



SCUOLA DOTTORALE IN BIOLOGIA
SEZIONE BIODIVERSITA' E ANALISI DEGLI ECOSISTEMI

XXVIII CICLO

Loggerhead turtles in the western and central Mediterranean Sea: habitat use, stock composition and sex ratios

La tartaruga marina comune, *Caretta caretta*, nel Mediterraneo centrale ed occidentale: habitat use, stock composition and sex ratios

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I declare that this thesis has been composed by myself, and has not been accepted in any previous application for a degree. It includes all papers that have been published in referred journals in which I was first and/or corresponding author and that have been written by myself. For those papers in which I was co-author, an extended summary of the research has been prepared, which mainly focuses on the specific contribution I provided to the study. Numbering of figures and tables is continuous per-chapter. All collaborations and sources of information have been acknowledged.

ACKNOWLEDGEMENTS

This thesis was made possible by the support and continuous provision of equipment and facilities from the Stazione Zoologica Anton Dohrn of Naples.

I would like to thank my tutor Prof. Marco Alberto Bologna for providing me with the opportunity to complete this PhD. Even though I spent so little time at the University, he has been actively interested in my work and available to help me whenever I needed. I appreciated all his contributions of time, ideas and advices.

I also thank my colleagues of the sea turtle rescue center of the Stazione Zoologica Anton Dohrn for being so dedicated to sea turtle conservation and for going always far beyond the call of duty.

Thanks to the many volunteers who enthusiastically participated in the fieldwork. I applaud your dedication and your altruism without which many of the results achieved in recent years would not have been possible.

Grazie alla mia famiglia, sostegno costante ed incredibile.

Grazie Sandra, tu mi rendi migliore. Condividere con te solo il merito di questa tesi sarebbe certamente riduttivo. Sono anni che hai abbracciato sta' croce e insieme abbiamo fatto cose meravigliose. Non vedo l'ora di scoprire ciò che saremo capaci di realizzare domani.

Grazie Samuel, sei la nostra felicità.

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1. GENERAL INTRODUCTION

1.1. ANCIENT SPECIES WITH MODERN PROBLEMS

Sea turtles are truly marvellous creatures, complex, successful and very worthy objects of research. The seven extant species may not seem like a horde compared to the tens of different marine mammal or marine bird species wandering in today's global oceans. However, if we measured evolutionary success by longevity, then sea turtles have scored a quite impressive ecological triumph.

The extant sea turtles are a monophyletic group (superfamily Cheloniodea) of the suborder Cryptodira (Figure 1.1).

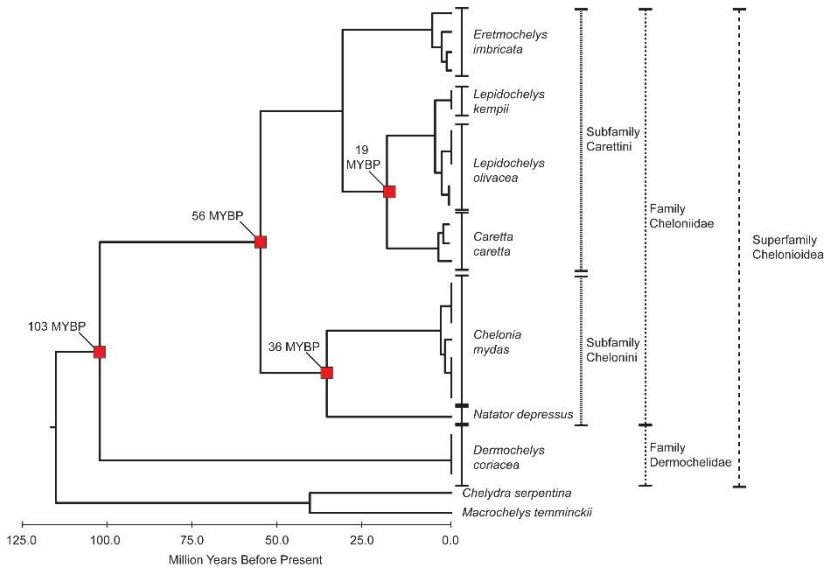


Figure 1.1. Chronogram for complete mitogenomic analysis of all sea turtle species. Red squares indicate nodes for which the time to the most recent common ancestor (TMRCA) was estimated. (Adapted from Duchene et al. 2012).

The oldest known members of this group are from the Early Cretaceous stage (about 110-120 million years before the present) (Hirayama 1998, Cervelli et al. 2003, Cadena & Parham 2015) when mammals were just tiny warm-blooded furry creatures and dinosaurs dominated both land and marine ecosystems. These ancient sea turtles had primitive paddle like flippers with movable digits as in freshwater turtles, but they already possessed some of

the key anatomical and physiological adaptations that we observe in today's species such as the hydrodynamic body shape and the salt excreting system (Hirayama 1998, Cadena & Parham 2015). For millions of years sea turtles have evolved and diversified but only two families survived until the present (Hirayama 1998, Lehman & Tomlinson 2004, Kear & Lee 2006). The Dermochelyidae with the leatherback (*Dermochelