juveniles and sub-adults of most species are believed to be pelagic, however their study in the open ocean is difficult and their habitat requirements are not completely understood (Wildermann et al. 2018). Upon reaching maturity, marine turtles migrate to breeding areas and females come to shore to nest. During the breeding season, adult turtles generally stay in inshore internesting habitats for approximately 3-4 months, laying eggs several times on the nearby nesting beaches during the period (Eckert et al. 2012). Marine turtles play important roles in ecosystems and many populations are currently of conservation concern, mainly due to anthropogenic threats such as overexploitation, fisheries bycatch, pollution and climate change (McClenachan et al. 2006, Soykan et al. 2008, Hawkes et al. 2009, Hamann et al. 2010, Fuentes et al. 2011, Duncan et al. 2017).

The leatherback turtle (*Dermochelys coriacea*) is the largest of the seven extant turtle species and has a circumglobal distribution (Fig. 1). Their diet is specialized on gelatinous zooplankton (Bjorndal 1997), which can have seasonal abundance in temperate and coastal areas (Gibbons & Richardson 2009). The turtles generally migrate vast distances between reproductive and foraging habitats, generally nesting on tropical beaches and also found foraging at high latitudes (James et al. 2005a, Stewart et al. 2011, Fossette et al. 2014). These areas can be thousands of kilometres apart, and leatherback turtles use navigational cues that may include geomagnetic fields and currents (Shillinger & Bailey 2015), allowing them to migrate to their natal nesting beaches (Dutton et al. 1999) and specific feeding grounds (James et al. 2005b).

Significant leatherback turtle nesting populations in the Atlantic Ocean exist in French Guiana-Suriname (Girondot & Fretey 1996, Hilterman & Goverse 2007) and Trinidad (Eckert et al. 2012), whereas the single largest rookery worldwide is located in Gabon in the eastern Atlantic (Witt et al. 2009). Nesting is currently widespread and occurs throughout the Caribbean on many island and mainland beaches (Ordoñez et al. 2007, Garner et al. 2017). In the Pacific, major rookeries are located in Costa Rica (Santidrián Tomillo et al. 2007) and in lesser extent in Mexico (Sarti Martínez et al. 2007). The two ocean basins currently present different population trends, with Atlantic populations being reported to have stable or increasing trends (Dutton et al. 2005, Stewart et al. 2011), while Pacific populations have declined drastically, with many existing at relictual levels today (Spotila et al. 2000, Mazaris et al. 2017). In the Western Indian Ocean, nesting occurs on the coast of South Africa (Hughes 1996). In the Southwest Atlantic Ocean, leatherback turtles are known to regularly nest only in eastern Brazil, on the coast of the state of Espírito Santo (Thomé et al. 2007). This rookery is genetically distinct (Dutton et al. 2013) and represents a unique Regional Management Unit (Wallace et al. 2010). This Southwest Atlantic Ocean subpopulation is regionally classified as Critically Endangered by the IUCN (Wallace et al. 2013), mainly due to the small population size and restricted geographic distribution.

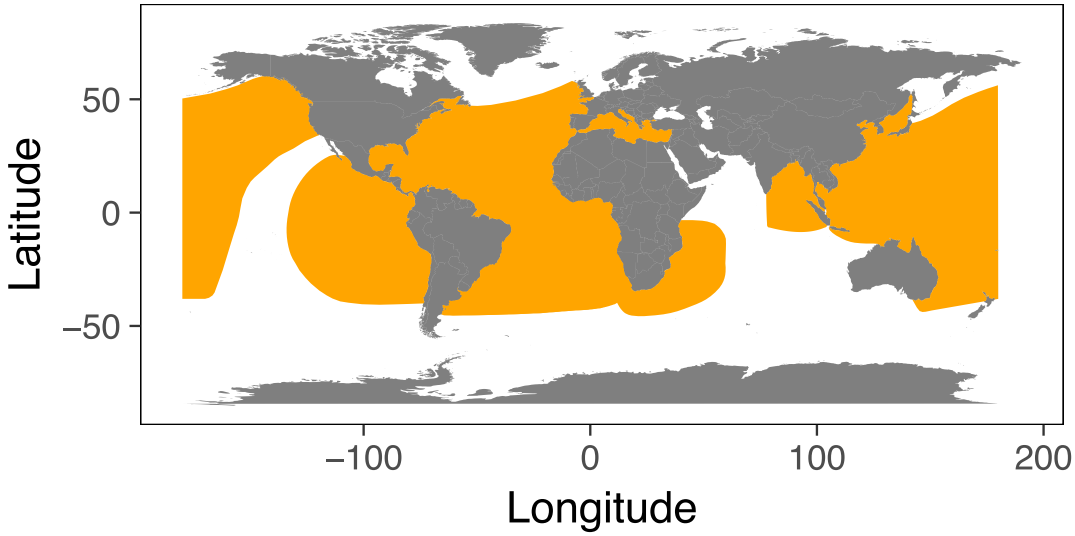

Figure 1. Global map of leatherback turtle (*Dermochelys coriacea*) distribution. Data from the International Union for the Conservation of Nature (IUCN, https://www.iucnredlist.org/species/6494/43526147).

In migratory, long-lived and late maturing species such as marine turtles (Avise et al. 1992, Zug et al. 2002), research has shown that effective conservation strategies should consider multiple techniques to understand ecology and threats (Seminoff et al. 2012, Fossette et al. 2014). Knowledge of key ecological parameters, such as population size and trends, distribution, movements and connectivity between habitats are essential to assess population viability, dynamics and resilience, as well as to evaluate conservation efforts and to identify and mitigate emerging threats.

In the present thesis, **'Ecology and conservation of the leatherback sea turtle (*Dermochelys coriacea*) nesting in Brazil'**, in four chapters, written as independent units of study, I use multiple techniques to explore the reproductive biology, nesting environment, population trends, threats and conservation of a globally important leatherback turtle population in Brazil. I look into their marine habitat use for a more complete understanding of the foraging strategies; and I use the case of four species of marine turtles nesting in Brazil to assess the potential impacts of anthropogenic threats, which may cause shifts in population dynamics and impact recovery rates (Santidrián Tomillo et al. 2017).

In the first chapter, "**Thirty years of leatherback turtle (*Dermochelys coriacea*) nesting in Espírito Santo, Brazil, 1988-2017: reproductive biology and conservation"**, I use a long-term dataset to investigate reproductive parameters and model population trends for leatherback turtles nesting in Brazil. My results show that a previous exponentially increasing population trend for this population was not sustained through time, and although increasing, the trend in the annual number of nests in this population is variable. Additionally, I show that there were no observable impacts from a large-scale mining accident that occurred in the region on hatching success of leatherback turtles in Espírito Santo.

In the second chapter, **"Stable isotope analysis and satellite tracking of Southwestern Atlantic leatherback turtles (*Dermochelys coriacea*): habitat use and female-offspring relationships"**, I investigate the marine habitat use of leatherback turtles in Brazil and infer animal movements. I use a forensic approach combined with satellite telemetry to gain insights into migratory strategies of this population. I identify a dichotomy in foraging strategies, with possible consequences for the susceptibility to threats in this population. I also offer additional methodological insights into using offspring tissues as proxies for assessing stable isotope data in female leatherback turtles. My findings underline the importance of understanding patterns of movements and can be directly applied into management, with further studies needed to better determine internesting and migratory habitat use and the location of feeding areas, potentially contributing to the establishment of protected areas.

In the third chapter, **“Estimating hatchling sex ratios of leatherback turtles (*Dermochelys coriacea*) in Brazil”**, I investigate incubation temperatures within nests in Espírito Santo and estimate, for the first time, the hatchling sex ratios being produced in this rookery. I use monitored temperatures to estimate the sex ratios for leatherback turtle hatchlings across three nesting seasons. My results suggest that, although with considerable interannual variation, this population is likely to be producing modestly female-biased hatchling sex ratios. A long-term decrease on the average incubation duration, however, suggests potential feminization, which may become a concern considering future scenarios of climate change.

In the fourth chapter, “**Assessing coastal artificial light and potential exposure of wildlife at a national scale: the case of marine turtles in Brazil”**, I use a long-term, nationwide data set on marine turtle nesting combined with available remote sensing imagery to assess the extent to which nesting populations of four marine turtle species - leatherback (*Dermochelys coriacea*), olive ridley (*Lepidochelys olivacea*), hawksbill (*Eretmochelys imbricata*) and two subpopulations of loggerhead (*Caretta caretta*) turtles - are potentially exposed to light pollution across the Brazilian coast. I show that light levels have increased in most nesting areas, with different species/subpopulations being impacted variably. Additionally, I identify conservation hotspots, which are majorly located in areas potentially exposed to light pollution. Interestingly, the status of all species/subpopulations has improved across the time period despite increased light levels. My findings suggest that conservation strategies in Brazil appear to have been largely successful, however, it is possible that impacts of artificial light on population recruitment may take longer to fully manifest in nesting numbers.

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Chapter I: Thirty years of leatherback turtle (*Dermochelys coriacea*) nesting in Espírito Santo, Brazil, 1988-2017: reproductive biology and conservation

This chapter is in the process of being revised following review for *Endangered Species Research*

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Abstract

In the southwestern Atlantic Ocean, leatherback turtles (*Dermochelys coriacea*) are only known to regularly nest in eastern Brazil, on the coast of the state of Espírito Santo. Here, we present an analysis of the nesting ecology, population trends and conservation status of this leatherback turtle colony between 1988 and 2017. We observed an increasing, although variable, trend in the annual number of nests, with the mean annual number of nests increasing from 25.6 in the first five years of the study to 87.0 in the last five years. Concurrently, there was also a significant decrease in the mean curved carapace length of the population, which we hypothesize was caused by recruitment of new females to the nesting population. Throughout the study period, nests were concentrated on the southern part of the 160 km long study area. No change was observed in the annual median nesting date. Mean annual hatching success was 66.0% and no significant effect on hatching success was detected after the major spill of mining tailings on the nesting area in 2015. We postulate that the local conservation actions that started in the 1980s have contributed to the gentle recovery of this population; however, given the small population size and restricted nesting geographical distribution, alongside the persistence of various threats - fisheries bycatch, climate change, pollution and coastal development - this population continues to be of conservation concern.

Introduction

Leatherback turtles (*Dermochelys coriacea*) are highly migratory animals, often moving vast distances between nesting and foraging habitats (James et al. 2005, Hays et al. 2006, Shillinger et al. 2008, Fossette et al. 2014, Horrocks et al. 2016). Estimating population size for widely-dispersed species can be feasible if these species form seasonal aggregations, as happens with seabirds (Patterson et al. 2008, Lynch et al. 2010), whales (Lindsay et al. 2016), and marine turtles (Stokes et al. 2014). Marine turtles congregate during the breeding season, with individuals generally staying in inshore internesting habitats for approximately 3-4 months, laying eggs several times on the nearby nesting beaches during the period (Eckert et al. 2012). Assessments of the numbers of nests laid on the nesting beaches can then be used as an indicator of population size (Spotila et al. 1996, Gerrodette & Taylor 1999).

The leatherback turtle is globally classified as Vulnerable by the International Union of Conservation of Nature (IUCN; Wallace et al. 2013b). The existence of distinct subpopulations (Wallace et al. 2010a) however, require specific data for the assessment of their conservation status. The Southwest Atlantic Ocean subpopulation is known to regularly nest only in eastern Brazil, on the coast of the state of Espírito Santo (Thomé et al. 2007). This population, which is regionally classified as Critically Endangered by the IUCN (Wallace et al. 2013b) and listed on the Brazilian government’s register of endangered species (Machado et al. 2008), is genetically distinct from others in the Atlantic (Dutton et al. 2013) and is considered a unique Regional Management Unit (Wallace et al. 2010a). Occasional leatherback nests, possibly by turtles from subpopulations other than the Southwest Atlantic Ocean one, are recorded elsewhere along the Brazilian coast (Soto et al. 1997, Barata & Fabiano 2002, Loebmann et al. 2008, Bezerra et al. 2014, Gandu et al. 2014).

Projeto TAMAR (the Brazilian Sea Turtle Conservation Programme) started monitoring marine turtle nesting on beaches in Espírito Santo in 1982, initially on Comboios beach and gradually extending towards the northern part of the state (Fig. 1). A previous study conducted by Thomé et al. (2007) presented the field methods used by Projeto TAMAR in the region and analysed leatherback nesting data from 1988 to 2003, suggesting this population was experiencing an exponential increase in size.

The study area was potentially impacted by a large-scale environmental disaster caused by the collapse, on 5 November 2015, of a tailing (mining waste) dam at the Fundão iron ore mine in the state of Minas Gerais, Brazil. The dam's collapse caused the death of 19 people and released an estimated 55 to 62 million m^3 of tailings into the Doce River watershed (Fernandes et al. 2016, Marta-Almeida et al. 2016), highly impacting the riverine fauna and flora (Carmo et al. 2017). This was the largest environmental disaster ever recorded in Brazil (Marta-Almeida et al. 2016). Given the scale and severity of the dam's collapse effects, this was a true socio-environmental tragedy. The river was loaded with toxic tailings mostly composed of Fe and other metals such as Mn, Cr, Zn, Ni, Cu, Pb and Co (Queiroz et al. 2018), which two weeks later reached the Atlantic Ocean in Espírito Santo, 660 km downstream from the fallen dam, impacting estuarine, coastal and open ocean environments (Carmo et al. 2017), including the marine turtle nesting grounds analysed here (Thomé et al. 2017). We hypothesize that the contamination of the nesting grounds could have impacts on leatherback turtle reproductive parameters such as hatchling success and output, through the alteration of the nest environment.

In the present study, we aim to determine if the population trend reported by Thomé et al. (2007) has continued to occur, and to update the results from monitoring with additional 14 years of data, expanding the temporal scale of the analyses to a total of 30 nesting seasons (1988 to 2017). The analysis of long-term datasets from conservation programmes is essential in evaluating their effectiveness. Furthermore, we aim to investigate the potential effects from the mining incident on this population, as well as to address a number of priority research questions (Rees et al. 2016), including: (1) the estimation of the population trend, (2) an analysis of reproductive parameters, and (3) an evaluation of the conservation situation of leatherback turtles nesting in Espírito Santo.

Materials and Methods

Study area

The study area is located on the coast of Espírito Santo state in Brazil, between latitudes 19°50’S and 18°36’S (Fig. 1a). It comprises 160 km of high-energy dynamic beaches, with coarse sand and is influenced by discharge from the Doce River in its southern part. The region is operationally divided into four sections, in a south-north direction: Comboios (37 km), Povoação (39 km), Pontal do Ipiranga (44 km) and Guriri (40 km) (Fig. 1). The entire area is divided by permanent marker posts at each kilometre, so the location of nests was recorded accordingly. A 15 km beach stretch just to the south of the Doce River mouth is located within the Comboios Biological Reserve, a protected area created by federal law in 1984 mainly to protect the marine turtle nesting grounds and the sandy coastal ecosystem (*restinga* in Portuguese); the 22 km of beach further to the south are within Indigenous Lands, protected by law, with restricted access and virtually no buildings; the areas north of the Doce River are not formally designated as protected, however local, state and federal laws and environmental regulations apply to the coastal zone in the region (Thomé et al. 2007). Baptistotte et al. (2003) described the climate and vegetation in the area. In addition to the nesting of leatherback turtles, Espírito Santo is also a major nesting site in Brazil for loggerhead turtles (*Caretta caretta*) (Baptistotte et al. 2003), with approximately 2500 nests recorded per annum in recent years (TAMAR, unpubl. data). Nests of olive ridley (*Lepidochelys olivacea*) and hawksbill turtles (*Eretmochelys imbricata*) are also recorded there in small numbers (Marcovaldi & Marcovaldi 1999).

Temporal patterns and field methods

The leatherback turtle nesting season typically runs from September to March. As each nesting season spans two calendar years, hereafter we refer to a season by the first of those two years, e.g., the season 2015-2016 is referred to as the 2015 season. Although TAMAR started working in Espírito Santo in 1982, the area has only been completely monitored since 1988, thus only data from 1988 onwards were incorporated into the analyses.

Monitoring procedures followed the standard TAMAR methodology (Marcovaldi & Marcovaldi 1999, Thomé et al. 2007). Morning patrols were conducted daily between 1 September and 31 March along the entire 160 km to assess nesting activity from the preceding night and to quantify the number of clutches laid. Night patrols were also undertaken opportunistically over the 30 km of beach between the Comboios and Povoação stations. The main reasons for the unequal effort on night patrols were associated to the high costs of maintaining this activity on such an extensive area. We used data from morning patrols to determine nest numbers and used data from night patrols to gain insights into female reproductive parameters. A small proportion of nests (2.9 %) were laid in the months April to August, but were recorded by TAMAR; these nests were included in the analyses.

Nests were located during morning patrols, marked with a numbered wooden stake and monitored during the incubation period. Nests were then excavated after the majority of hatchlings had emerged, or after 90 days (the longest incubation period shown for this population; Thomé et al. 2007). In 1998, 1999 and from 2008 on, when a crawl (a nesting emergence) was detected but a clutch could not be found, the entire area where the sand had been disturbed (termed a body-pit) was cordoned off. The presence of a clutch would then be confirmed by the emergence of hatchlings. Nevertheless, 25.8% of all nesting crawls observed in 1998, 1999 and from 2008-2017 were listed as “undetermined.” We assigned these undetermined crawls as either nests or false crawls (nesting crawls that did not result in egg deposition) based on the known percentage of all crawls that resulted in egg deposition in the 1988-1997 and in the 2000-2007 nesting seasons (66.0%). Thus, for each nesting season in 1998, 1999 and from 2008-2017, we assumed that 66.0% of all undetermined crawls were actual nests, and added these to the total number of confirmed nests

Nests that were in danger of beach erosion or tidal flooding were relocated either to more stable areas of the beach or, until 1997 in Povoação and 2000 in Comboios, to in-situ hatcheries. The entire area is divided by permanent marker posts at each kilometre, so the location of nests was recorded accordingly. We analysed the spatial distribution of nests based on the relative frequency of their occurrence in each kilometre; the spatial distribution was compared among decades through a chi-squared test for the equality of proportions (Dalgaard 2008).

Mark-recapture

Females were tagged on both hind flippers (Balazs 1999, Marcovaldi & Marcovaldi 1999) using monel tags until 1994 and inconel tags after 1995 (National Band and Tag Co., USA, style 681). Tag sites were checked for the presence of scars or calluses, which could indicate tag loss (Hughes 1996). Curved Carapace Length (CCL) was measured using a flexible measuring tape, from the centre of the nucal notch to the posterior tip of the carapace, alongside the central dorsal ridge, following the method described in Thomé et al. (2007).

Internesting intervals were calculated as the number of days between an observed egg laying and the subsequent observed egg laying (Broderick et al. 2002). Records of individuals observed nesting in different seasons were used to determine the remigration interval (number of years since the last observed nesting season). Although there is no clear record of the effort applied on night patrols across the years, they started to be conducted regularly (at least three times per week) from 2005 onwards, and thus here we calculate female reproductive parameters (internesting and remigration intervals) only with data from 2005 onwards.

Nest density

We created an indicator to measure the relative importance of each section of the beach in terms of nest density. The 160 km of the study area were subdivided into 16 sections of 10 km each. The average annual number of nests on each section for the period 2008-2017 (the last ten years of data) was calculated. Each section of the beach was then classified in terms of nest density as either high, medium or low, by locating the average annual number of nests on that section within the distribution of the 16 average numbers: high-density sections were those with average annual number of nests in the top 25% of the distribution; low-density sites were those in the lowest 25% of the distribution; and sites with densities in-between the two previous categories were classified as medium-density (Fuentes et al. 2016).

Hatching Success

Hatching success was calculated as the percentage of yolked eggs that produced live hatchlings, including live hatchlings encountered in the nest during excavation (Thomé et al. 2007). Nests with 0% hatching success (failed clutches) were also included in the analysis, and only *in situ* nests were considered. To evaluate possible effects of the mining event on the hatching success of leatherback turtles, we analysed the three seasons before (2012, 2013, 2014) and the three after (2015, 2016, 2017) the event.

Statistical analysis

Statistical analyses were conducted using R 3.5.1 (R Core Team 2018) applying significance level of 0.05. The trend in the annual number of nests was estimated using a Generalised Additive Model (GAM) with the function *gamm* of the R-package *mgcv* (Wood 2017). Annual variation in CCL was assessed using a local polynomial regression, by means of the R-package *locfit* (Loader 1999); for each female, only the first CCL record in each season was considered in the regression calculations. For statistical analyses concerning the CCL distribution in the whole study period (mean, SD), only the first record of each female among all seasons was considered. In the analysis of hatching success, non-parametric Kruskal-Wallis and Mann-Whitney tests were used (Hollander & Wolfe 1999). For the analysis of nesting dates, each season was considered to run between 1 August and 31 July. The date of each nesting event was converted to an absolute date (the time interval in days since the start of the season), and those were used to calculate the median nesting date (MND) of the season. The change in the MND over the seasons was analysed by means of an ordinary least-squares linear regression (Robinson et al. 2014). A detailed description of statistical analyses is presented in Appendices.

Results

Population trend and size

We estimate that 1608 leatherback turtle clutches were laid between 1988 and 2017. The GAM regression in Fig. 2 was significant (approximate significance of the smooth term: estimated degrees of freedom = 4.18, $F = 14.74$, $p < 0.00001$) and indicated a non-constant increasing trend in the annual number of nests. The mean annual number of nests over the first five years (1988-1992) was 25.6 nests, while during the last five years (2013-2017) it was 89.8 nests. Thus, we estimate that between 2013-2017 the annual nesting population consisted of between 15 and 18 females, assuming an average of between five and six clutches per female per season (Spotila et al. 1996, Eckert et al. 2012).

Turtle tagging and curved carapace length

Between 1989-2017, 143 individual nesting females were tagged in 372 encounters (no turtles were tagged in 1988). CCL at first capture ranged from 124.7 to 182.0 cm (mean 152.9 ± 10.0 SD, $n = 141$). There was a significant decrease in CCL across the 29 years, as no horizontal line (representing a constant CCL in the period) can be placed inside the 0.95 simultaneous confidence band in Fig. 3 ($n = 151$). In the five initial years of the period (1989-1993), the mean CCL was 166.3 cm (SD = 7.3 cm, $n = 12$), while in the last five years (2013-2017) it was 149.9 cm (SD = 9.1 cm, $n = 49$). There was a sharp downward shift in the CCL distribution from 2011 onwards (Fig. 3). In the seven-year period 2011-2017, the estimated quantile 0.05 of the CCL distribution was 134.3 cm, while it was 149.0 cm in the preceding seven-year period (2004-2010). Fig. 3 also shows the estimated quantile 0.95 in each of these seven-year periods; together with the quantiles 0.05, they allow us to visualise the marked downward shift in the CCL distribution from 2011 onwards. The two smallest leatherbacks ever measured while nesting in Espírito Santo were recorded in 2011 (CCL = 129.0 cm) and in 2015 (CCL = 124.7 cm) (Fig. 3).

No leatherbacks were ever found nesting in Espírito Santo bearing tags applied elsewhere, and no leatherbacks tagged in Espírito Santo have ever been reported nesting elsewhere. Individuals bearing only a single tag accounted for 6.1% of all recaptures, which provides a lower bound for the probability of tag loss in this population, since the loss of both tags could have gone unnoticed when encountering a turtle on the beach; flipper scars suggesting that both tags had been lost were found in one individual bearing no tags. Leatherbacks were recaptured between one and seven times in a given season; the maximum recapture distance along the beach in the same season was 65 km (mean 14.1 ± 11.0 SD, $n = 98$). Of the 143 females tagged, 48 (33.6%) were never recaptured. Our capture rates, or proportion of nests laid within a season that could be attributed to a known female, varied between 21.9 and 53.4% during the period 2013-2017.

Spatial distribution of nests

Clutches were distributed across the entire region; however, there was a higher concentration in the southern part of the study area: 75.2% ($n = 651$) of all nests were recorded between kilometres 10 and 60, which delimit the high-density nesting sites (Fig. 1). When comparing the spatial distribution among three decadal periods (1988-1997, $n = 209$; 1998-2007, $n = 456$ and 2008-2017, $n = 651$), there was no significant difference in the proportion of nests located between kilometres 10 and 60 (high-density areas) among the three periods (chi-squared = 2.12, $p = 0.35$). In the whole 30-year period, 25.1% of the total number of nests with recorded location ($n = 1316$) were located within the protected area of Comboios Biological Reserve (between kilometres 26 and 33, Fig. 1b). On the north side of the Doce River, the southern part of Povoação (between kilometres 38 and 60) is also an important nesting area, with 28.3% of the total number of nests with recorded location during the 30-year period (Fig. 1b).

Temporal distribution of nests

Of the total number of nests with recorded date of nesting (n = 1325), 66.9% were laid between November and December (Fig. 4a). There was no significant trend in the annual median nesting date (MND) over the study period (r^2 = 0.0040, p = 0.741, Fig. 4b) although the MND was highly variable among the seasons with a range of 48 days. Much of this range can be attributed to atypically late nesting that occurred in 1990, 1994 and 2005, and early nesting in 1997 and 1998, however we currently have no estimates as to whether those early and late nesting events could be driven by climate variables.

Internesting interval

The recorded internesting intervals ranged from 8 to 62 days (n = 148 intervals from 74 individuals; Fig. 5a). The multimodal pattern in Fig. 5a suggests that the internesting interval is defined by the range of 8-15 days, with recorded intervals of 18 days or greater likely representing one or more missed nesting events. For turtles showing internesting intervals within the range of 8-15 days (n = 88, or 59.5% of the total number of recorded intervals), the median interval was 10 days (mean 10.4 ± 1.2 SD).

Remigration interval

Remigration intervals were obtained from 9 females (6.3% of the 143 tagged ones), which contributed with n = 10 intervals, since one female was observed in three different seasons. The recorded remigration intervals ranged from 2 to 17 years. The most frequent interval was 2 years (n = 4), followed by 3 years (n = 2), and one record each of 4, 6, 8 and 17 years. The interval of 17 years likely represents several missed nesting events over some nesting seasons.

Hatching success and effects of the mining accident

The average hatching success for *in situ* clutches between 2000 and 2017 (18 seasons) was 66.0% (range = 0-100, SD = 26.2, *n* = 706 clutches). The annual average hatching success ranged from 38.8% (in 2010, *n* = 9) to 82.4% (in 2007, *n* = 35; Fig. 5b). Hatching success was not significantly different between years in the period 2012-2017, which include three seasons (2012, 2013, 2014) before the mining event and three others (2015, 2016, 2017) following it (Kruskal-Wallis test, *n* = 352, *p* = 0.18; Fig. 5b). The mining event occurred at the peak of the 2015 nesting season, however, no apparent changes in leatherback behaviour (spatial distribution of nests on the beach, and the frequency and timing of turtles coming ashore to lay eggs) were observed in that season following the accident. During the same period (2000-2017), 176 nests (19.9%, n = 882) were relocated due to risk of beach erosion, flooding or poaching, and were not considered in the analysis (Supplementary Table 1).

Discussion

This research provides valuable information regarding the population biology of leatherback turtles in the Southwest Atlantic and highlights the importance of maintaining long-term monitoring to better understand marine turtle ecology and inform conservation. Results have been obtained in four areas and we discuss them in turn as well as their conservation implications: (1) trends in annual nest numbers and average CCL of the population, (2) trends in the spatial and temporal distribution of nests, (3) reproductive parameters and (4) effects of the mining disaster on hatching success.

Population trends

The exponential increase in the annual number of nests in the period 1995-2003 observed by Thomé et al. (2007) now appears to be a part of a more complex temporal pattern in the annual number of nests (Fig. 2). Despite the generally increasing pattern, the high variability in annual nesting numbers limits a reliable prediction on population growth. The increase in the average annual number of nests from 25.6 in the period 1988-1992 to 87 in the period 2008-2017 is encouraging, however it should be interpreted with caution, since the relatively small annual numbers of nests and estimated annual number of nesting females - between 14 and 17 per year in the last five years of the study period - still make this population of conservation concern. We currently have no estimate of adult mortality for this population (see e.g. Groom et al. 2017), which hampers predictive abilities regarding population viability. Low mortality of adults and juveniles is required to maintain viable marine turtle populations (Crouse et al. 1987) and anthropogenic sources of mortality were suggested to be the main cause responsible for observed population declines in leatherback turtles in the Pacific Ocean (Eckert & Sarti 1997, Santidrián Tomillo et al. 2017).

Female size and recruitment

We hypothesize that the decrease in the CCL, observed mainly from 2011 onwards (Fig. 3), is due to the recruitment of younger and smaller females to the nesting population (Hughes 1996, da Silva et al. 2007, Bellini et al. 2013, Omeyer et al. 2017). The increase in nesting numbers that started around 1995 is possibly a result of egg and adult protection in the area since 1982. This suggests a time lag of *ca*. 13 years before the onset of any increase in the leatherback nesting population following the start of protective measures (as seen in Dutton et al. 2005, where a lag of 12-14 years was found), which would be consistent with the estimated average leatherback age of maturity of 13-14 years that has been proposed by Zug & Parham (1996), although more recent studies with skeletochronology suggested the age at maturity for leatherback turtles to be between 23-27 years (Avens et al. 2009). The wave of smaller females that seems to have recruited to the Espírito Santo nesting population from 2011 on could be the result of increased nesting numbers since 1995.

Spatial distribution of nests

The high-density nesting sites were concentrated in the south of the study area (between beach kilometres 10 and 60; Fig. 1a) and this remained constant across the study period. This region included both the Comboios Biological Reserve, a protected area, and Povoação, just north of the Doce River mouth, a region currently facing pressure from coastal development and there is no designated protected areas status. The mechanisms driving nest site selection and potential explanations for why these sites would be preferred by the turtles are not currently understood. Leatherback turtles are known to use a generally wider overall nesting area than other marine turtle species (Eckert et al. 2006, Stewart 2007, Almeida et al. 2011). Therefore, despite the importance of the protected area at Comboios, the need exists to expand the conservation areas to the north of the Doce River mouth (Almeida et al. 2011), since 75.2% of the recorded nests were observed in this region.

Remigration rates

The recapture rate for remigrant turtles in Espírito Santo (6.3% of individuals tagged) was low when compared to other leatherback turtle rookeries. In an increasing population at St. Croix, U.S. Virgin Islands, the average annual remigration rate in 1977-2001 was 43.5% (Dutton et al. 2005). In a small population in South Africa, the average annual remigration rate was 33.7% in 1984-1995 (Hughes 1996). Our low recapture rate could possibly have been influenced by the low encounter rates in our study area, and could also likely have been influenced by a combination of the following factors:

First, leatherbacks have high rates of external flipper tag loss - up to 50% between seasons (Garner et al. 2017). Flipper tags have always been used in Espírito Santo, however passive integrated transponders (PIT tags), which have never been used there, have proved to be more reliable in generating estimates of remigration intervals and survival (Balazs 1999, Dutton et al. 2005). Second, possible high at-sea mortality. Leatherback turtles have a wide oceanic distribution and are prone to interact with fisheries (Fossette et al. 2014), which is considered a major threat to the Brazilian leatherback turtle population (Sales et al. 2008). Dead and injured leatherbacks are found washed ashore along the Brazilian coast (Barata et al. 2004, Monteiro et al. 2016); genetic studies suggest that some of them could come from the Espírito

Santo population (Vargas et al. 2017). Recoveries of females tagged in Espírito Santo are scarce, however, three of them were found dead on the Brazilian coast (incidentally captured in fisheries around the Doce River mouth (TAMAR, unpubl. data)), one in Argentina (Alvarez et al. 2009) and one in Namibia, West Africa (Almeida et al. 2014). Third, turtles tagged in Espírito Santo could be nesting elsewhere. On the main nesting beaches in Brazil, however, morning patrols are conducted during the marine turtle nesting seasons, making it unlikely that leatherback tracks would be unnoticed. The points raised here evidence that more research is needed to fully understand remigration patterns for this population.

The multimodal internesting pattern observed in Fig. 5a, with groups of data with steadily diminishing numbers of records located around multiples of the mode of the first group of recorded nesting intervals, is a common feature of sets of internesting intervals obtained through incomplete tagging of turtles on a nesting site, see e.g. Mortimer & Carr (1987) and Bellini et al. (2013). In addition to the use of PIT tags for better returns from capture-mark-recapture studies, satellite telemetry could provide better insights not only regarding internesting intervals, but also internesting habitat use, remigration and clutch frequency (Tucker 2010, Weber et al. 2013, Rees et al. 2017).

Hatching success and potential impacts from the mining incident on leatherback turtles

The overall hatching success of leatherback turtle nests is low when compared to other marine turtle species, and exhibits considerable variability among nesting sites worldwide (Santidrián Tomillo & Swiggs 2015). Hatching success in Espírito Santo, 66.0%, is higher than those reported for other Atlantic populations. At St. Croix, US Virgin Islands, hatching success was 58.6% in the period 1982-2010 (Garner et al. 2017). In Matapica, Suriname, the annual average hatching success ranged between 52.7 and 56.0% in 1999-2005 (Hilterman & Goverse 2007). In the previous study by Thomé et al. (2007), the average hatching success for leatherbacks in Espírito Santo in 1994-2003 was found to be 65.1% (*n* = 185), similar to the 66.0% reported here for the period 2000-2017.

Although the mining event had catastrophic consequences for both the biodiversity and riparian human communities in the affected areas (Fernandes et al. 2016, Marta-Almeida et al. 2016, Carmo et al. 2017), no significant impact has been observed on the leatherback turtle hatching success in Espírito Santo. Given the large scale of this incident, it might be expected that turtles could avoid nesting in the areas, since coastal waters were contaminated with toxic tailings, however it seems that the mining event has caused no noticeable impact on the annual number of nests, as the figures in the years following the event (2016 and 2017) seem to follow a pattern in agreement with those observed in previous nesting seasons (Fig. 2).

Other impacts, however, might take longer to manifest themselves. Contaminated sediments could still find their way into the sand column at concentrations possibly high enough to harm the eggs during their incubation. Beach contamination could change the natural composition of the sand in terms of its colour and other physical or chemical properties, possibly altering san albedo and temperature, and so affecting the incubation of eggs. Furthermore, the sea in the region around the Doce River mouth continues (in November 2018) to receive contaminated water through the river discharge, and the effects of that contamination on both hatchlings and adult leatherback turtles while they swim through the surf zone and adjacent coastal waters are unknown and could include passively poisoning of the turtles through sea water. Predictions are hampered by the fact that the levels of contaminants in the region and dispersion mechanisms are still not completely understood (Marta-Almeida et al. 2016)

The reproductive parameters analysed here constitute a baseline for future comparisons when assessing long-term impacts of the burst mine tailing dam on leatherback turtles nesting in Espírito Santo.

Conservation implications

As with other marine turtle populations in different regions (Wallace et al. 2010b, Wallace et al. 2013a, Casale & Heppell, 2016), the incidental capture in fisheries has been pointed out as a major threat to the Espírito Santo leatherback turtle population (Thomé et al. 2007, Sales et al. 2008, Almeida et al. 2011). However, further threats also pose challenges for the conservation of this population: coastal development and industrial activities in the region could cause the loss or alteration of important nesting habitats. Possible management plans for the region are being discussed, with the participation of TAMAR and several different stakeholders: members of the local villages, local governments, federal and state governmental environmental agencies, universities and institutions managing recovery plans concerning the mining incident. The plans consider the different uses of the coastal and marine areas, public policies and the cultural traditions and economic needs of the local communities involved. The aim is to establish, in addition to the currently existing Comboios Biological Reserve, an environmentally protected area in a wider region around the Doce River mouth, in a way that would improve the conditions for the management and sustainable use of natural resources and the conservation of wildlife. This would be achieved through regulations in the use of coastal land and sea areas and through changes in fishing practices, aiming to reduce the incidental capture of marine turtles around the nesting area, and also through the development of further economic alternatives for people that live in the region, such as ecotourism and selected agricultural practices. Measures like these are expected to widen the local awareness towards conservation and to improved marine turtle conservation in the area.

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Ethical Standards

This research was submitted to SISBIO in Brazil (permit no. 47845-3) and subjected to approval by the ethical committee at the University of Exeter, UK, with ethical clearance received.

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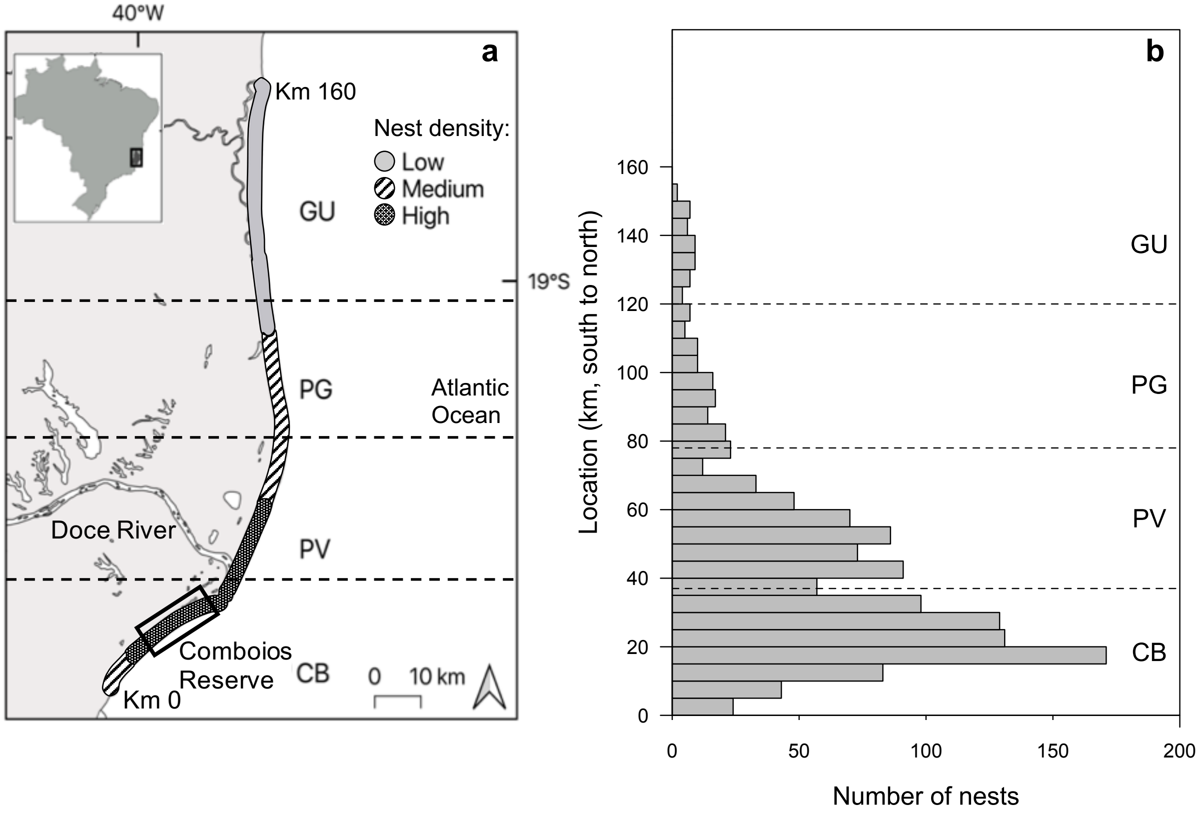

Figure 1. (a) Leatherback nesting areas in Espírito Santo, Brazil. The inset shows the location of the state of Espírito Santo in Brazil. Nest density classification (high, medium or low density) was based on the relative nest density of sixteen 10-km sections of the nesting area (see Methods). The black rectangle shows the limits of the Comboios Biological Reserve. **(b)** Spatial distribution of leatherback nests in Espírito Santo, 1988-2017 (*n* = 1316). Km 0 is the southernmost point of the study area. Dashed horizontal lines indicate the boundaries between TAMAR stations, with abbreviations: CB = Comboios, PV = Povoação, PG = Pontal do Ipiranga and GU = Guriri. The Doce River mouth is located at the boundary between CB and PV.

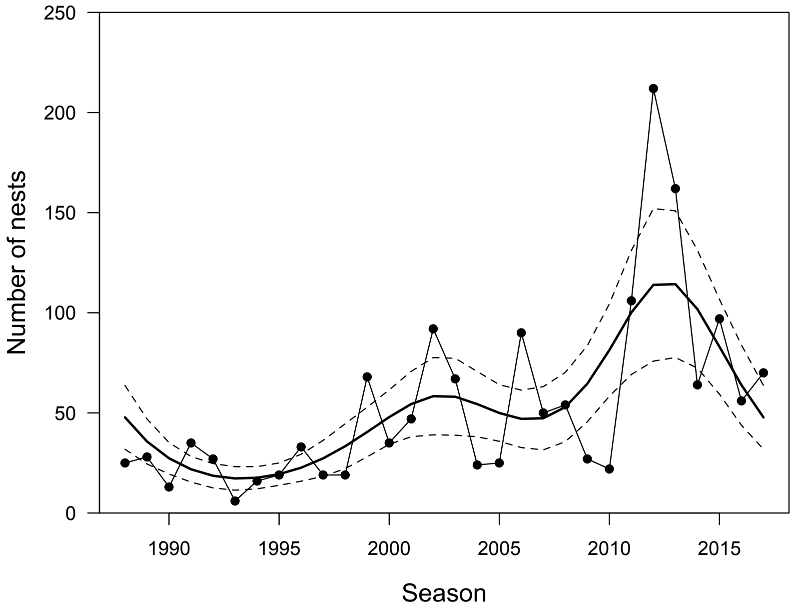

Figure 2. Annual number of leatherback nests in Espírito Santo, Brazil, 1988-2017 (1608 nests in total). The first year of each season is shown on the horizontal axis, e.g., 1995 represents the season 1995-1996. The connected dots show the raw data. The solid curve is a GAM (generalised additive model) regression, and the dashed curves indicate approximate 0.95 pointwise confidence intervals. For full details on statistical methods see Appendices.

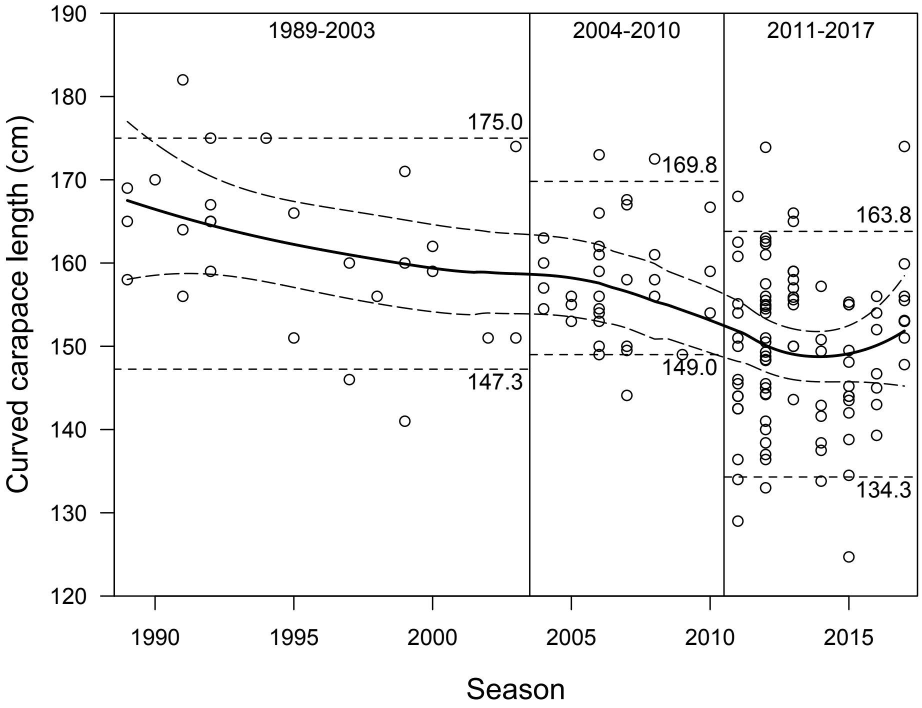

Figure 3. Curved carapace length (CCL) by nesting season, 1989-2017 (*n* = 151). The solid curve is a local polynomial regression, and the dashed curves delimit a 0.95 simultaneous confidence band. The vertical lines divide the overall time period into three smaller periods, indicated by the labels in the topmost part of the graph; in each of the three smaller time periods, the horizontal dashed lines indicate the estimated quantiles 0.05 and 0.95 of the CCL distribution in the period; the figures adjoining the dashed lines show the values of the quantiles. In the graph, to avoid superposition, the data points were jittered, that is, slightly randomly displaced from their true positions; for the regression calculations, the true values of CCL and season were used. For full details on statistical methods see Appendices.

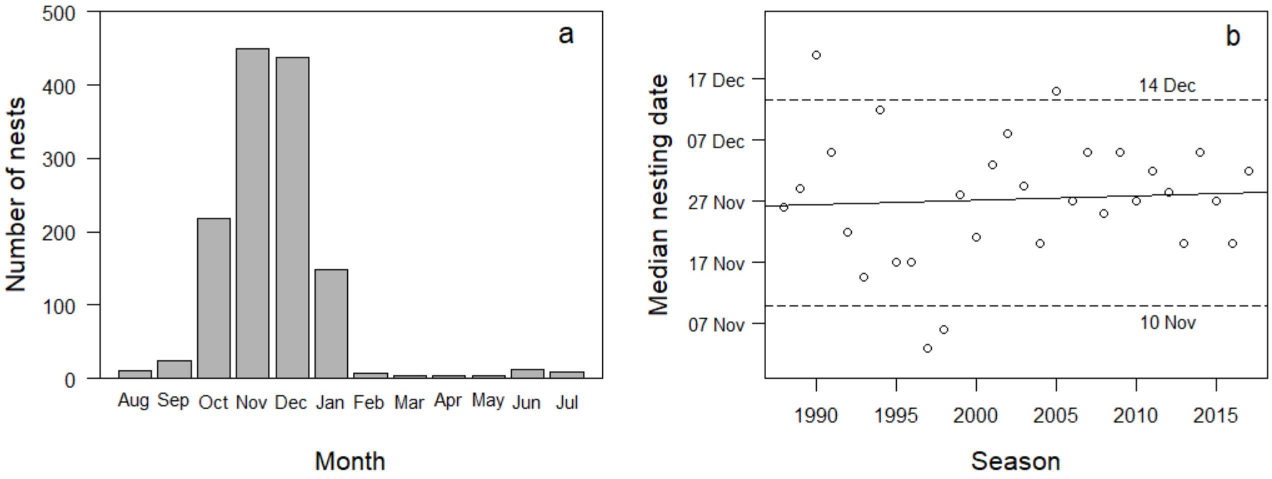

Figure 4. (a) Number of leatherback nests by month in Espírito Santo, 1988-2017 (*n* = 1325). **(b)** Annual median nesting date (MND) by nesting season; the solid line is an ordinary linear regression and the dashed lines represent the estimated quantiles 0.05 and 0.95 of the MND distribution.

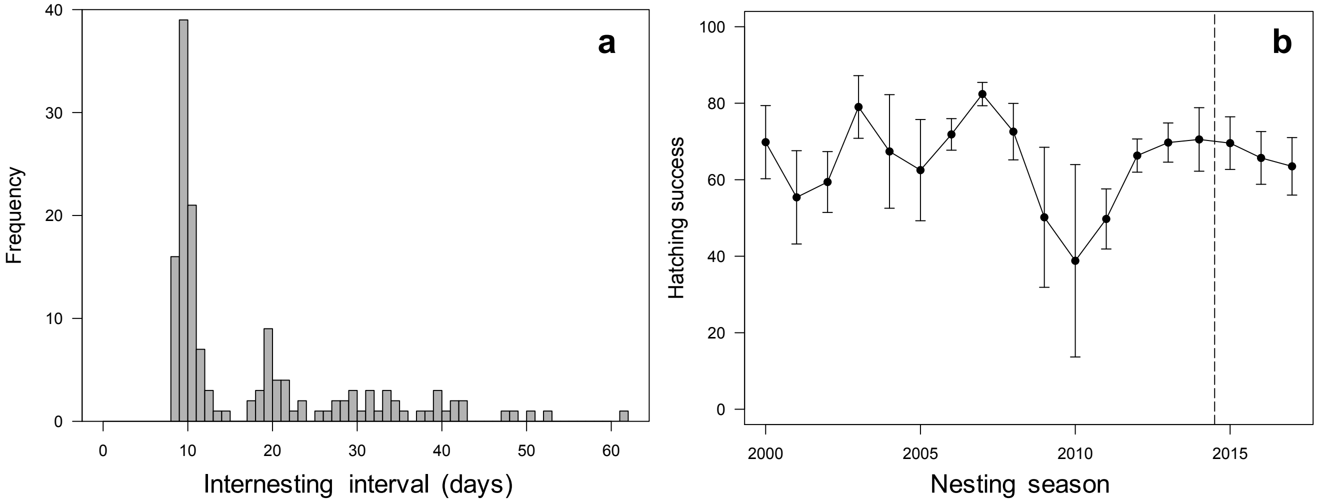

Figure 5. Reproductive parameters of leatherbacks in Espírito Santo, Brazil. **(a)** Observed internesting intervals, 2005-2017 (*n* = 148 intervals). **(b)** Average hatching success for *in situ* clutches per nesting season, 2000-2017 (*n* = 706 clutches); error bars indicate 95% confidence intervals; the dashed vertical line represents the mining incident at the beginning of the 2015 season.

Appendices

Supplemental Methods

Regarding the generalised additive model (GAM) used to analyse the trend in the annual number of nests: this is a kind of nonparametric regression where the shape of the trend curve is not predetermined but is estimated from the data (Wood 2017). This function allows for the existence of autocorrelation in a time series, a characteristic of nesting data that is sometimes readily observed (e.g. Bjorndal et al. 1999) and which has been considered in other studies of temporal nesting patterns of sea turtles (e.g. Chaloupka et al. 2008, Weber et al. 2014). Using loess regressions (Cleveland et al. 1993) with second order local polynomials and different values for the span parameter, we detrended the time series of annual number of nests; partial autocorrelation graphs of the different detrended series indicated the possible existence of second-order autocorrelation in the original time series. Thus, using the *gamm* function, we constructed different GAM models with negative binomial error distribution (with link function = log) and cyclic cubic regression splines, with combinations of different values for the dimension of the basis that is to represent the smooth term and of two different values for the order of the autocorrelation, 1 and 2. There seems to exist no established method for the assessment of goodness-of-fit of GAM models constructed with the *gamm* function, which could serve as a basis for model selection (Gilman et al. 2012; see also the mgcv.FAQ help page of the R-package *mgcv*). The function *gamm* operates internally by transforming the GAM model into another statistical model, a parametric mixed model (see Wood 2017), for which an approximate AIC statistic (Akaike information criterion; Anderson 2008) can be calculated. As our selection criteria, first we followed Zuur et al. (2009) and selected among the different GAM models those two models with the smallest AIC values; their AIC were nearly the same, and both GAM models included autocorrelation terms of second order. Second, we made a graphical diagnostic of the residuals of the two models (see Wood 2017) and chose the one with the best properties in that analysis, although in fact little difference was observed between the two models in that respect.

Regarding the local polynomial regression used to analyse the variation of the curved carapace length (CCL) over the years: the regression employed second degree polynomials. The method used allowed the construction of a simultaneous confidence band for the regression curve. The final smoothing parameter was selected by visual comparison of different regression curves obtained each with a different value for that parameter, so as to avoid both under- and over-smoothing of the data.

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Table S1. Hatching success for in-situ leatherback clutches in Espírito Santo, Brazil (2000-2017). Values are mean ± standard deviation, range and sample size (n). The number of in situ, relocated and total number of confirmed nests in each nesting season is also shown.

Year	Hatching success (%)	*n*	Confirmed in situ nests	Confirmed relocated nests	Total confirmed nests
2000	69.8 ± 23.4 (6.7-93.3)	23	30	5	35
2001	55.4 ± 23.3 (0-82.3)	14	37	10	47
2002	59.4 ± 29.5 (0-93.3)	53	78	14	92
2003	79 ± 22.9 (0-100)	30	61	6	67
2004	67.4 ± 30.3 (12.5-96.8)	16	20	4	24
2005	62.5 ± 26.1 (0-90.5)	15	22	3	25
2006	71.8 ± 15.5 (27.4-95.7)	54	69	21	90
2007	82.4 ± 9.2 (62.5-93.8)	35	42	8	50
2008	72.6 ± 17.7 (20-97.4)	22	30	9	39
2009	50.2 ± 39.6 (0-93.3)	18	20	4	24
2010	38.8 ± 38.5 (0-87.9)	9	11	8	19
2011	49.7 ± 32.3 (0-93.9)	65	73	17	90
2012	66.3 ± 25.5 (0-100)	133	142	26	168
2013	69.7 ± 21.2 (8.7-96.6)	66	74	31	105
2014	70.5 ± 25 (0-95.6)	35	40	11	51
2015	69.6 ± 27 (0-98.3)	59	61	11	72
2016	65.7 ± 19.3 (22.8-92.4)	30	33	4	37
2017	63.5 ± 20.7 (19.5-94.2)	29	39	13	52

Chapter II: Stable isotope analysis and satellite tracking of Southwestern Atlantic leatherback turtles (*Dermochelys coriacea*): habitat use and female-offspring relationships

This chapter is in preparation for submission to *Marine Ecology Progress Series*

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Abstract

In the Southwestern Atlantic Ocean, leatherback turtles (*Dermochelys coriacea*) nest mainly on the coast of Espírito Santo, eastern Brazil. This subpopulation is considered Critically Endangered by the International Union for the Conservation of Nature (IUCN) due to its small population size and restricted geographical distribution. In order to investigate the marine habitat use of nesting leatherback turtles and individuals washed ashore along the Brazilian coast, we performed stable isotopes analysis (SIA) of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) on skin tissue of 61 leatherback turtles (2012-2017). A *k*-cluster analysis revealed that nesting females segregate into at least two distinct groups based on their isotope values. Further evidence from satellite tracking of four individuals suggests differences in their choice of foraging habitats (oceanic *vs*. more coastal). The overlap in stable isotope signatures between the nesting population and individuals washed ashore suggests that some could have been sharing foraging areas; or pertain to the Brazilian nesting population. We also determined the stable isotope ratios of offspring tissues (egg yolk and hatchlings) from 16 assigned females (2015-2016). Egg-yolk and hatchling isotope values were correlated with nesting females for both $\delta^{13}C$ and $\delta^{15}N$, indicating that egg/offspring tissues are valuable proxies for assessing isotope data in leatherback turtles when female tissues are not available. Linear mixed-effects models showed that time in days since a female's first clutch was observed did not affect $\delta^{13}C$ or $\delta^{15}N$ in egg yolk or hatchlings in successive clutches. Our results provide key insights into habitat use and can inform conservation strategies, such as on the design and establishment of marine protected areas considering the distribution, movements and foraging strategies of this species in the South Atlantic Ocean.

Introduction

Tracking animal movements is an endeavour important to both theoretical ecology and conservation (Hobson 2007). Many marine species undertake regular seasonal or reproductive migrations (Hobson & Schell 1998, Rubenstein & Hobson 2004) and identifying links between breeding and non-breeding sites is important for species conservation (Hobson & Norris 2008). Animals such as marine vertebrates are inaccessible during parts of their life cycles and methods that rely on the recapture of the individual (such as the conventional tags, rings and marks) can be subject to sampling bias, particularly in species with extensive distributions (Hobson 2007). Satellite telemetry can provide real-time insights into animal movements, including the extensive seasonal migrations of marine vertebrates such as marine turtles (Hart & Hyrenbach 2009, Jeffers & Godley 2016), patterns of connectivity between genetic stocks, and identification of high-use habitats and consequently the establishment of protected areas for relevant species (Scott et al. 2012, Schofield et al. 2013, Revuelta et al. 2015). However, satellite tracking is expensive and sample sizes are generally quite small (Wilson & McMahon 2006, Godley et al. 2008).

In recent years, stable isotopes analysis (SIA) has been used as a powerful tool in ecological studies, providing information on trophic levels, identifying food sources and assessing migratory behaviour in species which are otherwise difficult to observe in their natural habitat (Arthur et al. 2008, Carpentier et al. 2015, Haywood et al. In Review). Stable isotopes in proteins of consumers tend to reflect those of their prey (DeNiro & Epstein 1978, Peterson & Fry 1987). The tissue of consumers is often enriched in nitrogen (^{15}N) in comparison to their prey, caused by the preferential excretion of lighter isotopes. Therefore, the stable isotope ratio of nitrogen ($\delta^{15}N$, $^{15}N/^{14}N$) is used to infer the trophic position within the food web (Peterson & Fry 1987). The stable isotope ratio of carbon ($\delta^{13}C$, $^{13}C/^{12}C$) is generally used to determine the diet of an animal and the original autotrophic carbon sources (DeNiro & Epstein 1978). Furthermore, the time-scale over which prey information is represented by a given isotope signature varies with tissue type and depends on the relative metabolic turnover rate of the tissue (Hobson & Sealy 1991, Petitet & Bugoni 2017). Stable isotope signatures of proteins with slow turnover are thought to represent an integration of dietary information over more extended periods (Tieszen et al. 1983, Godley et al. 1998).

Information regarding ecological and behavioural patterns, including identifying the location of foraging areas, are needed to address important research questions relative to marine turtles and to develop effective conservation strategies (Rees et al. 2016). The tissues of nesting female turtles can be sampled for SIA, providing information about their diet and habitat use prior to migration to the nesting beaches (Reich et al. 2010). Eggs and consequently neonate tissues are derived from maternal resources, which will influence their isotope composition (Pilgrim 2007). Sampling on nesting beaches can be logistically challenging, considering the small time-window available for sampling (during nesting) and the extensive distances that must be patrolled at some sites. In this context, if offspring isotope composition reflects that of the females, sampling offspring could represent a more feasible approach to study maternal foraging patterns, independent of female encounter, thus increasing the potential sample size (Frankel et al. 2012). Furthermore, stable isotope approaches to infer feeding areas can be validated through the isotope composition of satellite tracked animals (e.g. Jaeger et al. 2010, Seminoff et al. 2012, Bradshaw et al. 2017), allowing the tracking of a few individuals to be extrapolated to the wider breeding population (Zbinden et al. 2011).

Leatherback turtles (*Dermochelys coriacea*) are highly migratory animals, crossing ocean basins and covering great distances, dispersing widely between nesting sites and distant foraging areas (Horrocks et al. 2016, Robinson et al. 2016). They present variable migratory strategies, which can be ultimately related to different breeding intervals and reproductive output (Caut et al. 2008). It is believed that after leaving the nesting beaches, female leatherback turtles generally migrate to pelagic areas (Hays et al. 2006, Caut et al. 2008, Witt et al. 2011) in search of prey, which consists of gelatinous zooplankton such as coelenterates (class Scyphozoa and Siphonophora) and pelagic tunicates such as pyrosomas and salps (Bjorndal 1997, Davenport 1998, James & Herman 2001, Estrades et al. 2007, Witt et al. 2007, López-Mendilaharsu et al. 2009, Wallace et al. 2015). In the Southwestern Atlantic Ocean, leatherback turtles are known to regularly nest only in eastern Brazil, on the coast of the state of Espírito Santo, around latitude 19°S (Thomé et al. 2007, Colman et al. Chapter I), however sporadic nesting occurs along a large range of the Brazilian coastline (Soto et al. 1997, Barata & Fabiano 2002, Loebmann et al. 2008, Bezerra et al. 2014, Gandu et al. 2014). The rookery in Espírito Santo is genetically distinct (Dutton et al. 2013), presents high haplotype diversity (Vargas et al. 2017) and is considered a unique regional management unit (RMUs; Wallace et al. 2010). It is estimated that on average only between 10 and 20 females nest in Espírito Santo beaches per year (Colman et al. Chapter I) and this subpopulation is considered Critically Endangered by the International Union of Conservation of Nature (IUCN; Wallace et al. 2013).

Here we aim to (1) describe stable isotope ratios of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) for the Brazilian nesting population of leatherback turtles and for individuals washed ashore or bycaught in fisheries along the Brazilian coast; (2) use forensic techniques (SIA) to suggest potential foraging strategies for this population by extrapolating satellite tracking of a few individuals to the wider breeding population; (3) analyse stable isotope composition of offspring (egg yolk and hatchlings) to determine whether they can be used as a proxy for female isotope signatures; and (4) examine the variation in egg yolk and hatchling isotope ratios of females throughout the nesting season in order to determine if one sampling event would be representative of the entire nesting season.

Materials and Methods

Study area

Females were sampled in Comboios and Povoação beaches, in the state of Espírito Santo, Brazil (between latitudes 19°40'S and 19°25'S). These two beaches (total length = 60 km) are the leatherback turtle hotspots in the southwestern Atlantic Ocean and account for approximately 75% of all the leatherback turtle nests laid annually in Brazil (i.e., < 100 nests per year on average; Colman et al. Chapter I). Additionally, the southwestern Atlantic represents a mixed foraging area for leatherback turtles from different rookeries, including those probably originating from distant nesting grounds such as Gabon (Vargas et al. 2017). Dead and injured leatherback turtles, washed ashore or bycaught in fisheries are recorded along the extensive Brazilian coast (Barata et al. 2004, Monteiro et al. 2016) and were also sampled here (from Santa Catarina, in the south, to Ceará, in the north, between latitudes 28°38'S and 2°89'S).

Sampling methods

Monitoring procedures followed the standard methodology by TAMAR, the Brazilian sea turtle conservation programme (Thomé et al. 2007). From 2012 to 2017, during the nesting season (October to February), nesting females were tagged during night patrols using Inconel flipper tags (National Band and Tag Co., USA, style 681) and their curved carapace length (CCL) measured following the method described in Thomé et al. (2007). Tissue samples comprised of a small epidermal biopsy (6 mm) taken from the neck region and preserved in NaCl until sample preparation for SIA. Skin samples were also collected from individuals washed ashore or bycaught in fisheries, either dead or alive, along the Brazilian coast. In 2015 and 2016, samples were also taken from the offspring of clutches assigned to known females. Whenever possible, a 4 mm biopsy punch was used to sample the trailing edge of the front flipper in live and freshly-dead hatchlings (Dutton & Stewart 2013). One freshly-laid egg was collected at the time of egg laying and at least one unhatched whole egg was retrieved after hatchlings emergence (whenever possible). Sample size varied according to availability of hatchlings and unhatched eggs by the time of post-hatching nest excavation. Egg yolk was extracted with a syringe, placed in a vial and frozen until preparation for SIA (Carpentier et al. 2015).

Satellite tracking

Platform Terminal Transmitters (PTTs) were attached to four adult female leatherback turtles at nesting locations in Espírito Santo during the nesting season (October to February) in 2005-2006 (*n* = 2) and 2017-2018 (*n* = 2). The movements of the former two turtles were previously published in Almeida et al. (2011). For these, methods of turtle capture, transmitter type and attachment process are described in detail in Almeida et al. (2011). The transmitters in 2017-2018 were SPLASH10-295C (Wildlife Computers, USA). They were directly attached to the turtle's carapace during the egg laying process (Fossette et al. 2008, Witt et al. 2011). We located all turtles using the ARGOS satellite system (CLS 2011) and data were downloaded via the Satellite Tracking and Analysis Tool (STAT) (Coyne & Godley 2005). We removed all locations with accuracy class Z and 0 (Witt et al. 2010). Data were imported into the Geographical Information System (GIS) ArcMap 10.1 (ESRI, Redlands, USA http://www.esri.com) and visually assessed to determine migration routes.

Stable isotope analysis

We analysed the stable isotopes of carbon and nitrogen from leatherback turtle epidermal tissue (females and hatchlings) and egg yolk from freshly-laid and unhatched eggs. These tissues are assumed to reflect the diet and habitat of foraging grounds prior to breeding migrations. Tissue turnover rates in sea turtles are virtually unknown (Ceriani et al. 2014), however epidermal tissue and egg yolk are believed to reflect the diet from at least four months prior to sampling (Hamann et al. 2003, Reich et al. 2008, Ceriani et al. 2014).

Samples were prepared following a standard protocol (Ceriani et al. 2014), except that samples were dried at 60°C for 48 to 72 h. Skin samples were rinsed with distilled water and epidermis was separated from the underlying tissue and finely diced using a scalpel blade, prior to being oven-dried. Fresh egg yolk and contents from unhatched eggs were defrosted and then oven-dried for 48 to 72 h before being homogenised with pestle and mortar. Approximately 0.7 ± 0.1 mg of sample was weighed into a tin capsule, sealed and analysed for carbon and nitrogen isotopes. SIA was performed at Elemtex (Stable Isotope and & Elemental Analysis Expertise) in Cornwall UK, using a Thermoquest EA1110 Elemental Analyser linked to a Sercon 2020 stable isotope ratio mass spectrometer (IRMS) running in continuous flow mode. Stable isotope ratios are expressed using a conventional notation as δ values defined as parts per thousand or per mil (‰), according to the equation presented in Bond & Hobson (2012):

$$\delta X = [(\mathrm{R}\mathit{sample}/\mathrm{R}\mathit{standard}) - 1] \times 1{,}000$$

where X is ^{15}N or ^{13}C; R*sample* is the corresponding ratio of the heavier to lighter isotopes (^{15}N:^{14}N or ^{13}C:^{12}C) in the sample; and R*standard* is relative to the international standards of atmospheric nitrogen and Pee Dee Belemnite, respectively. Analytic precision for the isotope data was < 0.2 and determined for $\delta^{13}C$ and $\delta^{15}N$ from the reference materials $USGS_{40}$, $USGS_{41}$ and BLS.

Lipid extraction

To evaluate possible methodological biases, we conducted lipid extraction on a subset of 10 egg yolk samples using a 2:1 Chloroform:Methanol ratio in a Soxhlet apparatus to determine if lipid extraction was needed (Post et al. 2007). Paired Wilcoxon signed rank tests for non-parametric data were performed on untreated and lipid extracted samples with no significant differences found for $\delta^{15}N$ values (Z = -1.07, W = 38.5, *p* = 0.28). Statistically significant differences were detected between paired samples for $\delta^{13}C$ (Z = -3.09, W = 55, *p* = 0.002). The mean difference in $\delta^{13}C$ due to lipid extraction was -2.67‰ (range -3.76‰ to -1.53‰). This difference was higher than the mean difference between the two groups of nesting females identified in this study (-1.98‰). Lipid extraction was expected to cause an increase in $\delta^{13}C$ because lipids have considerably lower $\delta^{13}C$ values than other cell components, such as proteins and carbohydrates (DeNiro & Epstein 1977); therefore, tissues typically have higher $\delta^{13}C$ values after lipid extraction (Kaufman et al. 2014). We then used linear regressions following (Kaufman et al. 2014) to determine mathematical corrections for lipid extraction for $\delta^{13}C$ values in our data as it follows: $\delta^{13}C_{nonLE} = 0.74 \times \delta^{13}C_{LE} - 7.76$ ($r^2 = 0.79$, $p = 0.01$) and used the equation to correct values of the non-lipid extracted egg yolk samples (Fig. S1).

Statistical analysis

To test the existence of homogeneous subgroups among nesting females, we used a *K*-means clustering analysis, where the number of clusters, *K*, is specified and the *K*-means algorithm assigns each observation to one of the *K* clusters (James et al. 2013). We compared live *vs.*

fresh-dead hatchlings, and freshly-laid *vs*. unhatched eggs using paired *t*-tests. To compare among tissues, we used linear mixed-effects models (GLMMs) where the isotope of $\delta^{13}C$ or $\delta^{15}N$ was the dependent variable and tissue type (female, egg yolk or hatchling) was the independent variable. Individual turtles were introduced in the model as a random effect. We tested for the correlation between the isotope values of egg yolk and hatchlings with their respective nesting females using Pearson's correlation tests. We also developed voucher conversion equations between tissues through simple linear regression models (Vander Zanden et al. 2014, Petitet & Bugoni 2017). To investigate inter-clutch variability, we collected an egg and a hatchling per clutch from females that were encountered repeatedly over the course of the nesting season. We examined the variation in isotope ratios using linear mixed-effects models (GLMMs) in which the dependent variable was the stable isotope ratios of $\delta^{13}C$ and $\delta^{15}N$. The independent variable was the time in days since a given female's first clutch was observed, with time 0 corresponding to the day the first clutch was observed. Individual turtles were set as a random effect. Statistical analyses were carried out with the software R 3.5.1 (R Core Team 2018).

Results

Isotope assignation of nesting females

A total of 44 nesting leatherback turtles were sampled, ranging in size from 124.7 to 175.0 cm CCL (mean ± SD = 149.4 ± 9.1 cm). Epidermal tissue samples revealed that nesting turtles had a mean $\delta^{15}N$ signature of 12.7‰ (SD = 1.8; range 9.6 to 17.3‰) and a mean $\delta^{13}C$ of -17.0‰ (SD = 1.3; range -19.8 to -14.2‰; *n* = 44). There was a strong positive correlation between $\delta^{15}N$ and $\delta^{13}C$ ($F_{(1,43)} = 0.5$, $r^2 = 0.70$; $p < 0.01$, *n* = 44, Fig. S2). No significant correlation with CCL was found either for $\delta^{13}C$ ($F_{(1,43)} = 51.9$, $r^2 = 0.01$, $p = 0.95$) or $\delta^{15}N$ ($F_{(1,43)} = 25.1$, $r^2 = 0.03$, $p = 0.86$; *n* = 44). A cluster analysis using stable isotope signatures revealed the existence of two distinct groups (Fig. 1), with a partial overlap. The two groups were significantly different from each other (Kruskal-Wallis test, *n* = 44, $p < 0.001$), with one group having higher values of both $\delta^{13}C$ and $\delta^{15}N$ (named 'neritic group', mean $\delta^{13}C$ = -15.5 ‰, $\delta^{15}N$ = 15.4 ‰, *n* = 12) in comparison to the other group (named 'oceanic group', mean $\delta^{13}C$ = -17.4 ‰, $\delta^{15}N$ = 11.7 ‰, *n* = 32). In our study, 31.8% (*n* = 44) of the nesting females sampled had scars or visible signs suggesting past interaction with fisheries.

Satellite tracking

Four leatherback turtles (2005-2006 *n* = 2, 2017-2018 *n* = 2) were tracked from 110 to 409 days (Almeida et al. 2011, present study). The two satellite-tracked females for which we had stable isotope samples for (2017-2018), were classified by the *k*-means cluster analysis as pertaining to the 'oceanic group' (Fig. 1). They both moved to offshore waters during their post-nesting migrations (Turtles 3 and 4, Fig. 2a). Their isotope values for $\delta^{13}C$ were in the lowest 25% of the distribution considering all the sampled turtles. For $\delta^{15}N$, one turtle was close to the median value among all the females sampled and the other had a value within the lowest 25% of the distribution (*n* = 44; Fig. 2b-c). Considering the two turtles tracked in 2005-2006, one (Turtle 1, Fig. 2a) remained within continental shelf areas, moving south along the Brazilian coast towards the Rio de la Plata Estuary, in Uruguay. The other turtle (Turtle 2, Fig. 2a) travelled along the southern coast of Brazil before undertaking a transoceanic journey across the Atlantic Ocean until she reached waters off the coast of Angola, when transmissions ceased.

Stable isotope signatures from individuals washed ashore or bycaught in fisheries

We sampled 17 individuals found washed ashore (stranded) or bycaught in fisheries along the Brazilian coast, ranging in size from 109.1 to 206.0 cm CCL (mean ± SD = 142.2 ± 22.0). They had a mean $\delta^{15}N$ signature of 11.8‰ (SD = 1.9; range 8.2 to 15.5‰) and a mean $\delta^{13}C$ of -16.3‰ (SD = 0.7; range -17.5 to -14.3‰; *n* = 17; Fig. 3). Seven individuals were identified as males, three as females and four remained undetermined in relation to their sex due to advanced decomposition or impossibility to perform necropsies due to logistical reasons. Three individuals were classified as juveniles since they had CCLs smaller than the smallest nesting female ever recorded in Espírito Santo (124.7 cm; Colman et al. Chapter I). Those three individuals were sampled in Ceará (119 cm CCL, bycaught alive in an artisanal floating fishing weir), Rio de Janeiro (109.1 cm CCL, washed ashore dead in 2015) and Bahia (120.5 cm CCL, washed ashore alive and released after treatment in 2017, Fig. 3b). From the 17 individuals sampled, two were bycaught in fisheries and another two had visible signs of interaction with fisheries.

Comparing tissues

There was no significant variation in $\delta^{13}C$ (t_{14} = -0.10, p = 0.92, n = 15) or $\delta^{15}N$ (t_{14}= -1.57, p = 0.14, n = 15) values between live and fresh-dead hatchlings from the same clutch. There was also no significant variation in $\delta^{13}C$ (Z = -1.59, W = 191, p = 0.11, n = 23) or $\delta^{15}N$ (Z = -0.91, W = 107, p = 0.36, n = 23) values between freshly-laid egg yolk and egg yolk from unhatched eggs. We pooled the available tissues to increase the sample size, meaning each female would have a corresponding sample for hatchling (either a live or fresh-dead hatchling) and egg yolk (either a freshly-laid or unhatched egg).

We defined the overall pattern in the 16 females for which we had $\delta^{13}C$ and $\delta^{15}N$ values for the three different tissues (female epidermis, egg yolk and hatchling epidermis). $\delta^{13}C$ values varied significantly among tissues (F_{30} = 154.64, p < 0.001) while $\delta^{15}N$ values did not (F_{30} = 0.89, p = 0.42). Values of $\delta^{13}C$ showed the following pattern: egg yolk < hatchling < female (Fig. 4). The relative difference between the tissues is shown in supplementary figure S3. We found a significant positive relationship between female epidermis $\delta^{13}C$ and $\delta^{15}N$ and the corresponding hatchling epidermis ($\delta^{13}C$: $F_{(1,15)}$ = 0.99, r^2 = 0.85, p < 0.001; $\delta^{15}N$: $F_{(1,15)}$ = 1.31, r^2 = 0.86, p < 0.001, n = 16, Fig. 5a-b). A significant positive relationship was also found between female epidermis $\delta^{13}C$ and $\delta^{15}N$ and the corresponding egg yolk ($\delta^{13}C$: $F_{(1,15)}$ = 1.65, r^2 = 0.75, p = 0.001; $\delta_{15}N$: $F_{(1,15)}$ = 1.32, r^2 = 0.81, p < 0.001, n = 16, Fig. 5c-d). Tissue conversion equations are presented in Table 1.

Inter-clutch variation

We sampled egg yolk from 12 females that were encountered repeatedly over the course of the nesting season: 1 female sampled for 5 different clutches, 1 female for 4 clutches, 6 females for 3 clutches and 4 females for 2 clutches. We also sampled hatchlings from 9 females that were encountered repeatedly over the course of the nesting season: 5 females sampled for 2 clutches and 4 females for 3 clutches. The different clutches were not necessarily consecutive, i.e. one or more clutches might have been missed (not sampled) in between two sampling occasions. The GLMM showed that time in days since a female's first clutch was observed did not affect $\delta^{13}C$ or $\delta^{15}N$ in egg yolk ($t_{22} = -0.31$, $p = 0.76$ and $t_{22} = -1.21$, $p = 0.24$, respectively; Fig. 6a-b) or hatchlings ($t_{12} = -1.46$, $p = 0.17$ and $t_{12} = -0.75$, $p = 0.47$, respectively; Fig. 6c-d).

Discussion

We report here the stable isotopes composition from the Southwest Atlantic Ocean leatherback turtle population for the first time. We found that leatherback turtles nesting in Brazil are likely to present variable foraging strategies, with possible consequences for the susceptibility to threats for this population. Additionally, we found that offspring tissue can be used as a proxy to infer on female isotope signatures when female tissues are not available and can be applied in future studies investigating spatial and trophic ecology. This research highlights the importance of investigating habitat use for the species to better understand ecology and inform conservation. Our major findings offer insights that lead to recommendations on how to augment this work going forward to better understand connectivity among breeding and foraging grounds in the South Atlantic Ocean.

Isotope assignation of nesting females

Leatherback turtles are known to present varied foraging strategies (Hays et al. 2006, Wallace et al. 2006, Witt et al. 2007, Caut et al. 2008, Seminoff et al. 2012, Robinson et al. 2016), with individuals varying in the pattern of their movements (Hays et al. 2004), usually associated with local oceanographic conditions (Ferraroli et al. 2004). The Brazilian nesting population possibly also presents distinct foraging patterns, as indicated by the existence of at least two distinct groups based on their stable isotope signatures. They could reflect a foraging area dichotomy for this population, with possible differences in their choice of feeding habitats (offshore *vs*. more coastal) and foraging longitudes (Southeast Atlantic *vs*. Southwest Atlantic, respectively), with consequences to threats susceptibility (Fossette et al. 2014) and for the species conservation (Caut et al. 2008). The differences in foraging areas could also be associated with differences in remigration intervals, clutch frequency and other possible carry-over effects (Harrison et al. 2011, Zbinden et al. 2011, Ceriani et al. 2015) that can ultimately lead into further impacts on population dynamics (Caut et al. 2008).

Satellite tracking and foraging area dichotomy

Satellite tracking further corroborates the existence of varied foraging patterns, however the small sample size (*n* = 2 turtles with both tracking data and SIA) in our study hamper reliable conclusions regarding foraging strategies of this leatherback turtle population. The two turtles tracked in 2017-2018 moved to oceanic waters between latitudes -30°S and -40°S (Fig. 2), being classified to the 'oceanic group' by the cluster analysis. One other female, tracked after the nesting season in 2005-2006, remained in neritic waters, moving along the Brazilian coast and reaching southern waters around de Rio de la Plata Estuary, in Uruguay (Almeida et al. 2011), but was not sampled for isotopic analysis. Our classification of oceanic vs. coastal groups was based on distinct isotopic signatures and by the fact that oceanic individuals are generally depleted in both $\delta^{13}C$ and $\delta^{15}N$ in comparison with neritic individuals (e.g. Robinson et al. 2016), as $\delta^{13}C$ values generally increase from oceanic to neritic habitats, and from pelagic to benthic food sources (Hobson 2007, Koch 2007, Bradshaw et al. 2017).

The differences between the neritic and oceanic habitats are probably caused by the primary producers in each habitat. For carbon isotope ratios, differences can be linked to distinct productivities, since benthic and nearshore regions are supported by algae and seagrass, exhibiting higher $\delta^{13}C$ levels than less productive oceanic regions, supported by phytoplankton (Haywood et al. 2019). For $\delta^{15}N$ values, differences between the habitats can be related to the nutrient source of primary producers, nitrogen-based processes and isotopic fractionation

(Montoya 2007). Coastal areas can have increased $\delta^{15}N$ values in primary producers caused by processes such as anthropogenic waste and agricultural runoff, which can in turn be reflected in higher trophic levels and used as a marker for habitat use (Haywood et al. 2019).

From 44 turtles sampled for SIA in this study, 12 were classified to the 'neritic group' and 32 to the 'oceanic group', suggesting that, at least currently, the majority of the turtles from Espírito Santo would have oceanic foraging patterns. It is also possible that small scale fisheries (SSF), largely operating in coastal areas in Brazil, could represent a significant impact for leatherback turtles (Eckert & Sarti 1997, Alfaro-Shigueto et al. 2011), with individuals presenting neritic phenotypes possibly being more likely to be captured (Shillinger et al. 2008). The distinct foraging patterns could result in different susceptibility to threats and consequently to mortality rates, which could contribute to the higher number of "oceanic turtles" predicted by SIA. Further research with stable isotopes and satellite tracking would allow us to elaborate any dichotomy more clearly and would help us to better understand migratory connectivity (Zbinden et al. 2011) and identify important foraging areas in the South Atlantic Ocean.

Comparing nesting females with individuals washed ashore or bycaught in fisheries

Leatherback turtles are typically considered an oceanic species (Eckert et al. 2012), however they are known to forage in the Southwestern Atlantic coastal waters (López-Mendilaharsu et al. 2009), where high mortality rates are recorded (Monteiro et al. 2016). Our results show that the males and females washed ashore along the Brazilian coast are broadly falling within the clusters of nesting females, suggesting that turtles are likely sharing foraging areas with, or that some of the stranded animals could be part of the highly endangered Brazilian nesting population in the pelagic and neritic areas (Vargas et al. 2017). None of the juveniles, however, fell within the clusters, suggesting that they might have slightly different foraging patterns, possibly due to ontogenetic and dietary differences known to occur in other species of marine turtles (Arthur et al. 2008). To date, however, there is no information on the diet of immature leatherback turtles (Wildermann et al. 2018) to help us to clarify this matter.

Any adults not part of the Brazilian nesting population could have mixed contributions from several Atlantic rookeries, as has been demonstrated by genetics (Vargas et al. 2008, Dutton et al. 2013) and satellite tracking (Witt et al. 2007). Bycatch in fisheries has been pointed out as one of the main threats to leatherback turtles worldwide (Ferraroli et al. 2004, Roe et al. 2014) and regionally (Sales et al. 2008, Fossette et al. 2014). A significant proportion of turtles in this study (31.8% of nesting females and 23.5% of stranded animals) have possibly interacted with some sort of fisheries (observed bycatch or presenting net induced lesions, severed flippers or been found entangled in hooks or fishing lines), highlighting the magnitude of this threat for leatherback turtles.

Comparing tissues

We suggest that offspring (egg yolk and hatchlings) can be used as a proxy to estimate female isotope signatures when female tissues are not available. In Espírito Santo, extensive nesting areas (~ 160 km) and the small population size (< 20 nesting females per year, Colman et al. Chapter I) makes sampling nesting females logistically challenging. If offspring can be sampled during regular morning patrols, it allows wide sampling without requiring the intensive effort of night patrols. Furthermore, unhatched eggs or freshly-dead hatchlings can be obtained during nest excavation and can be a less intrusive method than sampling nesting females. However, as nesting beaches used by leatherback turtles are usually highly dynamic and unstable

(Kamel & Mrosovsky 2004), it is not possible to guarantee that all clutches would complete their incubation period, and thus it is recommended that females tracked or being analysed in other studies should have a fresh egg collected during egg-laying or skin sample taken (Ceriani et al. 2014).

We found that egg yolk was the most $\delta^{13}C$ depleted of all tissues sampled (female and hatchling epidermis, and egg yolk). A similar pattern was described for loggerhead turtle egg shells, where eggs were the most $\delta^{13}C$ depleted among the tissues sampled (blood, skin and egg yolk), however they were the most enriched in $\delta^{15}N$ (Ceriani et al. 2014), while in our study hatchlings were the most enriched in $\delta^{15}N$. The causes for variability are not completely understood and moving between tissues still represents a great challenge. Ideally, research with stable isotopes should favour the use of standardised tissues so to allow comparing intra- and inter-specific patterns (Ceriani et al. 2014, Haywood et al. in review).

Inter-clutch variation

In our study, both the $\delta^{13}C$ and $\delta^{15}N$ values in egg yolk and hatchlings were not significantly different among successive clutches of the same female, and thus a single sampling event would be representative of the entire nesting season. The same pattern was also found in loggerhead turtle eggs (Hatase et al. 2002, Ceriani et al. 2014), however a small but significant decrease in $\delta^{15}N$ values and a significant decrease in $\delta^{13}C$ values were found in successive clutches of loggerheads in the Mediterranean (Zbinden et al. 2011). In a study on leatherback turtles in French Guiana, $\delta^{15}N$ values in egg yolks were not significantly different among successive clutches of the same female, however $\delta^{13}C$ values tended to decrease from one clutch to another (Caut et al. 2008). It is not clear what might cause these differences, but some females could be feeding during the internesting period or just before the start of the nesting season, and incorporating this $\delta^{13}C$ into their egg yolk, causing the variation on the pattern observed among the different populations. From our analysis (Fig. 6), it seems that there is less variability in isotope values of hatchlings among successive clutches than that in egg yolk. The sampling of hatchlings, however, has proven to be more difficult as it will be influenced by factors such as hatching success and the ability to determine the exact hatching day so to excavate nests, a condition not always available during fieldwork on nesting beaches.

Conservation implications and conclusions

The patterns observed in isotope signatures among different leatherback turtle populations are far from a clear understanding. The existence of foraging area dichotomy and consequently distinct isotopic groupings within populations (Wallace et al. 2006, Robinson et al. 2016) highlights the complexity of the interactions that might be occurring among phenotypic plasticity, oceanographic conditions and the existence of prevailing marine isoscapes, which are spatial gradients in stable isotope values at the base of the food web (Hobson et al. 2010, Seminoff et al. 2012). On the other hand, a study with leatherback turtles foraging in Canadian waters found no significant differences among average stable isotope value of individuals according to breeding stock origin (Wallace et al. 2014). Complementary studies with data from other important Atlantic rookeries such as Gabon and Bioko, in West Africa, would improve our understanding of migratory connectivity and population dynamics in the Atlantic Ocean. Leatherback turtles bycaught in small-scale fisheries off the coast of Peru have proven to originate from multiple nesting grounds in Eastern and Western Pacific (Alfaro-Shigueto et al. 2011), further suggesting that shared feeding habitats could also exist in the Southwestern Atlantic (Vargas et al. 2017). Understanding patterns of movements in marine migratory animals is essential for their conservation. This research contributes to the understanding of some of the priority research questions addressed by Rees et al. (2016), and the priority actions listed on the National Plan for the species conservation in Brazil (ICMBio, Administrative Decree nº 287, 2018), however more research with increased sample sizes is needed to fully understand foraging ecology of leatherback turtles in the Southwestern Atlantic.

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Ethical Standards

Research was submitted to SISBIO in Brazil (permit no. 47845-3) and subjected to approval by the ethical committee at the University of Exeter, UK, with ethical clearance(s) received.

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Table 1. Tissue voucher conversion equations for leatherback turtles in Espírito Santo, Brazil (*n* = 16).

Tissue from	Tissue to	$\delta^{13}C$	r^2	p-value	$\delta^{15}N$	r^2	p-value
Yolk	Female	$\delta^{13}C_{egg} = 1.13 * \delta^{13}C_{female} -0.41$	0.75	0.001	$\delta^{15}N_{egg} = 1.06 * \delta^{15}N_{female} -0.85$	0.82	<0.001
Hatchling	Female	$\delta^{13}C_{hatchling} = 0.92 * \delta^{13}C_{female} -2.76$	0.85	<0.001	$\delta^{15}N_{hatchling} = 1.11 * \delta^{15}N_{female} -1.15$	0.93	<0.001

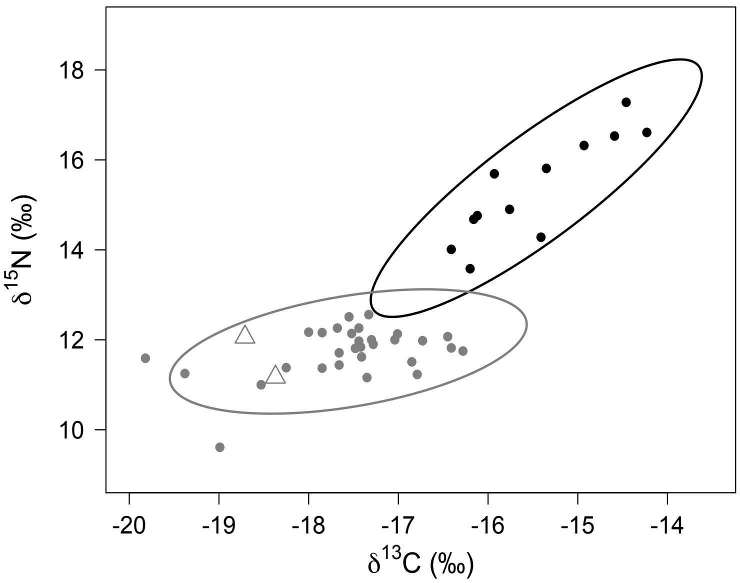

Figure 1. δ^{15}N and δ^{13}C values for leatherback turtles predicted to forage in: neritic environments (black circles, *n* = 12), or oceanic environments (grey circles, *n* = 32). Grey outlined triangles represent the two females tracked in 2017. Ellipses set at 95% CI (black line for the neritic and grey line for the oceanic turtles).

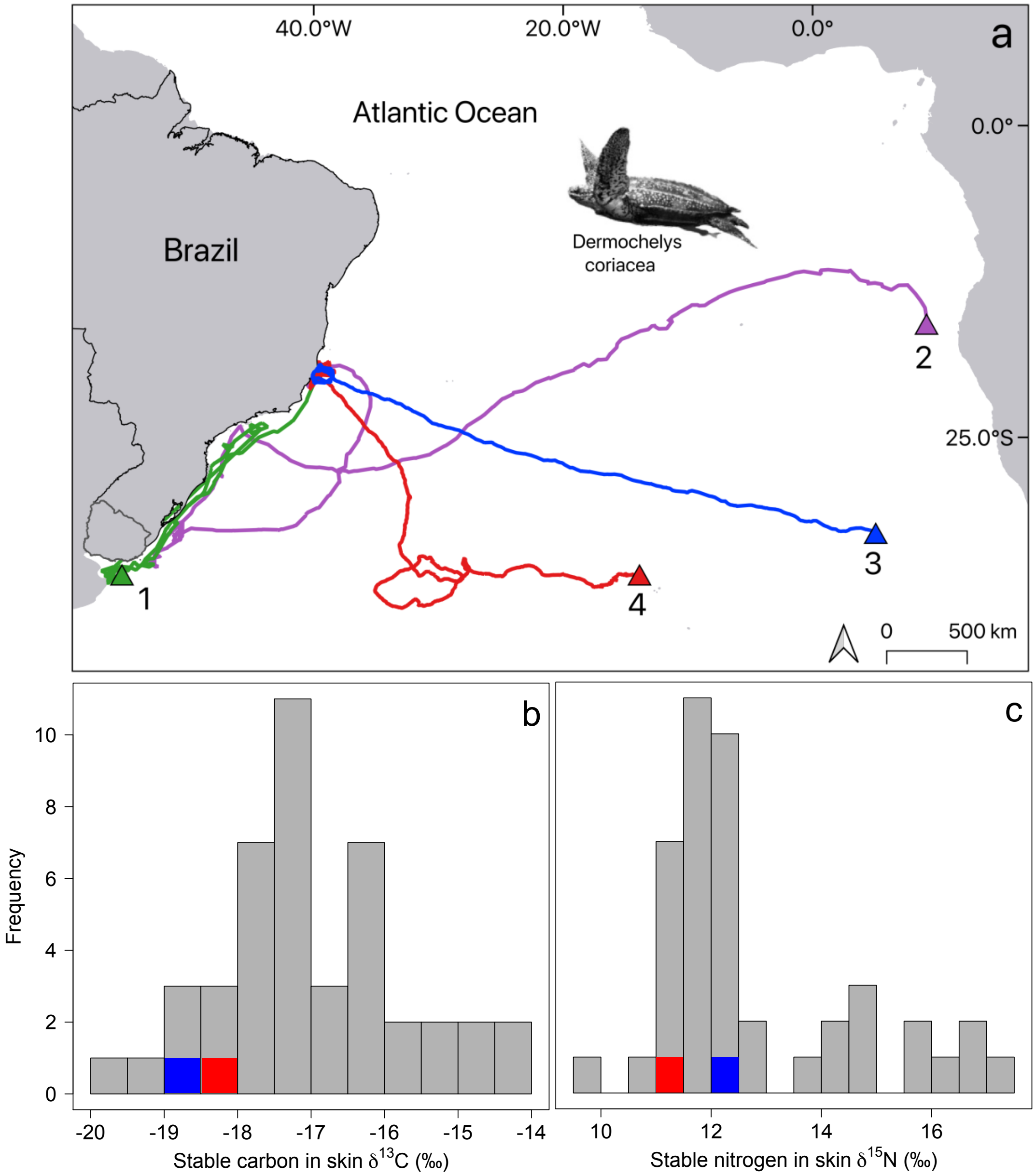

Figure 2. (a) Map of satellite-tracked post-nesting movements of four leatherback turtles from Espírito Santo, Brazil, overlaid with histograms of **(b)** stable carbon and **(c)** nitrogen isotope values for skin tissue of nesting females from the same population. Turtles were satellite tracked in 2005-2006 (turtles 1 and 2) and in 2017-2018 (turtles 3 and 4). Turtles were skin-sampled between 2012 and 2017 ($n = 44$). Blue (turtle 3) and red (turtle 4) track lines represent turtles for which we have both tracking and isotopes information. Their values are represented on the same colours within the $\delta^{13}C$ and $\delta^{15}N$ histograms **(b-c)**. Tracking data for turtles 1 and 2 are presented in Almeida et al. (2011).

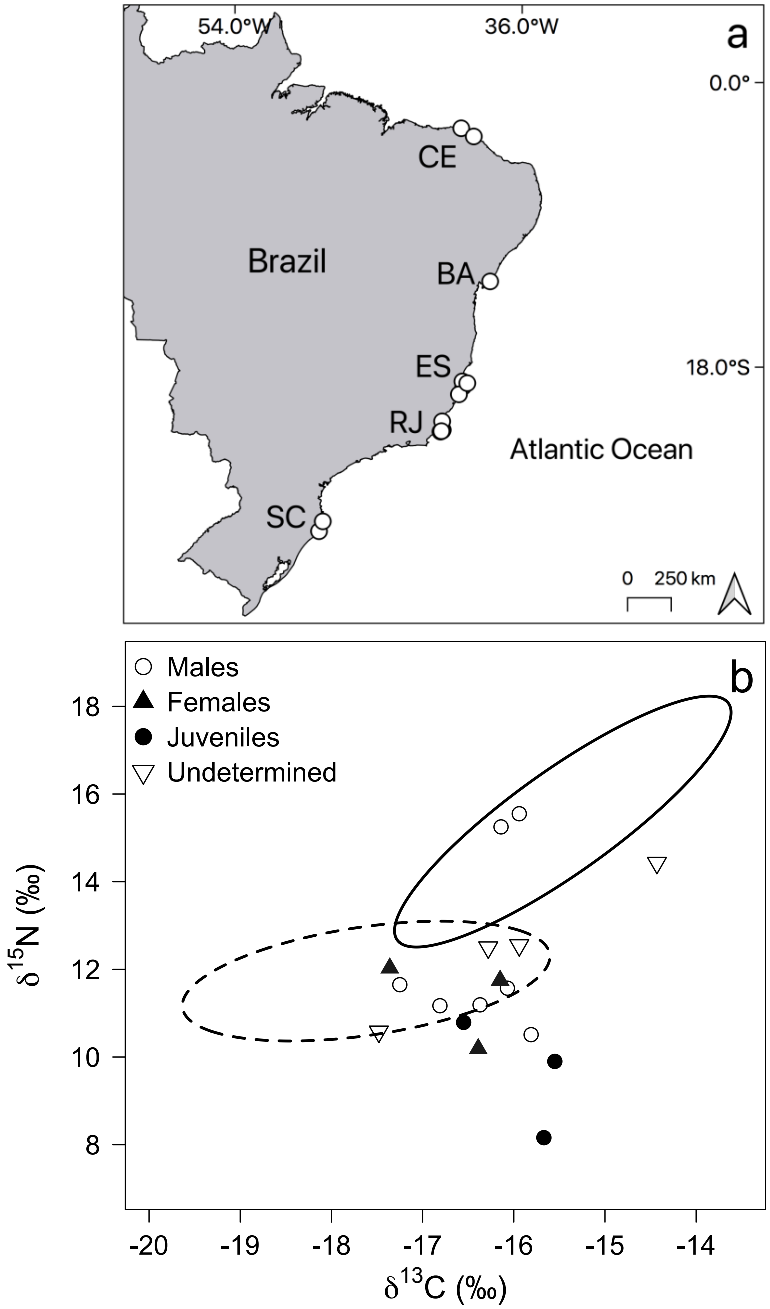

Figure 3. (a): Map of Brazil showing sampling locations (black outlined circles) of leatherback turtles washed ashore or bycaught in fisheries along the Brazilian coast: Santa Catarina (SC), Rio de Janeiro (RJ), Espírito Santo (ES), Bahia (BA), Ceará (CE) and **(b)**: stable isotope assignation from nesting females presumed to forage at neritic (full line ellipses) and oceanic (dashed ellipses) habitats, represented by their respective 95% CI ellipses from cluster analysis (*n* = 44). Individuals washed ashore/bycaught are presented in the figure and

classified as either males (black outlined circles, *n* = 7), females (black-filled triangles, *n* = 3), juveniles (black-filled circles *n* = 3) or undetermined (black outlined inverted triangles, *n* = 4).

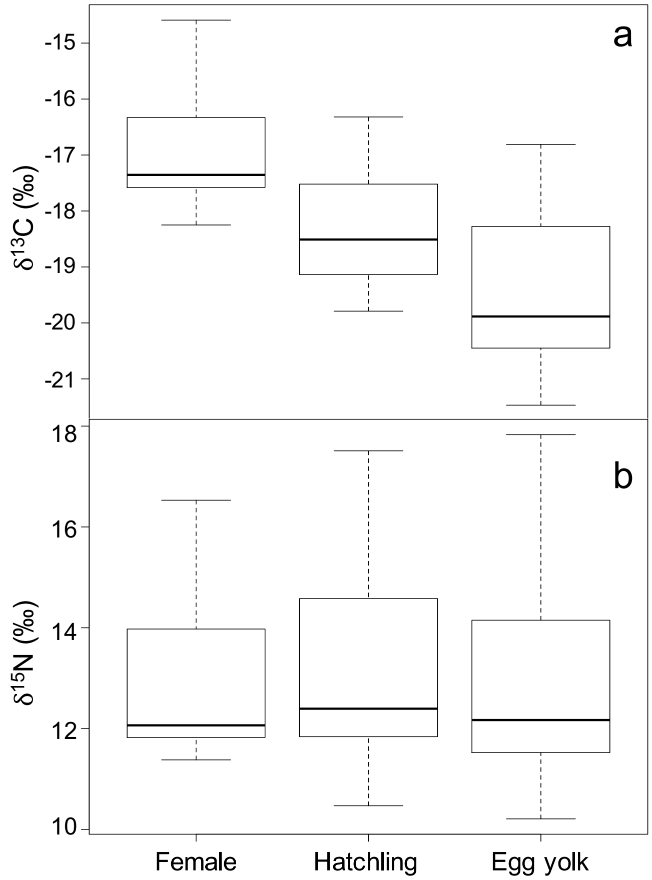

Figure 4. Boxplot summarizing the distribution of (**a**) δ^{13}C and (**b**) δ^{15}N ratios found in three tissues (female epidermis, hatchling epidermis and egg yolk) from 16 nesting leatherback turtles in Espírito Santo, Brazil. The box extends from the 25th to the 75th percentile; the central *line* indicates the median. The whiskers extend from the 10th to the 90th percentile.

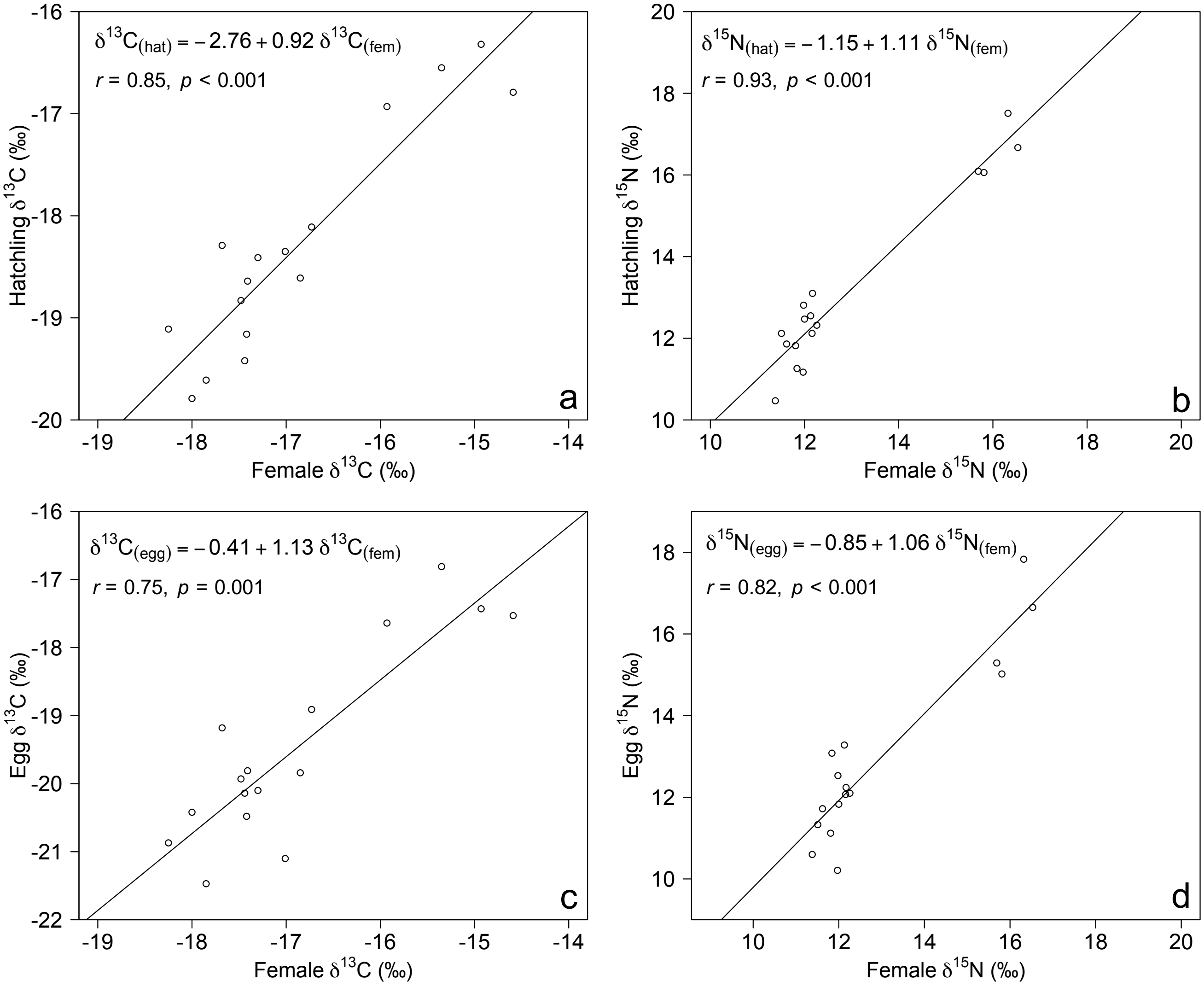

Figure 5. Relationships between female epidermis **(a)** $\delta^{13}C$ and **(b)** $\delta^{15}N$ values and those for hatchlings (n = 16) and for egg yolk **(c-d)**. The solid line is a linear regression. Equations within the graphs indicate the relationship between female and hatchling and female and egg yolk samples. *fem* female, *hat* hatchlings, *egg* egg yolk.

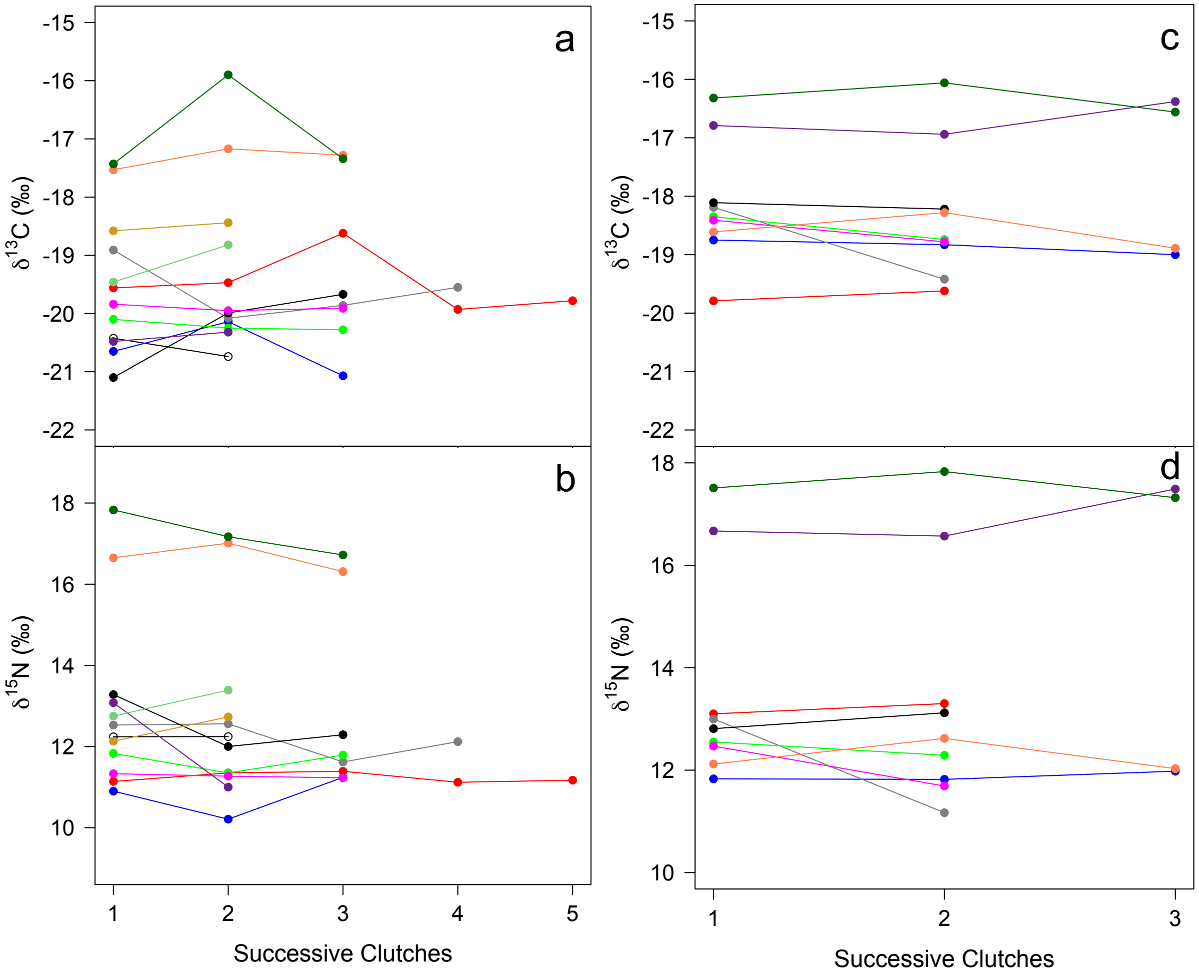

Figure 6. Inter-clutch isotope variability: trend in isotope values for **(a)** $\delta^{13}C$ and **(b)** $\delta^{15}N$ in egg yolk from 12 nesting leatherback turtles (*n* = 35 eggs) and **(c)** $\delta^{13}C$ and **(d)** $\delta^{15}N$ in hatchlings from 9 nesting leatherback turtles (*n* = 22 hatchlings). Each *point* represents a sampled clutch; each *line* connects two successive clutches laid by the same female. *Colours* correspond to specific females.

Chapter II: Supplementary information

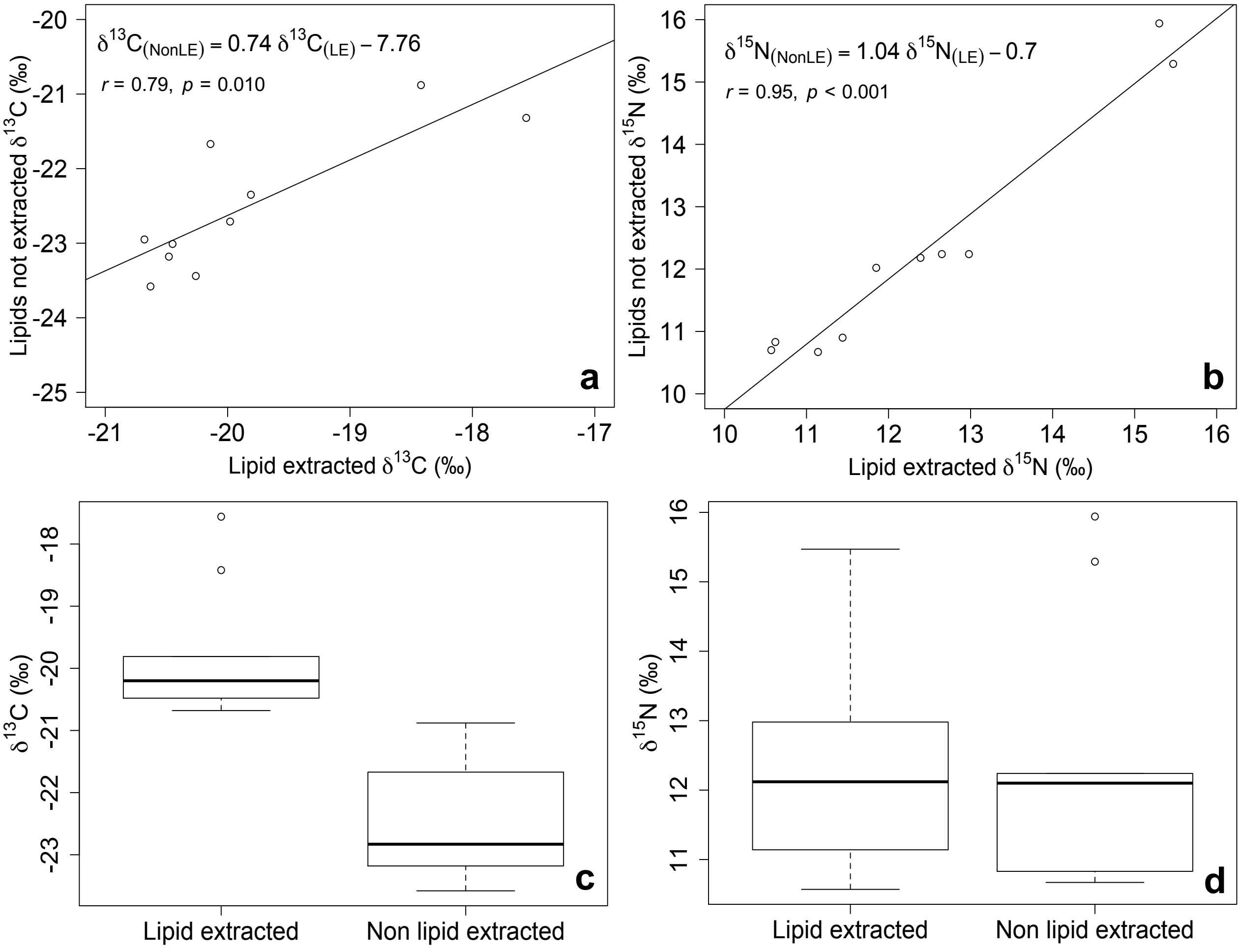

Figure S1. Relationship of stable isotope values between untreated and lipid extracted paired egg yolk samples for **(a)** $\delta^{13}C$ and **(b)** $\delta^{15}N$ (n = 10). Black solid line = regression line. Boxplots of **(c)** $\delta^{13}C$ and **(d)** $\delta^{15}N$ for lipid-extracted and untreated egg yolk samples (n = 10).

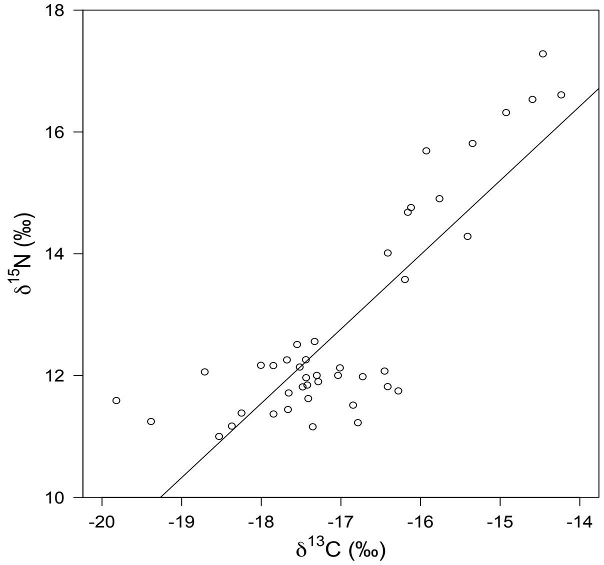

Figure S2. Correlation of $\delta^{15}N$ and $\delta^{13}C$ in nesting female leatherback turtles in Espírito Santo, from 2012 to 2017 (n = 44).

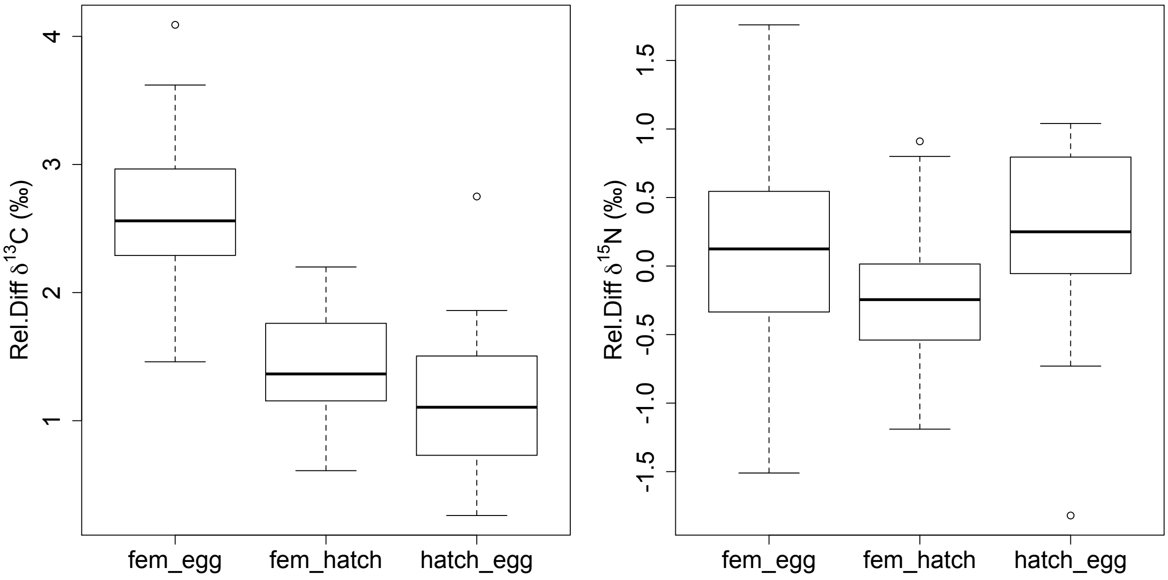

Figure S3. Boxplots summarizing the relative difference (Rel.Diff, y axis) between samples (*fem_egg* = female and egg yolk, *fem_hatch* = female and hatchling and *hatch_egg* = hatchling and egg yolk) for (**a**) δ^{13}C and (**b**) δ^{15}N ratios found in three tissues from *n* = 16 nesting leatherback turtles in Espírito Santo, Brazil.

Chapter III: Estimating hatchling sex ratios of leatherback turtles (*Dermochelys coriacea*) in the Southwestern Atlantic Ocean

This chapter is in preparation for submission to *Journal of Experimental Marine Biology and Ecology*

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Abstract

Knowledge on sex ratios of species with temperature dependent sex determination (TSD) is key to provide baseline information which can be used in management strategies and predictions of how climate change can affect populations. In marine turtles, increases in temperature can cause extreme sex ratio biases. Here we present a long-term analysis (30 years) on incubation durations of leatherback turtles (*Dermochelys coriacea*) in Brazil and estimate hatchling sex ratios. We deployed data loggers recording nest (*n* = 28) and sand (*n* = 6) temperatures from 2015 to 2017 and estimated sex ratios based on incubation temperatures during the thermosensitive period using the pivotal temperature described for the species in the literature. The average incubation duration for leatherback turtles in Brazil (1988-2017) was 66.3 days (range 52-91, SD = 6.8, *n* = 662), decreasing on average 2 days per decade and varying latitudinally across the nesting range of the population. When modelled to the entire nesting seasons, considering sand temperatures and metabolic heating, nests were estimated to have produced 83.9, 59.8 and 48.6% female hatchlings in 2015-2017, respectively (mean 64.1% females, *n* = 161). More balanced sex ratios were produced at the beginning of the season, being more female-biased as the season progressed. Studies with histological examination of hatchling gonads would be needed to confirm our predictions, however, the small population size makes this an unlikely option. This population is likely to be producing modestly female-biased sex ratios, however the decrease on the average incubation duration suggests potential feminization considering future scenarios of climate change. Irrespective, our results present baseline data on nest temperatures and estimated sex ratios for the region and are important to guide future management decisions for the Southwestern

Atlantic Ocean leatherback turtle population, considered Critically Endangered by the International Union of Conservation of Nature (IUCN).

Introduction

Knowledge on the primary sex ratio is key to assessing population viability and resilience (Melbourne & Hastings 2008, Mitchell et al. 2010). In species with temperature-dependent sex determination (TSD), offspring sex is determined by the incubation temperature during the thermosensitive period (TSP) and development of gonads (Bull 1983, Binckley et al. 1998). Temperature is known to be the main environmental driver of sexual differentiation in many reptiles (Wyneken and Lolavar 2015), including alligators (Yatsu et al. 2015), most turtles (Pieau et al. 1994) and some lizards (Charnier 1966). In marine turtles, all species studied to date exhibit this type of sex determination (Wibbels 2003). The adaptive significance of TSD is not completely understood, and theoretical models suggest that environmental sex determination (as in TSD) would be favoured by selection over genotypic sex determination (chromosome-based), when the environment during development distinctly influences fitness of females and males (Charnov-Bull model; Warner & Shine 2008).

Because the primary sex ratio is determined by the incubation temperature, the factors that influence this parameter play a central role in the dynamics of marine turtle populations (Hays et al. 1999). Extreme temperatures could result in the production of hatchlings of a single sex, thus making marine turtles particularly vulnerable to the impacts of projected global warming (Poloczanska et al. 2009, Fuentes et al. 2010, Mitchell & Janzen 2010, Fuentes et al. 2011). Most studies at marine turtle rookeries have estimated female-biased hatchling sex ratios, which are predicted to worsen following current predictions of climate change (Hawkes et al. 2007, Zbinden et al. 2007, Fuentes & Porter 2013, Marcovaldi et al. 2016). A few studies have reported male-biased or balanced primary sex ratios (Steckenreuter et al. 2010, Esteban et al.

2016, Patrício et al. 2017), and highlight the importance of these rookeries for the future conservation of marine turtles under the predicted climate change scenarios. It is yet uncertain whether marine turtles will be able to adapt to future climate changes. Highly female-skewed sex ratios being produced across several generations could lead to adaptive responses which would allow the continuity of the populations, or otherwise lead to population extinction (Mitchell & Janzen 2010). Many marine turtle populations have been reported to have increasing or stable population trends (Mazaris et al. 2017) with most of these recovering from a past of overexploitation following conservation efforts (McClenachan et al. 2006). The existence of other threats, such as fisheries bycatch, pollution and coastal development (Soykan et al. 2008, Wallace et al. 2010b, Duncan et al. 2017) might also act together with climate change, hampering population growth (Saba et al. 2012); identifying the impact of one threat when a population is recovering from the alleviation of another is a challenging task.

The potential impacts from climate change and increased temperatures on species with TSD calls for more research describing and predicting the primary sex ratios of distinct marine turtle species and populations (e.g. Hawkes et al. 2009, Fuentes et al. 2010, Hamann et al. 2013, Rees et al. 2016). Most studies have focused on loggerhead *Caretta caretta* and green turtles *Chelonia mydas*, with less data on the remaining species (Hawkes et al. 2009). Whilst there are a growing number of studies involving TSD and marine turtles (e.g. Hawkes et al. 2007, Marcovaldi et al. 2016, Patrício et al. 2017), there are still several basic and applied research questions concerning TSD which remain currently unanswered for leatherback turtles (*Dermochelys coriacea*). Few studies have considered long-term data to reconstruct past sex ratios, with most research using data that spans no more than one or two seasons (but see Sieg et al. 2011, Santidrían Tomillo et al. 2015). Studies describing the TSD curve and presenting pivotal temperatures and the range of intermediate temperatures producing both sexes, called transitional range of temperatures (TRTs) for leatherback turtles are known from only three rookeries: French Guiana in the Atlantic Ocean (Rimblot-Baly et al. 1987), Costa Rica in Eastern Pacific (Binckley et al. 1998) and Malaysia in the Western Pacific (Chen & Liew 1995). Recent studies have highlighted that more research with temperature data from different rookeries is needed in order to understand leatherback turtle TSD (Binckley & Spotila 2015). This knowledge is key to our ability to predict how changes in climate would interact with TSP and hatchling sex ratios, affecting the future of leatherback turtle populations.

Marine turtles have a wide geographical distribution, however the pivotal temperature (where a 1:1 sex ratio is produced) is believed to be relatively conserved among species and geographic locations, ranging from 28°C to 30°C (Mrosovsky 1994, Wibbels et al. 1998, Godley et al. 2002). For leatherback turtles, studies have shown a conservative range of pivotal temperatures (within 29.4-29.8 °C) in the Atlantic Ocean (Rimblot-Baly et al. 1987), Eastern Pacific (Binckley et al. 1998) and Western Pacific (Chan & Liew 1995). During the TSP, incubation temperatures above the pivotal result in a higher proportion of females, while lower incubation temperatures result in a higher proportion of males being produced (Mrosovsky & Yntema 1980), being the pivotal incubation duration the duration in days that would produce a 1:1 sex ratio. Chevalier et al. (1999) suggested that the temperature producing 50% of each sex (i.e. pivotal temperature) was not significantly different between the Atlantic and East Pacific rookeries. The range of intermediate temperatures producing both sexes (TRT), however, was significantly narrower for the French Guiana population, and variation in this parameter could have important effects in hatchling sex ratios (Binckley & Spotila 2015).

The Southwest Atlantic leatherback turtle population is known to regularly nest only on the northern coast of Espírito Santo state, in eastern Brazil (Thomé et al. 2007, Colman et al. Chapter I). The rookery in Espírito Santo represents a unique Regional Management Unit (Wallace et al. 2010a) and it is considered to be genetically distinct from other rookeries in the Atlantic (Dutton et al. 2013). This southwest Atlantic Ocean subpopulation is currently classified as Critically Endangered by the IUCN (Wallace et al. 2013). In Brazil, the species is included on the federal government's official list of endangered fauna (Machado et al. 2008). Despite its small population size - on average < 100 nests laid per year (Thomé et al. 2007, Colman et al. Chapter I) - this rookery is key, since it is located on the southernmost end of the species distribution in the Western Atlantic Ocean. Studies that investigated the sand temperatures and the primary sex ratios of loggerhead turtles being produced in Espírito Santo highlighted the importance of these male-producing beaches for the species (Baptistotte et al. 1999, Marcovaldi et al. 2016). Considering the predicted scenarios of climate change, this region may also become increasingly important for leatherback turtles, as other areas may become extremely female-biased or too hot to sustain hatchling production (Hays et al. 2017).

To date, no studies have investigated the sex ratios produced in the southwest Atlantic leatherback turtle population. As marine turtle hatchlings require gonadal histology to reliably determine sex (Broderick et al. 2000), indirect proxies such as incubation duration have been often used as an alternative to infer hatchling sex ratios (Marcovaldi et al. 1997, Marcovaldi et al. 2016). A study by Thomé et al. (2007) presented the incubation duration parameters for this population between 1988 and 2003. Here we build on this dataset and provide further ecological information regarding this parameter, and whether it has changed over time. We used indirect estimation methods based on the relationship between temperature and sex ratio determined in the laboratory to estimate hatchling sex ratios for this species (Binckley et al. 1998). We aim to contribute to the regional knowledge on leatherback turtle hatchling sex ratios and set out to (1) investigate long-term (30 years) incubation duration parameters and (2) use nest temperatures during the thermosensitive period (TSP) of incubation to estimate likelihood of sex ratios being produced in Espírito Santo.

Material and Methods

Study species and location

Espírito Santo state is located on the coast of Brazil between latitudes 19°40’S and 18°25’S (Fig. 1a). The leatherback turtle nesting areas comprise 160 km of high-energy and dynamic beaches, with coarse sand and are influenced by discharge from the Doce River in its southern part (Fig. 1b). On average about 90 nests are laid there annually, the majority (~ 80%) on the first southernmost 80 km (Colman et al. Chapter I). The leatherback turtle nesting season in the region typically runs from October to February, and as each nesting season spans two calendar years, hereafter we refer to a season by the first of those two years, e.g., the season 2015-2016 is referred to as the 2015 season. The climate and vegetation in the area were described in Baptistotte et al. (2003). There is no natural shading of the beach and virtually no human occupation or anthropogenic structures.

Monitoring procedures

Monitoring procedures followed the standard TAMAR methodology (Marcovaldi & Marcovaldi 1999, Thomé et al. 2007). The entire area is divided into 1 km sections for recording nest locations. Early-morning patrols were conducted daily, with constant effort, during the reproductive season along the entire study area (160 km). Nests were counted and marked with a numbered wooden stake, monitored during the entire incubation period and excavated after the majority of hatchlings had emerged. Incubation duration (ID) was calculated as the number of days between oviposition and the emergence of the first hatchlings (Thomé et al. 2007). In our analysis we included *in situ* nests only, from 1988 to 2017, where both oviposition and hatching dates were recorded.

Nest and sand temperatures

From October to February of the seasons beginning in 2015, 2016 and 2017 (encompassing the peak of the nesting seasons), hourly nest temperatures were recorded with Tinytag-TGP-4017 data loggers (Gemini Data Loggers; ± 0.3°C accuracy, 0.1°C resolution). Data loggers were deployed within nest chambers (*n* = 28 nests; 19, 6 and 3 in 2015, 2016 and 2017, respectively), during oviposition, at beaches on the first southernmost 60 km of the study area (Comboios and Povoação beaches, Fig. 1b). They were left there during the entire incubation period and retrieved upon hatching. Additionally, control dataloggers (*n* = 2 each year) were deployed in the sand, at a mean mid-clutch depth of 70 cm (TAMAR, unpubl. data) at Comboios beach (kilometres 24 and 29) to allow the reconstruction of seasonal sand temperature profiles and an estimation of the difference in sand temperatures associated with the metabolic heat produced by a typical clutch of eggs during incubation (Godfrey et al. 1997, Broderick et al. 2001). The initial four hours of temperature records were not considered, to enable data loggers to equilibrate with the surrounding sand (Broderick et al. 2001). Monitored nests were distributed along 38 kilometres, being up to 31 kilometres distant from control sites. All data loggers were compared to a calibrated datalogger before and after each nesting season in a constant temperature room (24 hours at 28°C) and had accuracy ≤ 0.3 °C. Clutches that had experienced disturbance of any kind (e.g. predation, relocation or destroyed by tidal flooding or beach erosion) were not considered in the analysis. For each nest we also recorded clutch size (by counting egg shells and unhatched eggs, with shelled albumin gobs (SAGs) frequently found in leatherback turtle nests, counted separately from yolked eggs and not considered in clutch size) and hatching success (percentage of yolked eggs that produced live hatchlings, including live hatchlings encountered in the nest during excavation; Thomé et al. 2007).

We used the annual mean control sites temperature to estimate sand temperatures during the nesting seasons in 2015, 2016 and 2017. Sand temperature was regressed against air temperature, obtained from the National Climatic Data Centre (https://www7.ncdc.noaa.gov/CDO/cdo; Vitória station, from between 100 and 250 km distant from nesting areas), to reconstruct the sand temperatures for the periods when data were missing (i.e. at the beginning and end of nesting seasons, when there were no data loggers recording temperatures; Supplemental materials). We estimated the middle third (ID_{mid}) mean incubation temperatures for nests laid from 30 September to 19 January for 2015, 2016 and 2017, by calculating a 22-d moving average of sand temperature at nesting beaches (22 d corresponding to the average duration of the ID_{mid} in the present study) and added mean metabolic heating (0.6 ± 0.5 °C; mean value for the present study; see below). Our control sites were located in a higher section of the beach, protected from beach erosion and storms. We calibrated the temperatures of mean control site and each clutch on the first five days of the incubation. Controls were on average 1°C warmer than clutches and as a result, to estimate mean incubation temperatures for the overall nests incubating throughout the nesting seasons, we adjusted sand temperatures by subtracting 1°C from the 22 d moving averages of the reference sand temperatures.

Sex ratio estimations

The mean incubation temperature during the thermosensitive period (TSP), believed to occur during the middle third of incubation (Binckley & Spotila 2015) was used to estimate the likelihood of production of both sexes. The proportion of females being produced was estimated using the following equation modified from Binckley et al. (1998) and Chevalier et al. (1999), using the pivotal temperature from Atlantic leatherback turtles (29.5°C; Rimblot-Baly et al. 1987), since there are no estimates of pivotal temperature for this population:

$$\text{Proportion of females} = \frac{1}{1+\exp(-(\mathrm{T}-29.5)/-0.2)}$$

Where T is the temperature during the $\mathrm{ID}_{\mathrm{mid}}$ and -0.2 is the S parameter, which defines the shape of transition from masculinizing to feminizing temperatures, calculated for leatherback turtles in the Pacific (Chevalier et al. 1999).

The sex ratio for the overall number of leatherback turtle nests laid on each season in Espírito Santo (including both in situ and relocated nests; the latter accounted for 17.0% of the total number of confirmed nests on the seasons from 2015-2017) was modelled as a function of the temperature of the sand, at nest depth (70 cm), during the $\mathrm{ID}_{\mathrm{mid}}$ of incubation. We then added 0.6°C of mean metabolic heating, which was estimated from our data, see Results. We corrected estimated sex ratios according to hatching success of each nest, in terms of the number of female hatchlings produced among the total number of live hatchlings, or whenever hatching success was not available for a given nest, we used the estimated hatching success for nests incubating on that bi-weekly interval, considering the average last five seasons. We only considered confirmed nests for the sex ratios estimates and not the total number of estimated nests (as in Chapter I), since in Espírito Santo approximately 25% of nesting emergences remain undetermined as to whether they were actual nests. We estimate that 66.0% of these nesting events are actual clutches, being then used to estimate the total number of nests in a given season. Therefore, we do not have the exact dates that those potential nests were laid, and cannot estimate their sex ratios.

Statistical analysis

The variation of incubation duration (until hatchling emergence) over the years was analysed with a local polynomial regression, using the R-package *locfit* (Loader 1999). To test for the difference among years, a Kruskall-Wallis test for non-parametric data was used (Hollander & Wolfe 1999). To analyse the relationship of incubation duration with geographical location of nests and the day in the season, we used loess regressions (Cleveland et al. 1993). Generalized Linear Models (GLMs) with Gaussian error structure and identity link function were used to test for the effect of clutch size (independent variable) on (1) mean ID_{mid} temperature and (2) hatching success (response variables). To assess the relationship of hatching success and incubation temperatures, we fitted logistic regressions with binomial errors and logit function, with hatching success as response variable and ID_{mid} mean temperature, mean and maximum temperature during the entire incubation as predictors. Statistical analyses were carried out with the software R 3.5.1 (R Core Team 2018). Estimates are presented as mean ± SD unless stated otherwise.

Results

Incubation durations

The overall average incubation duration for *in situ* clutches between 1988 and 2017 (*n* = 30 years) was 66.3 ± 6.8 days (range 52-91, *n* = 662 clutches). We estimate that 57.4% of the clutches incubated with longer incubation durations than the pivotal incubation duration (the incubation duration in days that would produce a 1:1 sex ratio; 63.9 days, Godfrey et al. 1996, Fig. 2a). The annual average incubation duration ranged from 61.5 days (in 1994, *n* = 4 and 2015, *n* = 49) to 78 days (in 1988, *n* = 1; Supplemental Table 1). Incubation duration was significantly different among years (Kruskal-Wallis test, *n* = 662, *p* < 0.001). There was a significant decrease in incubation duration across the 30 years as no horizontal line (representing a constant mean incubation duration in the period) can be placed inside the 0.95 simultaneous confidence band in Fig. 2b. Considering the decadal periods, the annual average incubation duration decreased from 69.5 ± 7.4 days (range 56-76, *n* = 75) in 1988-1997, 67.2 ± 6.6 days (range 52-90, *n* = 212) in 1998-2007 and 65.2 ± 6.5 days (range 53-91, *n* = 375) in 2008-2017. The incubation duration varied significantly with the date during the nesting season, as no horizontal line (representing a constant mean incubation duration in the period) can be placed inside the 0.95 simultaneous confidence band in Fig. 2c. Considering the geographic location of the nest, the incubation duration significantly varied with latitude (considered from south to north), as no horizontal line (representing a constant mean incubation duration in the area) can be placed inside the 0.95 simultaneous confidence band in Fig. 2d.

Nesting distribution

We recorded 72 leatherback clutches in 2015, 37 in 2016 and 52 in 2017. Mean peak nesting activity occurred between mid-October to mid-December (Fig. 3a), coinciding with higher mean precipitation levels (Fig. 3b). Mean peak hatching activity occurred between mid-December and mid-January (Fig. 3a).

Sand temperatures

We were unable to get sand temperatures for the beginning of the season in 2016 and 2017 and for the end of the season in 2017. For 2016 and 2017 respectively, we reconstructed these using the equations:

$T_{\mathrm{sand}} = 0.6\ \mathrm{T_{air}} + 14.2$ ($F_{1,112} = 115$, $p < 0.001$, $r^2 = 0.5$)

$T_{\mathrm{sand}} = 0.7\ \mathrm{T_{air}} + 12.4$ ($F_{1,98} = 84.3$, $p < 0.001$, $r^2 = 0.5$)

where T = temperature (°C) (Supplemental Fig. S1). Considering November and December (months with the highest concentration of nests), mean sand temperatures were significantly different among years (Kruskal-Wallis test, $x^2 = 34.3$, $p < 0.001$, Fig. 3c). The mean sand temperature (2015-2017) during the nesting season varied from 28.1°C to 31.9 °C (Fig. 3d) and it was below or close to the pivotal temperature until the beginning of December, increasing gradually along the season to temperatures higher than the pivotal temperature (Supplemental Fig. S2).

Nest temperatures

From 2015 to 2017 (n = 3 years) we gathered temperatures of n = 19, n = 6 and n = 3 nests that successfully hatched at Comboios and Povoação beaches, in Espírito Santo (Fig. 1b; Table 1). In 2015, the mean temperature during the $\mathrm{ID_{mid}}$ of incubation was 30.3 ± 0.7 °C (range

29.2-31.8, *n* = 19). In 2016, the mean temperature during the ID_{mid} of incubation was 29.8 ± 1.4 °C (range 28.2-31.9, *n* = 6). In 2017, the mean temperature during the ID_{mid} of incubation was 28.9 ± 1.2 °C (range 27.4-30.5, *n* = 3). The mean incubation temperature during the ID_{mid} varied slightly with the date during the nesting season, being relatively constant until mid-November, increasing until mid-December and then decreasing (Fig. 4). Clutch size (72.1 ± 12.6, *n* = 28, $F_{1,26} = 0.9$, $p = 0.4$) was a poor predictor of ID_{mid} mean incubation temperature. Mean sand temperature was a better predictor of ID_{mid} incubation temperature ($F_{1,26} = 59.4$, $p < 0.001$, *n* = 28) than incubation duration ($F_{1,26} = 19.8$, $p = 0.001$, *n* = 28, Fig. 5).

Metabolic heating

The metabolic heating during incubation ranged from -3.6 to 4.1 °C and was more pronounced during the last third of incubation, when the average metabolic heating was 1.9 ± 0.9 °C (range -2.6 to 4.1 °C). During the middle third of incubation, the average metabolic heating was 0.6 ± 0.5 °C (range -3.6 to 2.3 °C). During the first third of incubation, the average metabolic heat was 0.2 ± 0.4 °C (range -1.7 to 2.0 °C). Illustrations of the typical relationship between control site and within nest temperatures are given in Fig. 6 for each one of the years throughout the incubation period. We can note a lack of diel fluctuation and a steady increase in temperatures towards the end of incubation, except for the nest on Fig. 6a, where we recorded a wash over by the tides following a storm event during the TSP period, as indicated in the figure.

Estimated sex ratios

The mean primary sex ratios estimated for clutches with monitored temperatures was 62.9 ± 19.7% females (*n* = 28 nests; 3 seasons). The estimated proportion of female hatchlings produced varied between 89% in 2015 (*n* = 19), 58.4% in 2016 (*n* = 6) and 41.3% in 2017 (*n*

= 3) (Table 2). When we combined estimates of nests with modelled sand temperatures, the numbers were quite similar. The sex ratios estimated for the overall clutches in the season varied among years (2015: 83.9% females, *n* = 72; 2016: 59.8% females, *n* = 37; 2017: 48.6% females, *n* = 52), with a mean 64.1 ± 14.7% female offspring for the period 2015-2017 (*n* = 161, Fig. 7, Table 2).

Relationship of nest temperatures with incubation duration and hatching success

Hatching success in monitored nests varied between 38.3 and 98.3%, with a mean of 73.3 ± 18.5% and we found no significant relationship with clutch size ($F_{1,26}$ = 3.1, *p* = 0.09, *n* = 28). The relationship between hatching success and each one of the three predictors (mean temperature during ID_{mid}, mean and maximum temperature during the entire incubation period) was not significant (Fig. 8a,b). Hatching success was not affected by the maximum temperature during incubation (Fig. 8c). The sex ratios estimated for modelled nests varied from 50.5 to 81.4% female per year (mean 64.6 ± 12.8%), and it was virtually the same when we accounted for hatching success (mean 64.1 ± 14.7%), ranging from 48.6 to 83.9% (Supplemental Figure S3).

Discussion

Small populations are of conservation concern; however, it can be challenging to research key life-history parameters due to small data volume. Here we utilize an important long-term dataset to gain insights into the thermally related leatherback turtle hatchling sex ratio production at an important nesting ground for the species in the southwest Atlantic Ocean (Thomé et al. 2007, Colman et al. Chapter I). Our major findings are three-fold, offering clear insights that lead to recommendations on how to augment this work going forward to better understand the scope of population recovery, resilience and potential for adaptation in the face of climate change.

Temporal and spatial variation in incubation duration

Incubation conditions varied both temporally and spatially, with the decrease in the average annual incubation duration throughout the years being suggestive of potential feminization of the population. The seasonality of nesting, however, has not changed during the same period (Colman et al. Chapter I). The latitudinal variability in incubation duration indicates leatherback turtles might use strategies to produce a broader range of offspring sex ratio and consequently be more resilient to climate change, having improved chances of adaptation (Fuentes et al. 2013, Abella Perez et al. 2016). The slight decrease in incubation durations seen towards the northern part of the area could also be caused by different sand properties, such as colour, size and other parameters (Hays et al. 1999) not investigated in the present study.

Estimated sex ratios of leatherback turtle hatchlings

Different leatherback turtle nesting populations are believed to produce greatly different sex ratios. While the populations in the Pacific produce overall female-biased sex ratios (83.2% females observed in Costa Rica from 1998 to 2007, Sieg et al. 2011), a well-studied leatherback turtle nesting colony in the western Atlantic Ocean, Suriname, when researched two decades ago, was estimated to be producing modest female-biased sex ratios (60.5 - 69.4% females, Godfrey et al. 1996, Mrosovsky et al. 1984). Our estimates ranging between 62.9 – 64.1% female hatchling sex ratios are similar to the ones reported in Suriname. Further research with more representative sample sizes and spanning more seasons, together with information on other important Atlantic rookeries such as Gabon, in West Africa, would help us to better understand patterns of sex ratios for leatherback turtles in the Atlantic.

The pattern of earlier nests in the season having more balanced hatchling sex ratios while as the nesting season peaks, female-biased or completely female-producing incubation temperatures are recorded, was also observed in other leatherback turtle nesting grounds (Rimblot-Baly et al. 1987, Godfrey et al. 1996, Sieg et al. 2011). Incubation studies suggest that most field temperatures produce either all females or males, given the narrow TRT of leatherback turtles (Spotila & Binckley 2015). This could also be the case with the leatherback turtles in Brazil, where the vast majority of nests were estimated to have produced predominantly female or male hatchlings and a few nests to have produced balanced sex ratios.

Metabolic heating was more pronounced during the last third of incubation and showed heterogeneity among clutches, suggesting there might be too much variation to provide a reliable estimation of parameters other than nest temperatures. The large variation in metabolic heating can also be caused by wash over events during the incubation period, which can have a marked cooling effect on nests, allowing seasonally improbable male-producing temperatures (< 29.4°C) to be produced (Houghton et al. 2007). Metabolic heating was found to be less pronounced within leatherback turtle nests in Costa Rica (+0.20 °C, Sieg et al. 2011) and higher in Suriname, being on average 0.82 °C during the thermosensitive period (Godfrey et al. 1997). Causes for variation could be related to clutch size (Broderick et al. 2001, Zbinden et al. 2007), position within the nest (Godfrey et al. 1997) and the proportion of decomposing embryos within nests. Irrespective, metabolic heating should still be considered in predictions of sex ratios (Broderick et al. 2001). Further studies investigating nest temperatures in Brazil should consider the metabolic heating produced within the different months of the nesting season. The variation in metabolic heating is also more likely to be detected with a long-term dataset of temperature measurements (Sieg et al. 2011).

Incubation duration as a proxy to estimate sex ratios

The estimation of clutch sex ratios has largely been based in indirect proxies such as temperature or incubation duration (e.g. Marcovaldi et al. 1997, Glen & Mrosovsky 2004, Hawkes et al. 2007). This is due to the challenges of sexing marine turtle hatchlings, most reliably determined through the histological examination of gonadal tissue (Mrosovsky et al. 2009, Wibbels 2003, Yntema & Mrosovsky 1980), often requiring the sacrifice of hatchlings to achieve reasonable sample sizes. The relationship between sex ratio and incubation duration may become uncoupled in some nests, especially those most likely producing mixed-sex offspring (Mrosovsky et al. 2009). Our results showed that incubation durations were a poor predictor of temperature during the ID_{mid} and therefore sex ratios. The variability in the period between hatching and nest emergence can also add error to estimation of sex ratio using incubation durations. Additionally, it is believed that i are also likely to be driven by the temperature across the whole incubation period and not only during the thermosensitive period.

One important concern is the potential direct effects of increased temperatures on successful embryonic development. In highly female-skewed marine turtle populations, the increase in temperatures are predicted to cause hatchling mortality and decrease the natural growth of populations, threatening their long-term survival (Laloë et al. 2017). In our study area, hatching success was higher near the pivotal temperature (between 29 and 30 °C), and mean incubation temperatures in 2015-2017 were well within the thermal range. One possibility is that nest temperatures were likely not excessively hot to have negative effects on hatching success, and thus correcting the sex ratios considering the hatching success did not impact the overall conclusions in our analysis.

Conservation implications

The increase seen on nesting numbers for the species is encouraging (Colman et al. Chapter I). The decrease on average incubation duration, together with a modestly female-biased hatchling sex ratio over the decades could, however, have consequences to the recovery of this population. The female-biased sex ratios could contribute to population growth, since it would ultimately increase the number of nesting females (Hays et al. 2017). On the other side, climate change and other threats such as fisheries bycatch, pollution and coastal development could act synergistically, hampering population growth (Brook et al. 2008, Saba et al. 2012). Due to temporal and spatial variability and variable relationship of incubation duration with temperature, broad predictions for this population in terms of sex ratios being produced are not recommended.

To better understand patterns of leatherback turtle sex ratios being produced in Espírito Santo and allow future demographic modelling, further research with nest and sand temperatures should be conducted. Increased sample sizes would allow more refined estimates of sex ratios and better understanding of the thermal nesting environment. Studies comparing the different beach sections and zones (open sand, dunes and vegetation) could investigate the within-population variability in the sex ratios. A more detailed understanding of the thermal profile of the region would benefit our knowledge of potential resilience and adaptation to future scenarios of climate change. Studies with histological examination of hatchling sex ratios are needed to confirm our predictions, however the small annual number of nests makes these studies currently not justifiable. New methods to estimate the sex of hatchlings based on steroids extracted from eggshell, a non-lethal sexing method (Xia et al. 2011, Kobayashi et al. 2015) are promising, however the approach remains to be validated.

Better knowledge of beach temperatures and records of incubation duration contribute to an increase in our understanding of natural sex ratios for marine turtle populations and have implications for conservation practices (Mrosovsky & Yntema 1980), such as the potential effects of nest relocation. In Espírito Santo, on average 20.4% of nests during the last five years were considered “doomed”, as they were laid too close to the sea, and were relocated further up the beach to a safe spot. This practice could interfere with the incubation process and embryonic development, altering the sex ratios produced (Sieg et al. 2011). For a Critically Endangered subpopulation as the one in the present study, such conservation practices, when used cautiously, are still valuable as they reduce the loss of egg clutches and potentially contribute to population recovery.

In the present study we used the temperatures during the middle third of incubation to estimate sex ratios. We acknowledge that models considering the stages of embryonic development in response to temperature to estimate the incubation temperatures during the TSP should be used (Girondot & Kaska 2014), however those require key parameters (such as embryo development sizes and mean hatchling size) which are currently not available for this population. Future work should test between the two approaches, however a study conducted with green turtles in West Africa has suggested they do not vary significantly (Patrício et al. 2017). Irrespective, our results present baseline data on nest temperatures and estimated sex ratios for the region and are important to guide future management decisions for the southwestern Atlantic leatherback turtle population.

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Ethical Standards

This research was submitted to SISBIO in Brazil (permit no. 47845-3) and subjected to approval by the ethical committee at the University of Exeter, UK, with ethical clearance(s) received.

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Table 1. Leatherback turtle nests with monitored temperatures by month of oviposition in Espírito Santo, from 2015 to 2017 (*n* = 28). Oct = October, Nov = November, Dec = December.

	Month			
Season	**Oct**	**Nov**	**Dec**	**n**
2015	3	7	9	19
2016	1	3	2	6
2017		2	1	3

Table 2. Estimated number of female leatherback turtle hatchlings produced in Espírito Santo in monitored nests and then estimated overall for each of the 2015, 2016 and 2017 nesting seasons.

Season	Estimated proportion of female hatchlings					
	Monitored nests			Predicted overall season		
	Live hatchlings	Female hatchlings	(%)	Live hatchlings	Female hatchlings	(%)
2015	1042	927	89.0	3723	3122	83.9
2016	301	176	58.4	1934	1156	59.8
2017	135	56	41.3	2660	1293	48.6

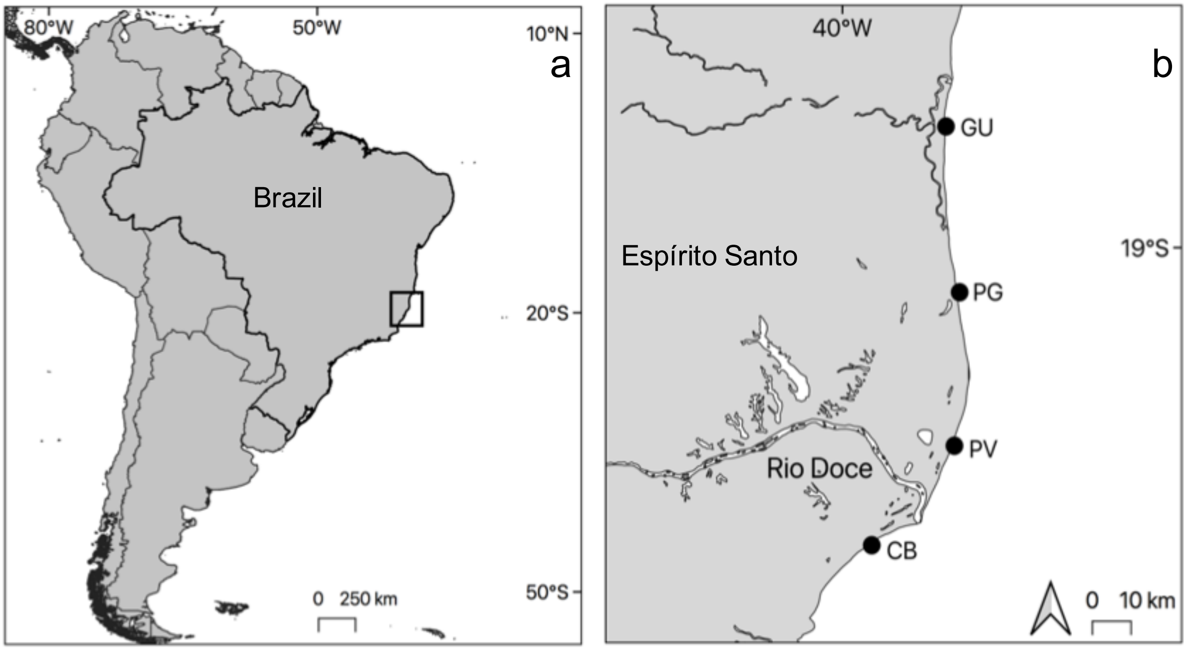

Figure 1. (a) Map of Brazil: the leatherback turtle nesting areas in Espírito Santo state are depicted by the black frame. **(b)** Map of the coast of Espírito Santo state, Brazil. Black circles represent the TAMAR stations where the data were collected. From south to north: CB = Comboios, PV = Povoação, PG = Pontal do Ipiranga, GU = Guriri. Rio Doce = Doce River.

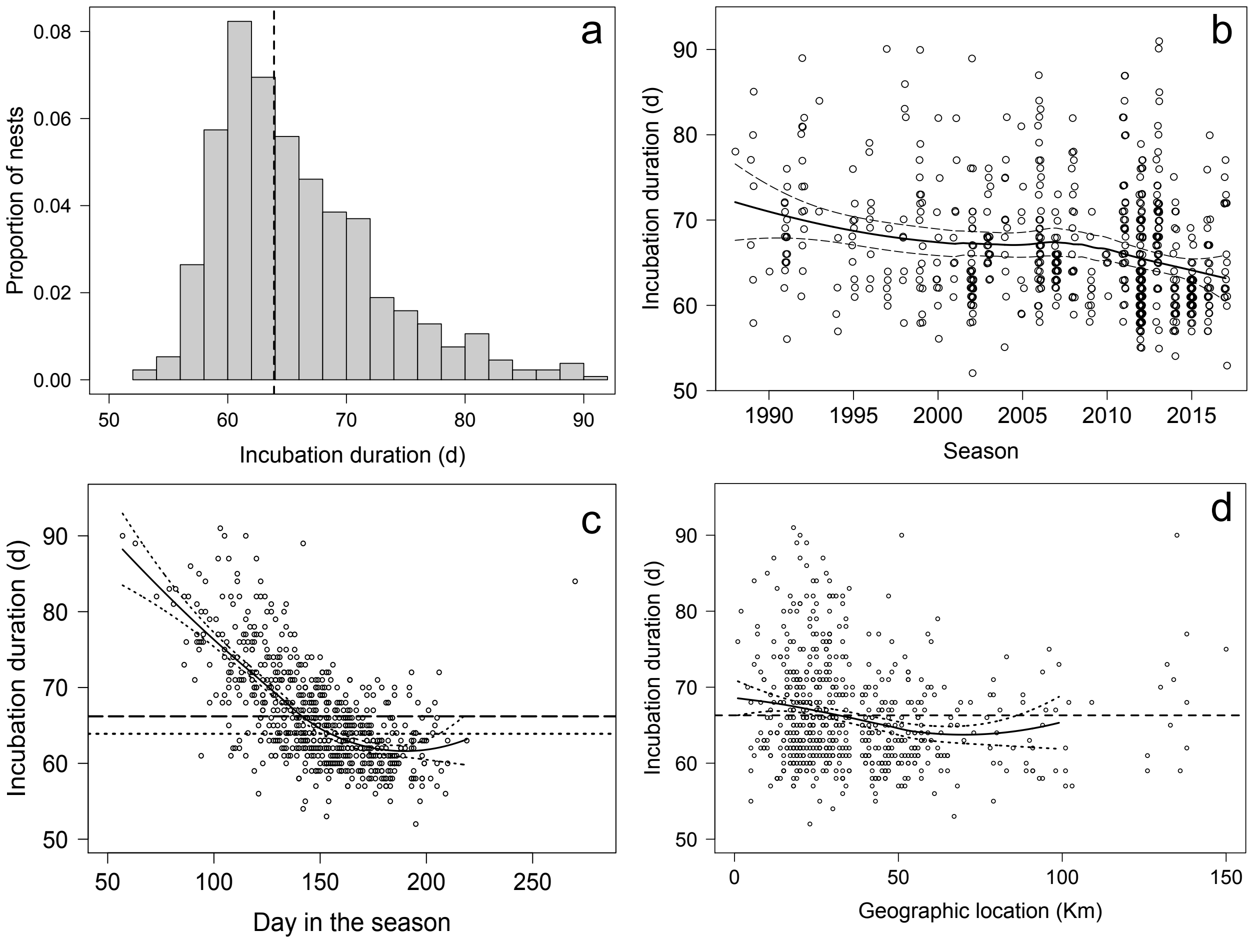

Figure 2. Incubation duration (days) of *in situ* leatherback turtle nests from Espírito Santo, from 1988 to 2017. **(a)** Proportion of nests and incubation duration (*n* = 662). The dashed vertical line represents the pivotal incubation duration (63.9 days) described in Godfrey et al. (1996). **(b)** Incubation duration by nesting season, 1988-2017 (*n* = 662). The solid curve is a local polynomial regression, and the dashed curves delimit a 0.95 simultaneous confidence band. In the graph, to increase clarity, the data points were jittered. For the regression calculations, the true values of incubation duration and season were used. **(c)** Incubation duration by oviposition date (in each season, July 1 = day 1) (*n* = 662). The solid line curve is a loess regression (*n* = 661), outer curves (dashed lines) show approximate pointwise 95% confidence intervals. The rightmost data point was not considered in the loess regression because of the insufficient number of points to reliably estimate the regression curve for that section of the

graph. The dashed horizontal line represents the average incubation period in this study (66.3 days). The dotted horizontal line represents the pivotal incubation duration (63.9 days) described in Godfrey et al. (1996). **(d)** Incubation duration by geographical location. The solid line curve is a loess regression (*n* = 640). Outer curves show approximate pointwise 95% confidence intervals. The 18 rightmost data points were excluded from the loess computations, because there was an insufficient number of points to reliably estimate the regression curve in the rightmost region of the graph. The dotted horizontal line represents the average incubation period in this study (66.3 days).

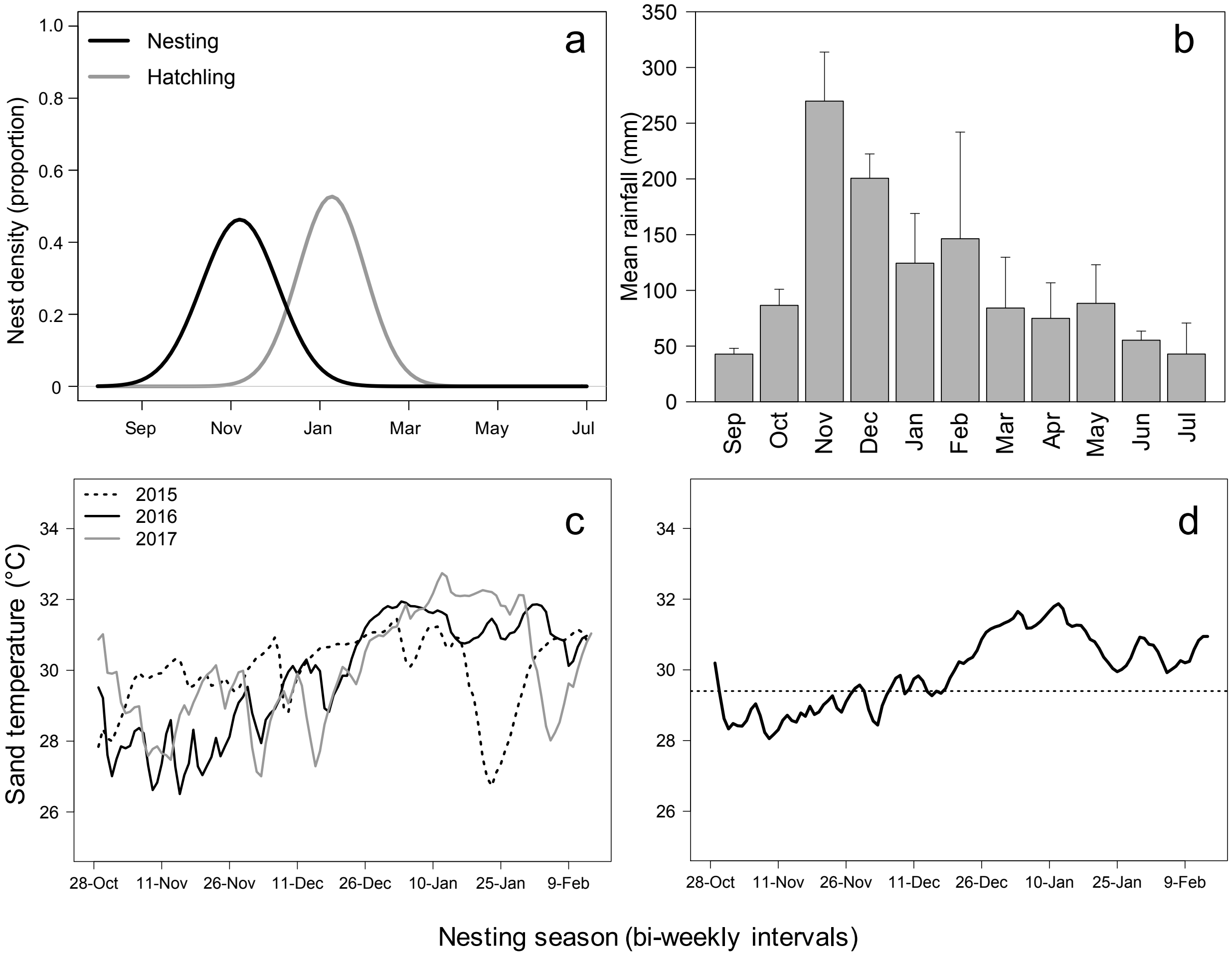

Figure 3. (a) Seasonality of nesting (black line) and hatchling (grey line) season of leatherback turtles in Espírito Santo, Brazil, from 2015 to 2017, *n* = 661. The lines represent the normal distribution for each variable (nesting and hatchling). **(b)** Mean precipitation (mm) in Vitória (between 100 and 250 km from nesting beaches), Brazil, over the nesting seasons from 2015 to 2017 (https://www.worldweatheronline.com/espirito-santo-de-vitoria-weather-averages/espirito-santo/br.aspx). The error bars indicate upper 95% confidence interval (CI). **(c)** Interannual variation in sand temperatures (°C) at nest depth (70 cm) across the nesting season and **(d)** Mean sand temperatures (°C) in 2015-2017 (bi-weekly intervals), at control sites in Espírito Santo, Brazil. The dotted horizontal line represents the pivotal incubation temperature (29.5 °C) presented in Rimblot-Baly et al. (1987).

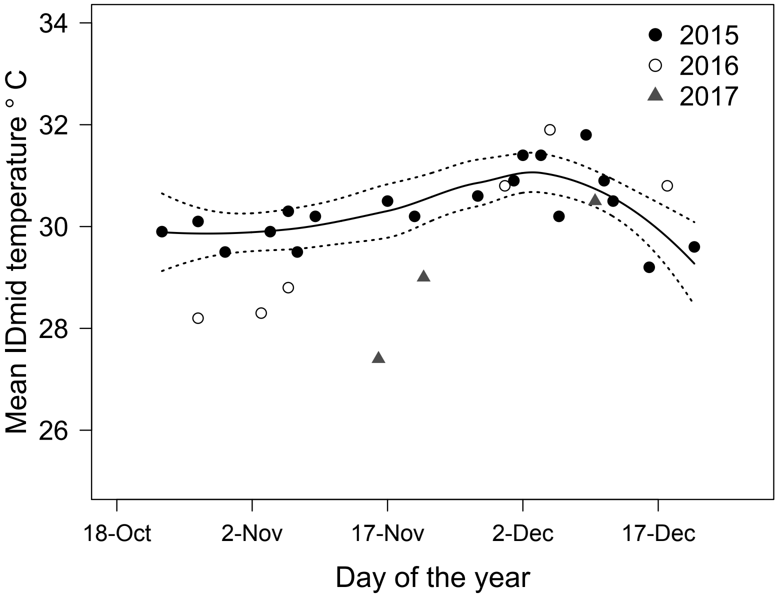

Figure 4. Mean ID_{mid} incubation temperature by day of the year (*n* = 28). The solid line curve is a loess regression and outer curves (dashed lines) show approximate pointwise 95% confidence intervals. The loess regression curve was calculated with the 2015 data points only (*n* = 19).

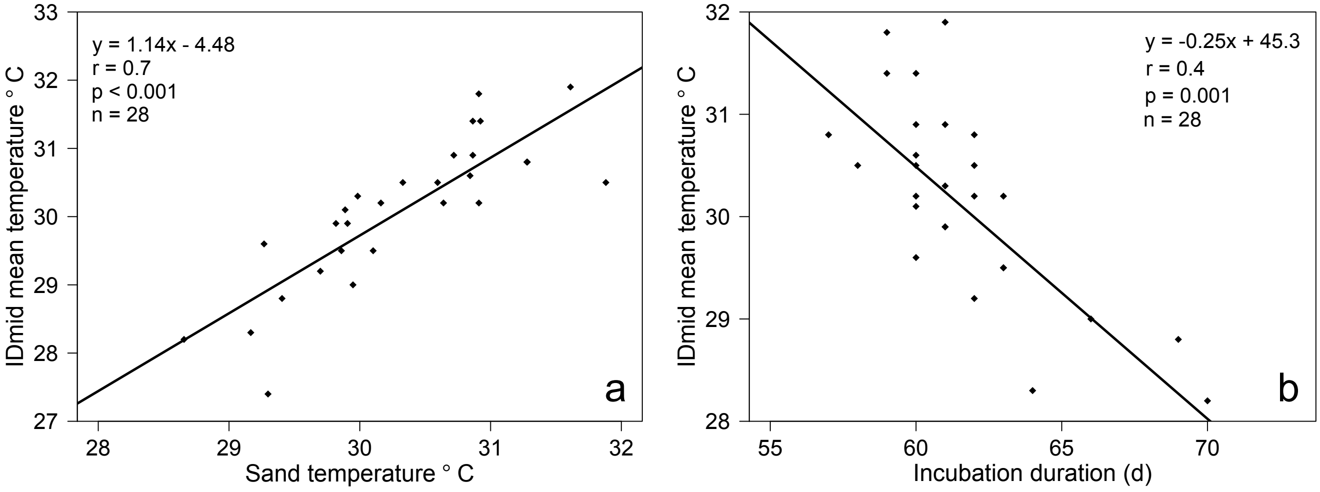

Figure 5: Linear regressions between clutch mean ID_{mid} incubation temperature and **(a)** mean sand temperature and **(b)** incubation duration in *n* = 28 monitored leatherback turtle clutches in Espírito Santo (2015-2017).

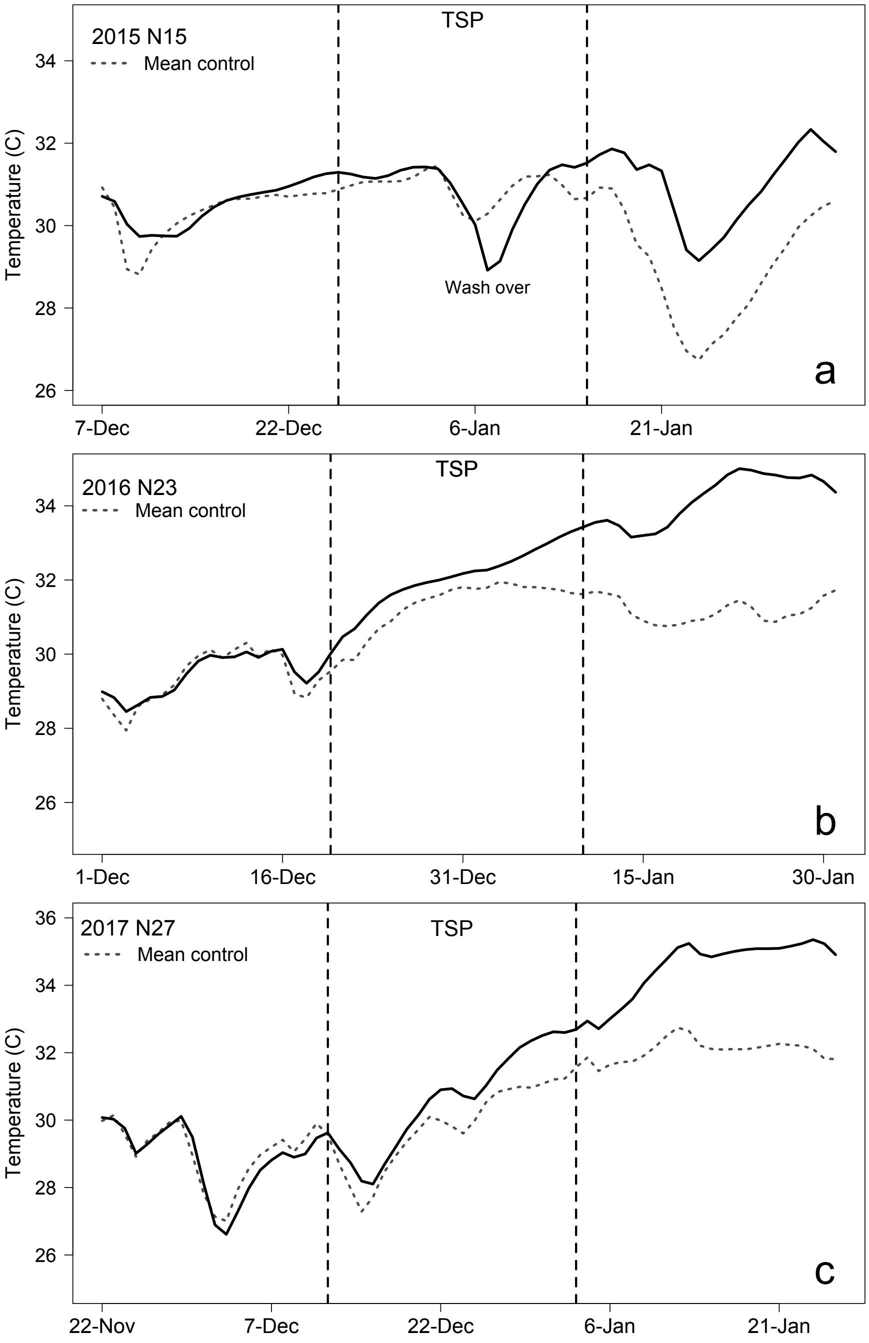

Figure 6. Comparison of daily mean control site and nest temperatures recorded for one nest in **(a)** 2015, **(b)** 2016 and **(c)** 2017. Dotted grey lines denote mean temperature within control sites; solid black lines denote mean temperature within nests. Dashed vertical lines represent the beginning and end of the TSP of incubation.

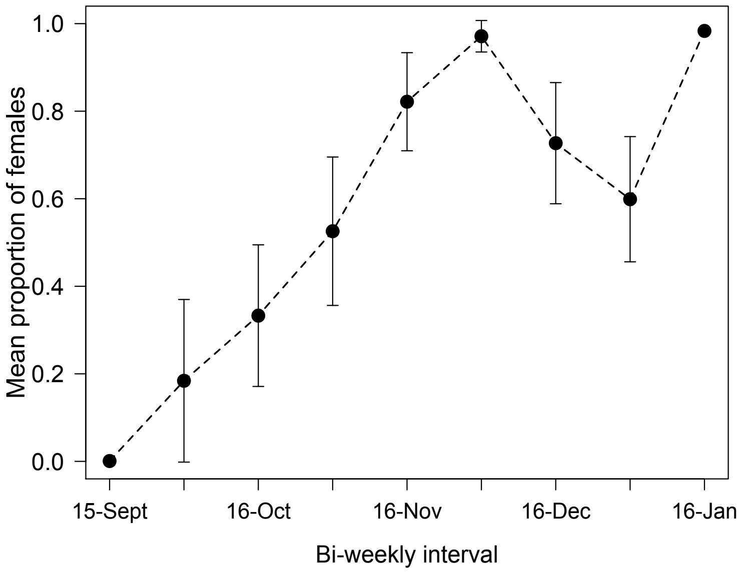

Figure 7. Mean estimated proportion of females (%) produced considering the overall number of leatherback turtle nests laid in Espírito Santo in 2015-2017 (*n* = 161). Error bars indicate 95% confidence intervals.

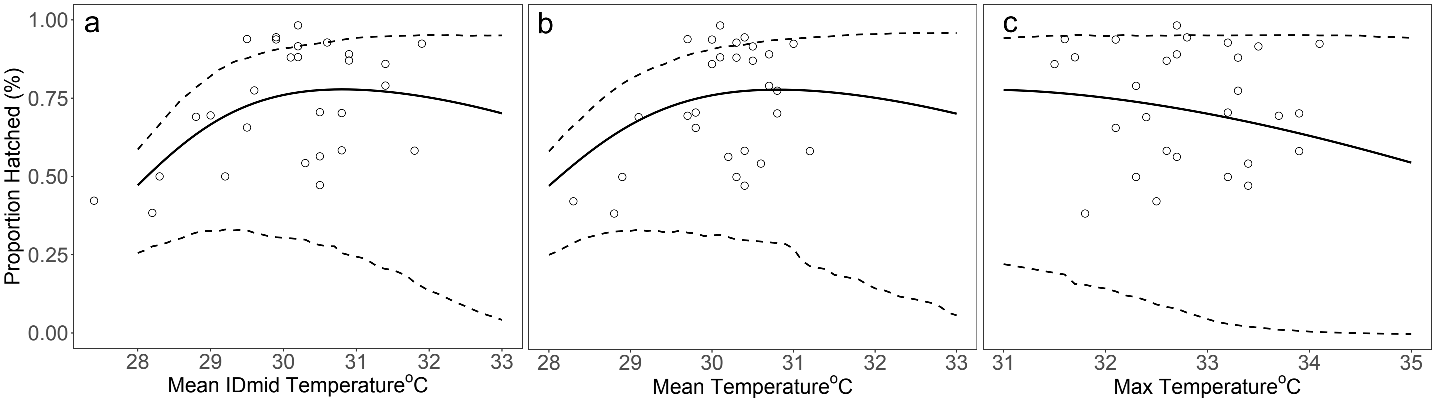

Figure 8. Relationship of hatching success of leatherback turtle nests with **(a)** mean ID_{mid} incubation temperature, **(b)** mean and **(c)** maximum temperature during the entire incubation period for monitored nests in Espírito Santo (2015-2017, *n* = 28). In all plots, curves show fitted logistic regression and dashed lines represent 95% confidence interval (CI). Significance of fit: **(a)** *p* = 0.34, **(b)** *p* = 0.4 and **(c)** *p* = 0.9.

Chapter III: Supplemental Material

Table S1. Incubation duration of leatherback turtle nests per year in Espírito Santo, from 1988 to 2017 (*n* = 662). The number of in situ, relocated and total number of confirmed nests in each nesting season is also presented.

Incubation duration (d)				Confirmed in situ nests	Confirmed relocated nests	Total confirmed nests
Season	**Mean ± SD**	**Range**	**n**			
1988	78.0 ± NA	(78-78)	1	8	17	25
1989	72.0 ± 9.7	(58-85)	7	11	17	28
1990	64.0 ± NA	(64-64)	1	13	0	13
1991	67.6 ± 4.6	(56-76)	19	25	10	35
1992	74.4 ± 7.8	(61-89)	14	21	6	27
1993	77.5 ± 9.2	(71-84)	2	4	2	6
1994	61.5 ± 4.8	(57-68)	4	7	9	16
1995	67.5 ± 4.5	(61-76)	11	13	6	19
1996	70.0 ± 5.9	(62-79)	9	23	10	33
1997	67.0 ± 10.6	(60-90)	7	12	7	19
1998	71.3 ± 9.9	(58-86)	9	15	2	17
1999	68.9 ± 8.2	(58-90)	19	37	18	55
2000	66.1 ± 7.4	(56-82)	11	30	5	35
2001	69.2 ± 6.8	(61-82)	9	37	10	47
2002	64.2 ± 6.0	(52-89)	43	78	14	92
2003	68.0 ± 3.7	(63-76)	21	61	6	67
2004	69.4 ± 7.1	(55-82)	13	20	4	24
2005	67.4 ± 7.9	(59-81)	8	22	3	25
2006	68.8 ± 7.2	(58-87)	45	69	21	90
2007	65.0 ± 2.7	(60-73)	34	42	8	50
2008	69.0 ± 6.9	(59-82)	20	30	9	39
2009	62.5 ± 3.9	(58-71)	11	20	4	24
2010	64.6 ± 2.1	(61-66)	5	11	8	19
2011	70.3 ± 7.4	(58-87)	39	73	17	90
2012	63.5 ± 5.4	(55-79)	118	142	26	168
2013	69.9 ± 7.3	(55-91)	53	74	31	105
2014	62.4 ± 5.1	(54-75)	32	40	11	51
2015	61.5 ± 2.4	(57-69)	49	61	11	72
2016	64.0 ± 5.5	(57-80)	28	33	4	37
2017	65.7 ± 6.3	(53-77)	20	39	13	52
All seasons	**66.3 ± 6.8**	**(52-91)**	**662**	**1071**	**309**	**1380**

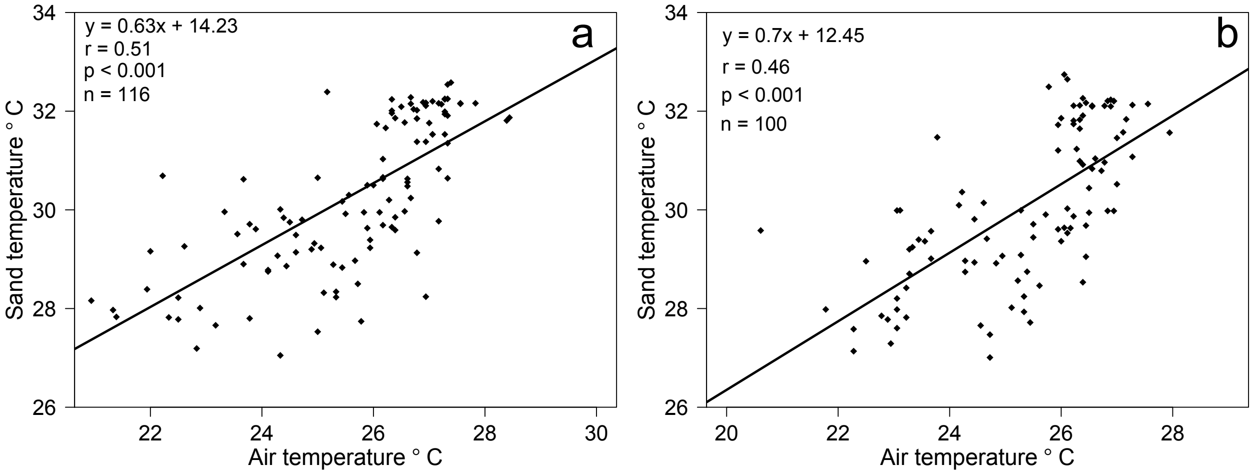

Figure S1: Linear regressions between mean daily sand temperature recorded at the control sites and air temperatures in Vitória, Espírito Santo, in **(a)** 2016 and **(b)** 2017 (https://www7.ncdc.noaa.gov/CDO/cdo).

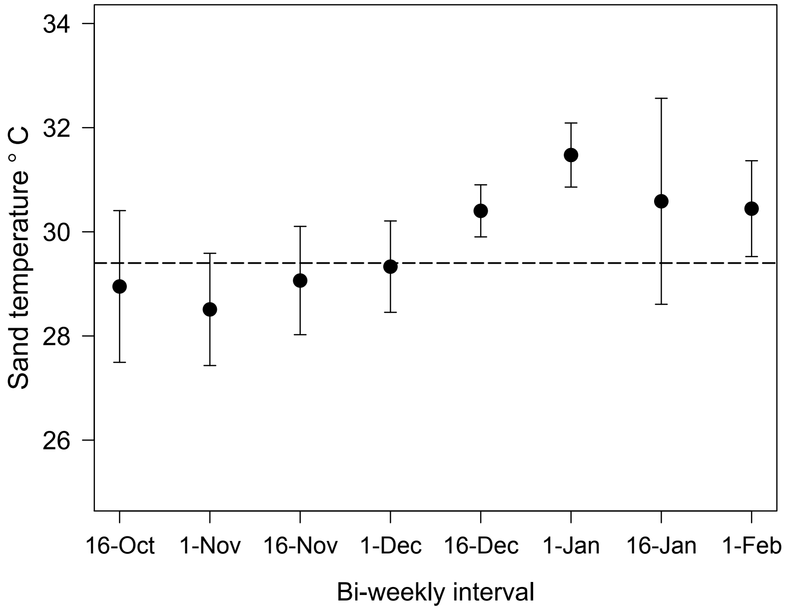

Figure S2: Mean sand temperatures at control sites by bi-weekly intervals (2015-2017). Each point represents one bi-weekly mean sand temperature value. Error bars indicate 95% confidence intervals. The dashed horizontal line represents the pivotal temperature described for the species (29.5 °C).

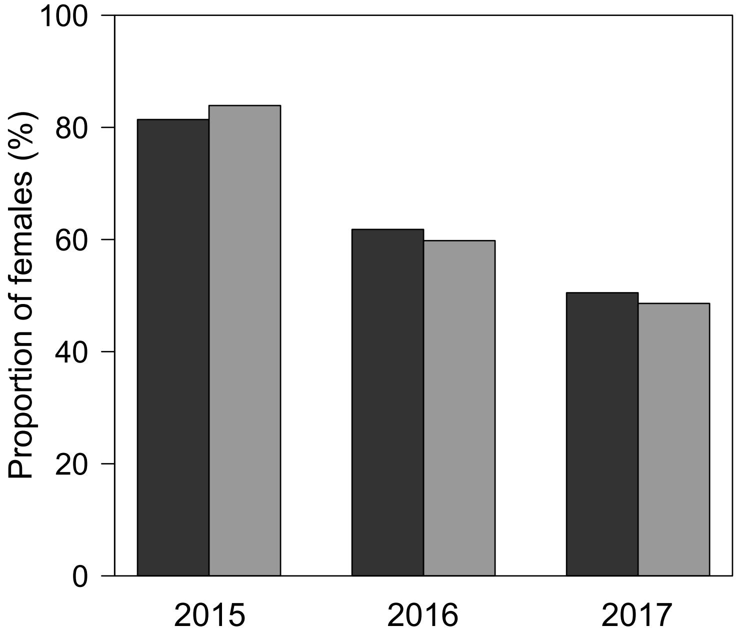

Figure S3: Annual proportions of female leatherback turtle hatchlings in Espírito Santo (2015-2017, *n* = 161) estimated from modelled nests (dark grey, 81.4, 61.8 and 50.5%) and corrected for hatching success (light grey, 83.9, 59.8 and 48.6%).

Chapter IV: Assessing coastal artificial light and potential exposure of wildlife at a national scale: the case of marine turtles in Brazil

This chapter is in the process of being revised following review for *Biological Conservation*

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Abstract

Light pollution impacts species, ecosystems and their processes. Coastal areas provide critical nesting habitat for marine turtles and understanding how artificial light might impact particular populations is key to effectively guide management strategies. Here we assess the extent to which nesting populations of four marine turtle species - leatherback (*Dermochelys coriacea*), olive ridley (*Lepidochelys olivacea*), hawksbill (*Eretmochelys imbricata*) and two subpopulations of loggerhead (*Caretta caretta*) turtles - are potentially exposed to light pollution across 604 km of the Brazilian coast. We used yearly night-time stable light composite satellite images from two different 5-year periods (1992–1996 and 2008–2012) from the US Air Force Defense Meteorological Satellite Programme (DMSP) Operational Linescan System (OLS) to determine the proportion of nesting areas experiencing detectable levels of artificial light and how this has changed over time. Observed increase in brightness was experienced in 63.7% of the 604 km of coastline monitored. Based on nest densities we identified 54 reproductive hotspots: 62.9% were located in areas potentially exposed to light pollution and 64.8% experienced increasing light levels. Light levels appeared to have a significant effect on nest densities of hawksbill turtles and the northern loggerhead turtle stock, however high nest densities were also seen in areas with high light levels. The status of all species/subpopulations has improved across the time period despite increased light levels. These findings suggest that (1) nest site selection is likely primarily determined by variables other than presence/absence of light and (2) conservation strategies in Brazil appear to have been largely successful in contributing to reducing impacts on nesting beaches. There is, however, the possibility that light affects hatchlings not only on the beach, but also in coastal

waters, and any impact on population recruitment may take a longer period to fully manifest in nesting numbers.

Introduction

Life on Earth is guided by daily, lunar and seasonal cycles of natural light (Foster & Roenneberg, 2008; Bradshaw & Holzapfel, 2010). The introduction and proliferation of artificial light at night, however, has transformed the night-time environment over significant portions of our planet's surface (Longcore & Rich, 2004). Light pollution can affect organisms in different ways, impacting their ecosystems and the processes that occur within them. The effects on wildlife can include changes in orientation systems and attraction or repulsion from the altered light environment, which ultimately might have consequences for foraging, reproduction, migration and communication (Longcore & Rich, 2004).

Remote sensing has been used as a proxy to measure artificial night-time light (Cinzano et al., 2001) and its effects on wildlife, including invertebrates, birds, reptiles and mammals (Longcore & Rich, 2004; Aubrecht et al., 2008; Kamrowski et al., 2012; Mazor et al., 2013; Weishampel et al., 2016). Worldwide measurements of artificial light have been collected by the US Air Force Defense Meteorological Satellite Program (DMSP) Operational Linescan System (OLS) since 1992 (Elvidge et al., 2007). These data can be downloaded from the NOAA's National Geophysical Data Center (NGDC) and represent cloud-free composite images created from multiple nightly orbits by the DMSP satellites for each year (Elvidge et al., 2001, 2007).

In marine turtles, nesting distributions are determined by several environmental factors that operate at different spatial scales. The range of suitable nesting areas is most likely determined primarily by temperature (Pike, 2013). At regional scales, nest density appears to be strongly linked to how well the offshore migrations of hatchling marine turtles is facilitated, that is, coastal areas in close proximity to favourable currents for transporting hatchlings to nursery habitat would have higher nest densities than other less favourable areas (Putman et al., 2010a; Putman et al., 2010b, Okuyama et al., 2011; Shillinger et al., 2012, Putman, 2018). At local scales, site-specific topographic features such as vegetation, sand dunes, beach slope, substrate and lighting become increasingly important (Price et al., 2018).

Light pollution can impact fundamental biological processes for marine turtles. After emerging from nests, hatchlings are generally guided by several cues to find the ocean, including beach elevation and natural brightness (Lorne & Salmon, 2007; Pendoley & Kamrowski, 2015). The presence of artificial lighting can cause disruption to sea-finding, increasing predation risk and also possibly causing exhaustion, dehydration and the loss of energy that is vital to their initial migration towards the open sea, resulting in higher mortality (Witherington & Bjorndal, 1991). Artificial lighting at nesting beaches is also believed to potentially disrupt adult turtle nesting (Pendoley & Kamrowski, 2015; Silva et al., 2017) and females probably avoid particularly lit areas (Salmon, 2003). The presence of artificial light at beaches and near shore waters may also alter the predator-prey dynamics, influencing the behaviour of species that predate on sea turtle egg and hatchlings (e.g. Silva et al., 2017), as well as attracting hatchlings dispersing from natal beaches (Thums et al., 2016).

To evaluate the potential effects of artificial light on marine turtle nesting, we use remote sensing and information on globally important marine turtle nesting grounds in Brazil. The Brazilian coast and offshore islands hold nesting grounds for five sea turtle species: the loggerhead (*Caretta caretta*), hawksbill (*Eretmochelys imbricata*), olive ridley (*Lepidochelys* *olivacea*), leatherback (*Dermochelys coriacea*) and green turtles (*Chelonia mydas*) (Marcovaldi & Marcovaldi, 1999). Conservation efforts started by TAMAR (the Brazilian Sea Turtle Conservation Programme) in 1982 (Marcovaldi & Marcovaldi, 1999) contributed to the increasing or stable trends seen in nesting numbers for all the five species (da Silva et al., 2007; Marcovaldi et al., 2007; Marcovaldi & Chaloupka, 2007; Thomé et al., 2007; Bellini et al., 2013). Despite the possible recovery from past exploitation seen for the marine turtles in Brazil, the persistence of various threats - fisheries bycatch, coastal development, pollution and climate change – means these populations remain subject of conservation concern. Furthermore, since the satellite data has been available, the human population in Brazil has grown considerably, with much of the population growth seen along coastal areas (Instituto Brasileiro de Geografia e Estatística, 2013). The consequent coastal development and associated artificial lighting represent a potential threat to marine turtles in Brazil.

Here, we sought to assess the extent that nesting habitats of marine turtles in Brazil are exposed to artificial light at a large national scale. We analyse spatial patterns of artificial night-time lighting across the major marine turtle nesting grounds for multiple species along the Brazilian coast and how this has changed over time. We determine the proportion of nesting marine turtles that are likely to be potentially exposed to coastal light pollution. We identify reproductive hotspots within Brazilian nesting grounds. In particular, we assess what level of exposure to light pollution they potentially experience. Finally, we discuss the findings considering the conservation efforts and recent population growth for the turtle management units in the region.

Methods

Light data

We downloaded yearly night-time stable lights composite images from two different 5-year periods (1992–1996 and 2008–2012, *n* = 10 years) from the National Geophysical Data Centre (USA; http://ngdc.noaa.gov/eog/dmsp/downloadV4composites.html). They were created with data from the Defense Meteorological Satellite Program (DMSP) Operational Linescan System (OLS) and represent Average Visible, Stable Lights and Cloud Free Coverage images. These images are nominally at 1 km resolution; however, they are re-sampled from data at an equal angle of approximately 2.7 km resolution at the equator. Each pixel is represented by a digital number (DN) between 0 and 63, with zero representing total darkness and bright-lit areas generally saturating at values of 63. Because there is no on-board calibration of the satellites, the images were inter-calibrated and drift-corrected according to Bennie et al. (2015). This method for cross-calibration included correcting for geolocation errors between the years and posterior intercalibration of the images using a 6th order polynomial regression on the median (for full description of calibration methods see Bennie et al. 2015). Even after being corrected, the annual light data should be interpreted with caution, especially considering the variation in sensitivity among years and the saturation in urban areas. A more precautionary approach is to use average values for 5-year periods. In this way, DMSP/OLS night-time lights remain a valuable source for detecting long-term trends in the distribution of artificial light at night (Bennie et al., 2015). The OLS sensors capture light that is consistent with wavelengths believed to disrupt adult and hatchling marine turtles (within the 440 to 700 nm range), with longer wavelengths (approximately 580 nm) showing greatest sensitivity for adults (Levenson et al., 2004) and shorter wavelengths (from 350 to 540 nm) for hatchlings (Witherington & Bjorndal, 1991; Witherington & Martin, 2000).

Marine turtle nesting areas

The georeferenced location of nesting areas in Brazil were obtained from TAMAR and included areas between latitudes 10° 34’ 29”S and 22° 05’ 32”S (*n* = 604 ~1 km segments). The coast of four Brazilian states were encompassed, from north to south: Sergipe (SE), Bahia (BA), Espírito Santo (ES) and Rio de Janeiro (RJ) (Fig 1). The northern part of the area, along the coasts of Sergipe and northern Bahia, represent key nesting areas for the northern loggerhead turtle stock, olive ridley and hawksbill turtles, across 347 km (da Silva et al., 2007; Marcovaldi et al., 2007; Reis et al., 2010; Marcovaldi et al., 2016). The southern part of the area holds important nesting grounds for the genetically distinct southern loggerhead turtle stock, across 257 km of the northern coast of the states of Rio de Janeiro and Espírito Santo (Baptistotte et al., 2003; Lima et al., 2012). Leatherback turtles have their only known regular nesting area in Brazil in the northern coast of Espírito Santo, along 159 km (Thomé et al., 2007; Colman et al. Chapter I) (Fig 1). Green turtles have their major nesting areas restricted to the offshore oceanic islands, with only a small number of nests seen per year on continental beaches (Almeida et al., 2011; Bellini et al., 2013), thus this species was not considered further in the analysis.

Nesting data

To determine the proportion of nesting marine turtles that are likely to be potentially exposed to coastal light pollution, we used data on nest counts collected by Projeto TAMAR. During the nesting seasons (September to March), morning patrols conducted daily by trained personnel would monitor the beaches and record nesting activity by identifying tracks left by nesting females on the previous night (Marcovaldi & Marcovaldi, 1999). We used the average annual nesting numbers per species during the most recent period (2008–2012) as a representative of current nesting levels and also because the monitoring effort over the nesting grounds was considered constant throughout the period. As it is not possible to determine species from all nests in the field, to estimate the total number of nests per species laid in each season at each site, the number of nests from unknown species for a given year was divided proportionally in relation to the proportion of the known nests by species for that same year. This estimation was applied on the annual data from each area, as they present heterogeneity in species distribution, caused by the large latitudinal variation on nesting areas analysed in this study (see Fig 1b). In each area, we assume that the nests for which the species was known represented a random sample of all the nests (from known and unknown species) in that area (Marcovaldi et al., 2007).

Nest densities

Nest density per species/subpopulation was calculated as nests$\cdot$km$^{-1}\cdot$yr^{-1}. An indicator to measure the relative importance of each section of the beach in terms of their nest density was created. As part of TAMAR standard methodology, the entire 604 km of the study area are divided by permanent marker posts at each kilometre. The average annual number of nests on each one of the kilometres during the period 2008–2012 (five years of data) was calculated. Each kilometre of the beach was then classified in terms of nest density as either high, medium or low, by locating the average annual number of nests on that kilometre within the distribution of the 604 average numbers, relative to each species or subpopulation: high-density sections were those with average annual number of nests in the top 25% of the distribution; low-density sites were those in the lowest 25% of the distribution; and sites with densities in-between the two previous categories were classified as medium-density. A very high-density category was assigned to the top three beach segments for each species/subpopulation (Fuentes et al., 2016). Each species had its own value of density per segment, irrespective if the segment was used by multiple species (i.e. one segment could have both a low nest density for one species and a very high nest density for another).

Processing

We created an average calibrated image for both the first (1992–1996) and the last (2008–2012) five years of data, as well as one with the difference or change between them. Pixels were considered potentially exposed to detectable artificial light when they had values higher than 5.5 DN units. Observed changes in brightness were considered when pixels increased or decreased by more than a threshold of 3 DN units of difference between the mean of each of the first and last five years of the surveyed periods. Values of 5.5 and 3 can be used as reliable thresholds for exposure and change in artificial light, considering the noise within the dataset (Bennie et al., 2015; Duffy et al., 2015). We then overlaid the information on nesting areas with the DMSP data for both periods (1992–1996 and 2008–2012). We used the 'spatial analyst sample tool' in ArcMap 10 to determine the nearest pixel value associated to each monitored segment (*n* = 604 ~1 km segments). The proportion of nesting areas potentially exposed to artificial light, or changes in brightness, was calculated dividing the number of exposed pixels or pixels with observed change by the total number of pixels within each area. We then compared the two different periods (1992–1996 and 2008–2012) to assess potential changes and trends in brightness considering the nesting areas and periods analysed.

Nest densities and relationship with light levels

The relationship between nest densities (nests·km^{-1}·yr^{-1}) by species/subpopulation and the average light levels during 2008–2012 was analysed using a generalised additive model (GAM) and the function *gamm* (generalised additive mixed modelling) of the R-package *nlme* (Pinheiro et al., 2018). The response variable (nest density) was determined as a function of two continuous covariates: average light levels and latitude. Each ~ 1 km beach segment contributed to the distribution only once (n = 347 for the northern loggerhead turtle stock, hawksbill and olive ridley turtles, n = 257 for the southern loggerhead turtle stock, n = 159 for leatherback turtles). Statistical analyses were carried out using the software R 3.5.1 (R Core Team, 2018) and for statistical tests, the significance level was alpha = 0.05.

Reproductive hotspots

Based on nest densities (nests·km^{-1}·yr^{-1}) during the most recent period (2008–2012), we identified two types of reproductive hotspots for each species/subpopulation: (1) nesting areas with very high- or high-nest density and high exposure to artificial light (> 5.5 DN) and (2) nesting areas with very high- or high-nest density and low exposure to artificial light. Those areas were grouped into beaches with variable extents (2 to 37 km), according to standard methodology adopted by TAMAR. We used TAMAR's dataset (2008–2012) to classify these beaches according to (1) the type of management strategy used: clutches could be either left in situ*,* relocated to another section of the beach or, on a lesser extent, to open-air hatcheries. Nest relocations are required in some areas to protect clutches from beach erosion, risk of flooding, fox predation or illegal egg take by humans, or other anthropogenic impacts such as light induced misorientation, in places where there is extensive coastal development (Marcovaldi et al., 2005), (2) the existence of documented cases of hatchling orientation problems, identified during beach patrols through examination of hatchling tracks. The 54 sites were classified regarding the existence of recorded hatchling orientation problems according to the scores: 0 = no cases or 1 = recorded cases of hatchling orientation problems. The proportion of sites with hatchling orientation problems was calculated considering the sites that had recorded orientation problems divided by the total number of sites and (3) if the presence of artificial light influences the management strategy used locally, defined as whether clutches on a given beach or section of a beach needed to be relocated because of the presence of light or not. A given beach was considered potentially exposed or to have had observed changes in brightness when more than 50% of the hotspot segments were located in areas potentially exposed to light pollution (> 5.5 DN) or had observed changes in brightness (> 3 DN, Table 1).

Results

All species/subpopulations showed an increase in the proportion of nesting areas potentially exposed to light pollution (> 5.5 DN) between the periods (1992–1996 and 2008–2012). The overall proportion of nesting areas potentially exposed, considering those nesting areas have not changed over time, increased from 34.1% during the first period (1992–1996) to 61.1% during the most recent period (2008–2012; Fig 2 and Table 2). Overall, increase in brightness (> 3 DN) was experienced in 63.7% of the area. None of the nesting areas experienced a decrease in brightness (> 3 DN). Considering the annual nesting numbers during the most recent period (2008–2012), a variable proportion of nests for the different species/subpopulations are located in areas potentially exposed to light pollution (Table 2; 28.7-80.5%).

We identified 253 ~ 1 km segments which had either very high- or high-nesting density for any of the species/subpopulations. Those reproductive hotspots were grouped into 54 sites, important for one or more marine turtle species (Table 1). Of those very high- and high-nesting density sites, 37.1% were located in areas with no/low exposure to light, the remaining 62.9% were in areas potentially exposed to light pollution (*n* = 54) and 64.8% of hotspots had experienced increasing light levels. The proportion of reproductive hotspots potentially exposed to light pollution varied among each species/subpopulation, ranging from 26.7% in olive ridley turtles to 75.9% in hawksbill turtles (Table 3). In 55% of reproductive hotspots there are documented cases of hatchling orientation problems and in 42.6% the presence of artificial light influences local management strategies, however in most cases it means a small proportion of nests located in specific areas, rather than those across an entire beach or whole

~1 km segment (n = 54; Table 1). The proportion of reproductive hotspots potentially exposed to light pollution also varied considering the different states, being 46.1% in Sergipe, 60.7% in Bahia, 50% in Espírito Santo and 100% in Rio de Janeiro.

The GAM models suggested that light had a significant effect on nest densities of hawksbills and the northern loggerhead turtle stock (approximate significance of the smooth term: estimated degrees of freedom = 1.05, $F = 11.58$, $p = 0.0001$ and estimated degrees of freedom = 5.39, $F = 15.34$, $p < 0.0001$, respectively, Fig 3). Latitude had a significant effect on nest densities of all species (olive ridley turtles estimated degrees of freedom = 7.7, $F = 18.30$, $p < 0.0001$; leatherback turtles estimated degrees of freedom = 6.51, $F = 38.98$, $p < 0.0001$; southern loggerhead turtle stock estimated degrees of freedom = 5.31, $F = 18.47$, $p < 0.0001$, northern loggerhead turtle stock estimated degrees of freedom = 2.88, $F = 29.49$, $p < 0.0001$, hawksbill turtles estimated degrees of freedom = 8.38, $F = 25.70$, $p < 0.0001$; Fig 3).

Discussion

The majority of turtle nesting areas analysed here experienced increases in brightness between 1992–1996 and 2008–2012 (Fig 2), suggesting the increasing trend in artificial lighting has the potential to represent a significant anthropogenic impact for coastal habitats and marine turtles in Brazil. The human population in Brazil has grown considerably throughout the study period and much of the population growth was seen along coastal areas, where the largest urban centres are currently located (Instituto Brasileiro de Geografia e Estatística, 2013). With an extensive and diverse coastline, coastal development and artificial lighting has evolved distinctly among regions in Brazil, mostly driven by tourism (Lopez et al., 2015), industrial activities or the development of the communities themselves.

Long-term conservation efforts started by TAMAR in the 1980s is thought to have contributed to recent increases in nesting numbers for all four mainly continentally nesting species, indicating population recovery (Baptistotte et al., 2003; da Silva et al., 2007; Marcovaldi et al., 2007; Marcovaldi & Chaloupka, 2007; Thomé et al., 2007). This is in parallel with many other marine turtle populations globally (Mazaris et al., 2017). In Brazil, the estimated annual growth rate is different among species/subpopulations, ranging from 4 to 12% per year (TAMAR, unpublished data). The fact, however, that both variables (annual nesting numbers and average light levels) are increasing suggests that if light is managed well, turtles and humans can co-exist in Brazil. As coastal development continues to progress, future conflicts could arise, and continued management of artificial light will be key. Conservation interventions could either have reduced the impact of other important disturbances factors, or direct lighting, since interventions such as light shielding will not be picked up by satellite imagery. We know that in northern Bahia, beach resorts with ‘turtle-friendly’ lighting structures ensure high marine turtle nesting densities are sustained while relatively high average light levels were recorded by the satellite images. Identifying the impact of one disturbance when a population is recovering from the alleviation of another is a challenging task.

Other studies have highlighted the importance of detecting temporal changes in artificial light exposure of marine turtle nesting areas (Kamrowski et al., 2012, 2014). Additionally, here we identified hotspots (Table 1), areas with high reproductive importance, which may either be impacted by artificial light, thereby representing zones requiring management attention, or dark areas which are likely candidates for future and continued protection (Fuentes et al., 2016). The majority of the reproductive hotspots identified here are located in areas considered potentially exposed to artificial light, however, those areas are generally bright as a result of skyglow, which is the scattering of upwardly reflected artificial light in the atmosphere and reflection by clouds (Davies et al., 2014). Despite the existence of specific legislation prohibiting direct light incidence on nesting beaches (IBAMA normative nº 11, from 31 January 1995), across globally important nesting areas for hawksbill and loggerhead turtles such as northern Bahia (Marcovaldi & Chaloupka, 2007; Marcovaldi et al., 2007) and Rio de Janeiro coasts (Lima et al., 2012), relocation is used as a strategy for managing nests in lit areas, although it can affect hatchling sex ratios (Godfrey & Mrosovsky, 1999) and could cause long-term genetic consequences (Mrosovsky, 2006).

Light levels and nesting density had variable relationships across the species/subpopulations, reflecting the interactions between nest site selection and the variable patterns of artificial light along the Brazilian coast. Olive ridley and leatherback turtles have their core rookeries within long established dark and protected areas – Santa Isabel and Comboios Biological Reserves, established in 1988 and 1984, respectively. Those species are still of conservation concern, however, as coastal development continues to pressure the surroundings of the protected areas. An increase in nesting numbers caused by population recovery could also result in broader geographical distributions, as turtles could re-colonise previous nesting areas, creating conflicts with humans.

For hawksbills and both the southern and northern loggerhead stocks, the relationship between nest density and average light levels at the individual beach scale varied, with high nesting density also seen in areas where there were relatively high average light levels (Fig 3e-g). This suggests that other drivers for nest site selection, such as temperature gradients, beach and horizon elevation and sand moisture (Wood & Bjorndal, 2000; Pendoley & Kamrowski, 2015), may play a more important role than the presence/absence of artificial light. Cloud cover and lunar phase are also important factors influencing hatchling orientation, and site-specific topographic features such as vegetation, sand dunes and beach slope can help hatchlings in finding their way to the ocean, and females might be more likely to emerge to nest, as those features may shield nesting areas from light. Another possibility is that turtles may not be exposed to as much light as suggested by the satellite data, i.e. it may be due to the coarse scale of the light data not allowing for differences at the local beach scale to be considered. Loggerhead, green and leatherback turtle nest densities, however, were negatively influenced by artificial light levels at the individual beach scale at other nesting sites such as Florida (Weishampel et al., 2016; Hu et al., 2018) and eastern Mediterranean (Mazor et al., 2013). In Brazil, in heavily industrialised coastal areas such as Rio de Janeiro state, all reproductive hotspots identified are located in areas potentially exposed to light pollution

(Table 1). The existence of high nesting densities in lit areas suggests turtles can tolerate quite high levels of disturbance (Kamrowski et al., 2012). Further research should investigate the potential interaction and cumulative effects of these other factors affecting the response variable, in this case nest density, operating in finer scale (e.g. species-specific nest site selection, physical characteristics of the beaches and spatial autocorrelation of the nesting data) in comparison to artificial light.

Despite being of great utility to marine turtle management and conservation in Brazil, interpretation of our findings requires a number of considerations of the spatial, temporal and spectral resolution limitations of the DMSP OLS sensors data as a proxy to estimate the effects of light pollution on marine turtles. Firstly, the 1 x 1 km pixel scale is much coarser than the width of most Brazilian beaches, including brightness that can be inland. Secondly, the measure of light as viewed from the sky might not represent the light as perceived by turtles from the beach. On-ground assessments of the impacts of light pollution are needed to confirm the identified levels of exposure (Kamrowski et al., 2014) and to establish thresholds of exposure at which light affects individual turtles and their populations (Fuentes et al., 2016, Lara et al. 2016).

Thirdly, the Stable Light DMSP product represents an annual average, including periods outside the nesting season. Beach use increases during the Austral summer months, coinciding with the peak of the nesting season (December and January). If the resolution of the data were finer, those seasonal changes could be captured more precisely. A newer product with finer spatial resolution, the Visible Infrared Imaging Radiometer Suite (VIIRS), has been introduced as a successor to DMSP-OLS in 2012, and used to assess impacts of light pollution and coastal development over sea turtles in Florida (Fuentes et al., 2016; Hu et al., 2018). Future assessments of temporal changes in light adjacent to marine turtle nesting areas should use these finer-resolution monthly nightlight data (Kamrowski et al., 2014), even though the archive only starts in 2012 (Miller et al., 2013), currently preventing the assessment of long-term changes. Finally, in this study we did not use habitat or landscape features other than light and nesting locations. Consequently, some important explanatory variables might have not been considered, and there is the possibility that light covaries with measures that we are not yet accounting for. This would be worthy of further investigation.

Coastal artificial light constitutes a potential threat for marine turtles, however offshore lighting, such as from oil rigs, is known to attract fish of commercial interest (Marchesan et al., 2005), and research has shown that marine turtle hatchlings could also be attracted, increasing predation risk (Thums et al., 2016) and potential impacts on population recruitment. There is need for regional assessments to evaluate the impacts of offshore and near shore lighting on marine turtle hatchlings dispersing from natal beaches in Brazil. Their dispersion routes are very likely to overlap with productive oil and gas fields, located near Rio de Janeiro, Espírito Santo and Sergipe coasts (Fig 2 b to e), representing a potential ecological sink for hatchlings.

Light pollution can affect marine turtles and their habitats and quantifying the impacts at a population-level remains a challenge to ecologists (Davies & Smyth, 2018). If lighting is unsuitable during the hatchling emergence period, hatchlings could have direct fitness consequences, such as reduced energy during their frenzy period offshore, which appears essential for reaching the “favourable currents” that facilitate reaching nursery habitats (Scott et al., 2017). A study conducted in Mediterranean nesting sites estimated that nightlight could result in an additional reduction of recruitment of up to 6% (Dimitriadis et al., 2018). For species with late maturity such as marine turtles, with relatively uncertainty regarding typical time to reach sexual maturity, the impact on population recruitment could take many decades to fully manifest in nesting numbers.

Human-wildlife conflicts where coastal development overlaps with nesting areas can be effectively managed with the establishment of dark and protected areas, the existence and enforcement of specific legislation or ultimately with nest relocation. The reproductive hotspots identified here can be used as guidance in future management decisions considering marine turtles in Brazil, identifying areas where intervention is needed and those candidates for continued protection. Perhaps most notable is the fact that conservation strategies used by TAMAR in Brazil during the last 35 years have heavily relied on the involvement of local communities, with the development of varied environmental awareness activities, adapted to the socio-environmental evolving contexts of the different locations (da Silva et al., 2016). Evaluating the effects of anthropogenic factors on sea turtles habitats was one of the 20 questions pointed out as research priorities for marine turtles (Hamann et al., 2010), and was considered still insufficiently addressed in a recent review of the peer-reviewed literature (Rees et al., 2016). As coastal development increases, not only in Brazil, but worldwide, the use of satellite imagery is valuable to monitor medium to long-term trends in light and to evaluate potential impacts of light on marine turtles and their habitats.

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Table 1. Reproductive hotspots in Brazil (*n* = 54 beaches). SE = Sergipe, BA = Bahia, ES = Espírito Santo, RJ = Rio de Janeiro, LO = olive ridley, NS = northern loggerhead stock, SS = southern loggerhead stock, EI = hawksbill, DC = leatherback. I = in situ, P = relocated to other section of the beach, T = relocated to open-air hatcheries.

State	Beach	Species	Hotspot beaches	Potentially Exposed to light (%)	Increase in brightness (%)	Manag. strategy	Hatchling orientation problems (%)	Light influences manag. (%)
SE	Ponta dos Mangues	LO, NS	4	0	0	I, P, T	0	0
	Pirambu	LO, NS	5	60	60	I, P, T	20	60
	Abais	LO, NS	4	75	100	I, P, T	50	100
BA	Mangue Seco to Coqueiro	LO	2	0	0	I	0	0
	Dunas to Costa Azul	LO, NS	4	0	0	I	0	0
	Siribinha to Ribeiro	LO, NS	2	100	50	I, P, T	0	0
	Baixios to Mamucabo	NS, EI	2	0	0	I, P	0	0
	Subauma to P. do Sauipe	LO, NS, EI	4	25	50	I, P	25	25
	Costa do Sauipe to Imbassaí	NS, EI	2	100	100	I, P	0	0
	Praia do Forte	LO, NS, EI	1	100	100	I, P	0	0
	Itacimirim to B. do Jacuípe	NS, EI	4	100	100	I, P, T	75	100
	Berta to Arembepe	NS, EI	2	100	100	I, P	50	50
	Santa Maria to Busca Vida	NS, EI	3	100	100	I, P	0	33.3
	Buraquinho to Itapuan	NS, EI	2	100	100	P	100	100
ES	Povoação	SS, DC	3	66.7	66.7	I, P	0	25.0
	Comboios	SS, Dc	1	0	0	I, P	0	0
RJ	Tatagiba to P. do Sul	SS	3	100	100	I, P, T	67	67
	Chapeu do Sol	SS	2	100	100	I, P	100	100
	Maria Rosa to Atafona	SS	4	100	100	I, P, T	60	80

Table 2. Summary of nesting areas (~ 1 km segments; some of the segments are used by more than one species), proportion of areas potentially exposed to light pollution (> 5.5 DN) during the 1992–1996 and 2008–2012 periods, areas with observed changes in brightness (> 3 DN) and estimated annual number of nests per species/subpopulation (2008–2012, range, mean ± SD), showing the proportion of nests that would be potentially exposed per species/subpopulation.

Species/ subpopulation	~ 1 km segments	Area exposed (%)		Change (%)	Nesting numbers per year mean (± SD, range)	Exposed (%)
		1992-1998	2008-2012			
Northern loggerhead stock	347	37.8	58.2	57.6	4254 (± 410.9, 3275 – 4675)	76.0
Olive ridleys					4776 (± 1446, 3645 – 7215)	28.7
Hawksbills					1411 (± 185.9, 1220 – 1663)	80.5
Southern loggerhead stock	257	28.8	64.6	71.6	2588 (± 489.2, 1954 – 3084)	68.9
Leatherbacks	159	13.8	48.4	57.2	82 (± 77.2, 21 – 207)	46.1
Overall (all nesting areas)	604	34.1	61.1	63.7	-	-

Table 3. Nest densities (nests·km^{-1}·yr^{-1}) based on average nesting numbers per year (2008–2012, range < 0.1 – 190.8 nests·km^{-1}·yr^{-1}) per species/subpopulation. Nest density classifications of low, medium, and high were created according to quartile ranks. A very high-nesting density classification was assigned to the three beaches for each species/subpopulation with the highest values within the range.

Species/ subpopulation	~ 1 km segments	Average density	Very high	High	Medium	Low	Hotspots exposed (%)
Northern loggerhead stock	347	12.3	155.4 - 190.8	12.0 - 109.8	1.3 - 11.9	< 1.1	73.6
Olive ridleys		13.8	124.6 - 169.9	15.5 - 111.7	0.9 - 15.4	< 0.9	26.7
Hawksbills		4.06	41.0 - 52.7	4.2 - 39.8	0.6 - 4.1	< 0.5	75.9
Southern loggerhead stock	257	10.1	42.6 - 46.1	12.4 - 41.4	3.7 - 12.2	< 3.6	71.4
Leatherbacks	159	0.5	1.8 - 2.9	1.0 - 1.8	0.2 - 0.9	< 0.1	53.7

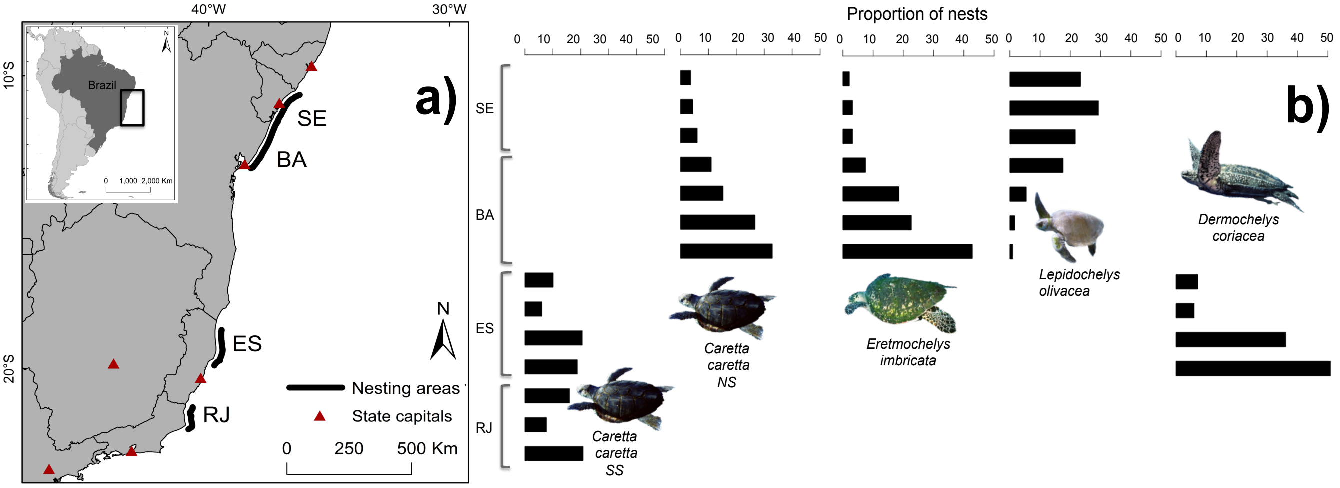

Figure 1. Distribution of turtle nesting. **(a)** Map of Brazil with marine turtle nesting areas considered in this study denoted by black lines (inset shows Brazil and study region within the context). **(b)** Proportional spatial distribution of nests by species (*n* = 15 areas in 4 regions). We considered the estimated annual average number of recorded nests per area and species during 2008–2012. Labels: Sergipe (SE), Bahia (BA), Espírito Santo (ES) and Rio de Janeiro (RJ). For *C. caretta*, BA + SE represent the northern stock (NS) and ES + RJ represent the southern stock (SS).

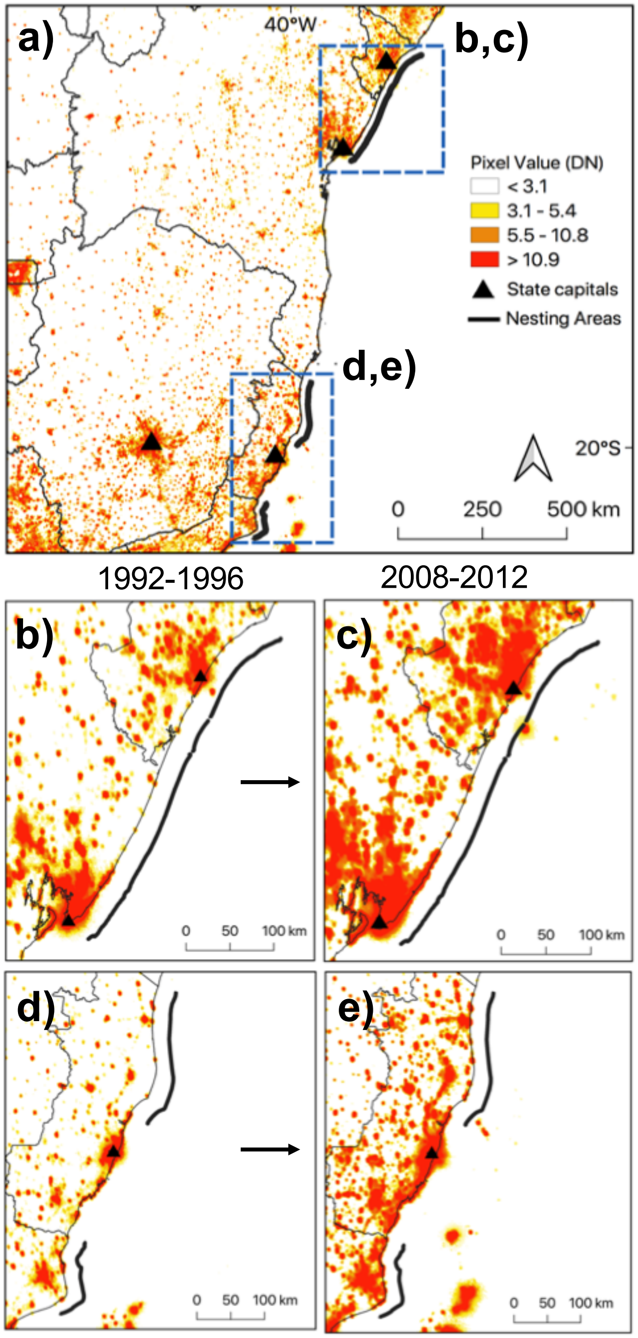

Figure 2. Change in brightness seen across nesting areas in Brazil. A digital number (DN) between 0 and 63 represents each pixel. Zero represents darkness, while brightly lit areas saturate at values of 63. **(a)** Presents a national overview. **(b)** and **(c)** Represent Bahia and Sergipe, nesting grounds for the northern *Caretta caretta* stock (NS), *Eretmochelys imbricata* (EI) and *Lepidochelys olivacea* (LO) turtles. **(d)** and **(e)** Represent Espírito Santo, nesting grounds for the southern *Caretta caretta* stock (SS) and *Dermochelys coriacea* (DC) turtles,

and Rio de Janeiro, nesting grounds for the southern *Caretta caretta* stock (SS). The zoomed in areas represent the average calibrated images from 1992–1996 **(b, d)** and 2008–2012 **(c, e)**.

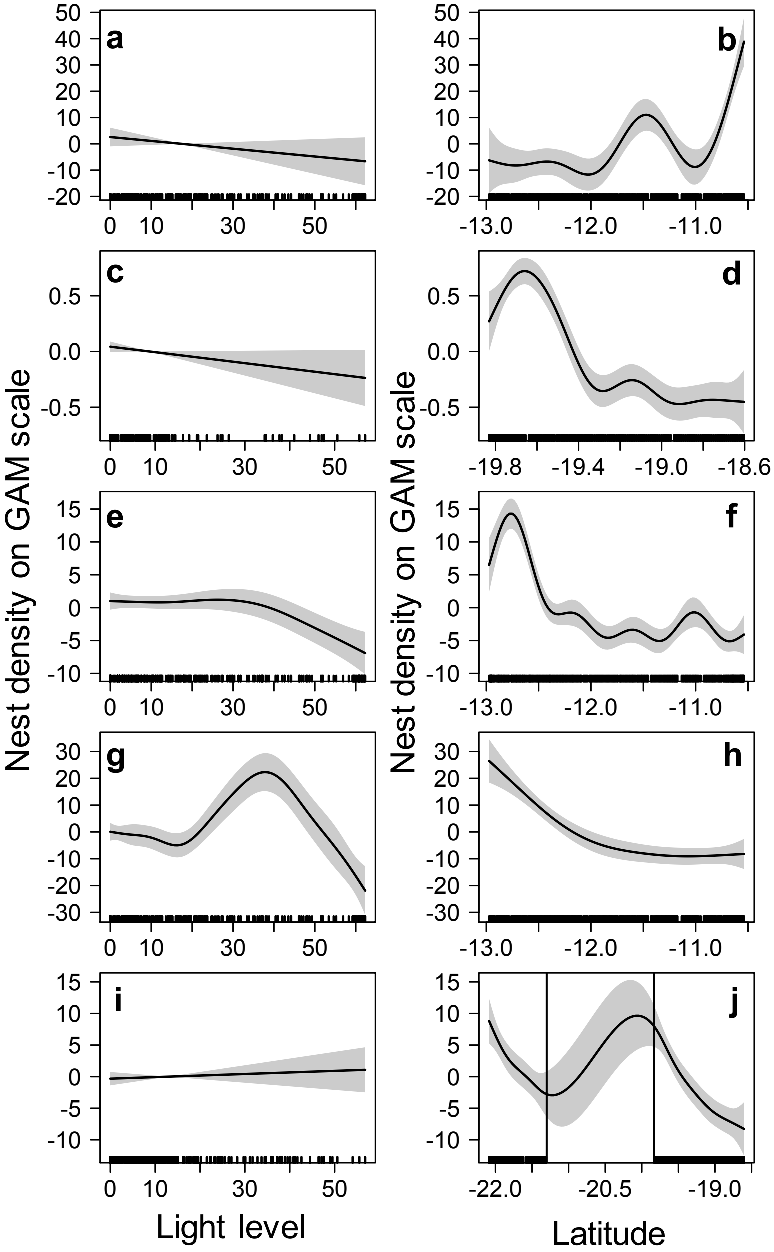

Figure 3. Graphical summary of GAMM model fits for nesting densities. Covariates are shown on x-axis: average light levels in DN (left side) and latitude (right side) for **(a,b)** olive ridley turtle (n = 347), **(c,d)** leatherback turtle (n = 159), **(e,f)** hawksbill turtle (n = 347), **(g,h)** northern loggerhead turtle stock (n = 347) and **(i,j)** southern loggerhead turtle stock (n = 257). Note different x and y-axis scales. In **(j)**, the region between the two vertical lines represent latitudes where no nesting data was collected.

Final remarks

In this thesis I investigated several important ecological parameters of the Southwest Atlantic Ocean leatherback turtle subpopulation nesting in Espírito Santo, Brazil. Among others, some interesting findings resulting from my research were that: i) long-term monitoring at nesting beaches shows signs of population recovery, however, the variable trend in nesting numbers and the small population size indicate this leatherback turtle population remains of conservation concern; ii) leatherback turtles nesting in Brazil are likely to present variable foraging strategies, with possible consequences for the susceptibility to threats for this population; iii) this rookery is likely to be producing modestly female-biased hatchling sex ratios, however a long-term decrease in incubation durations suggests the potential feminization of the population may become a concern considering future scenarios of climate change. Additionally, using the case study of four marine turtle species, I found that both nesting numbers and artificial light levels have been increasing in Brazil. Light levels affect species/subpopulations variably and any impacts on population recruitment may take longer to fully manifest in nesting numbers.

Here I provide some applied and fundamental insights, identifying knowledge gaps and ways this work can go forward to better understand the scope of recovery, resilience and potential for adaptation in the face of current threats to this population. In addition to contributing to advance the knowledge of this most important leatherback turtle population with scientific findings and publications originating from my PhD, this research has also directly contributed to the conservation of marine turtles in Brazil.

Conservation implications

The fieldwork in Brazil was conducted in collaboration and under the supervision of Projeto TAMAR (the Brazilian Sea Turtle Conservation Programme), and with the participation of the local communities from Regência and Povoação, in Espírito Santo. Marine turtles have always been part of the local culture and traditions, and the long-term work maintained by TAMAR in the region involves the people in the conservation activities. The protection of marine turtles contributes to the development of economic activities and brings income to local people, including tourism and direct employment. As the local communities impacted by the large-scale mining accident that occurred in the region in 2015 recover from the profound environmental and socio-economic impacts suffered, marine turtle conservation work will be part of the planning of recovery strategies for the region. Knowledge obtained from studies such as the present one can provide scientific evidence to evaluate potential impacts and support management decisions.

During the three fieldwork campaigns I worked together with local community members, as well as with many Brazilian undergraduate students and volunteers helping with the turtle monitoring activities. I worked together with TAMAR promoting their training in sampling and monitoring techniques, as well as leading meetings where we would present the results of our research and discuss the biology and conservation of marine turtles and their environment. These young people are key to biodiversity conservation, as they will be the future field biologists leading conservation work and also decision making in Brazil.

Results from scientific research also directly contribute to conservation management. The variable population trend observed in Espírito Santo was a compelling argument to justify the maintenance of the ‘Critically Endangered’ status for leatherback turtles in Brazil during the latest national conservation assessment organised by the Brazilian Government and Ministry of Environment for the species in October 2018. Several research questions addressed in this study were pointed out as priority research questions on the National Action Plan for the Conservation of Marine Turtles in Brazil (ICMBio 2011), with results on movements and foraging strategies being particularly relevant for the establishment of protected areas, with closure to fisheries. Additionally, this is the first study investigating the potential impacts of the mining accident on marine turtles in the region, and although we conclude that so far there is very little of an effect, the mechanisms are still unclear; and our data will constitute baseline for future analyses.

Finally, the dissemination of the scientific outputs of this thesis, through peer-reviewed publications, presentations at international conferences and social media platforms, will emphasize the importance of this highly endangered leatherback turtle population. Those strategies help to promote awareness and potentially facilitate the establishment of future partnerships that could result in continued funding for marine turtle and biodiversity conservation.

Future research

Results from Chapter I suggest that the long-term work on the leatherback turtle population nesting in Espírito Santo has potentially contributed to the increase in nesting numbers following the continued protection of eggs and females. Using this dataset, we gained important insights into key population parameters, however, to fully understand population dynamics, continued monitoring and further applied research is needed. The use of more reliable methods to study capture-mark-recapture, such as passive integrated transponders (PIT tags), would be recommended for this population. Those could generate better estimates of important parameters such as remigration intervals and survival (Balazs 1999, Dutton et al. 2005), essential for the assessments of population size and conservation status (Marcovaldi & Chaloupka 2007, Roberts & Hamann 2016). Additional telemetry would offer better insights into nest site fidelity and clutch frequency (Weber et al. 2013).

Several studies have pointed out that leatherback turtles present multiple foraging strategies (Hays et al. 2006, Seminoff et al. 2012, Robinson et al. 2016). Results from Chapter II suggest that this is also the case with leatherback turtles in Brazil. Our small sample size (only two females tracked contributed with post-nesting movement data) hampers more conclusive analyses of their foraging areas location and habitat use, and further studies should aim to continue sampling nesting females for SIA and tracking more individuals, which could help us to better understand their spatial ecology. The stable isotope signatures for this population are described here for the first time, and these results could be used in future research to understand connectivity among breeding and foraging grounds in the South Atlantic Ocean. Future studies should also incorporate diet information into stable isotopes analysis, as prey items could be used as endpoints to infer regarding offshore and coastal habitats (Petitet & Bugoni 2017).

The results obtained in Chapter II will also be further developed through the analysis of additional satellite tracking data available for three females during the nesting season in 2017 and existing dataset from Almeida et al. (2011). These studies will be key to better define internesting habitats and high-use areas during the nesting seasons, being particularly important to design protected areas and possibly define spatio-temporal fisheries closure. Future research should also consider oceanographic data on ocean productivity and currents, to understand leatherback turtle movements and define foraging areas. Another promising area is the investigation of leatherback turtle hatchling dispersal using oceanographic models (Scott et al. 2017). That could provide preliminary insights into the habitat use for this understudied life-stage.

Chapter III presents important data on nest and sand temperatures that will set out the baseline for future comparisons of temperature and sex ratios considering the predictions of climate changes. Further work should continue monitoring temperatures and have more representative sample sizes, both temporally and spatially, of control sites and within-nest dataloggers, in order to refine our predictions on sex ratios being produced in the region. The use of non-lethal methods to determine hatchling sex ratios would also be valuable to confirm our predictions (Xia et al. 2011, Kobayashi et al. 2015).

Results from Chapter IV suggest that identifying the impact of one disturbance when a population is recovering from the alleviation of others is a challenging task. Further studies should conduct on-ground assessments of the impacts of light pollution to establish thresholds of exposure, at which light would affect marine turtle populations (Fuentes et al. 2016). The use of satellite imagery with finer spatial resolution (VIIRS, Visible Infrared Imaging Radiometer Suite) should be useful to study seasonal changes, since the human occupation of coastal areas grows considerably during the summer in Brazil, and results from the annual average light levels could be significantly different from the ones obtained monthly during the nesting season. Future studies should also look at the potential effects of nearshore artificial lighting, proven to attract hatchlings with possible consequences on hatchling survival and recruitment (Thums et al. 2016). Further studies to better understand nest site selection patterns could also be valuable to understand how the presence of artificial light influences nest site selection. The use of habitat and landscape features in future analyses could also add important explanatory variables to the results obtained, since light could covary with several variables currently not considered in our study.

Finally, the continued long-term monitoring in Espírito Santo is key to achieve a more complete understanding of the dynamics of this population, and further applied research should help us know more about this population, especially considering the emergence of new threats such as the contamination of the marine and coastal area of this nesting ground by the mining accident in 2015. Providing key baseline data for the species contributes to the discussion of several questions recognised as priority research by Rees et al. (2016) and scientific data can be used as evidence to support the design and implementation of protective measures for the species and its environment.

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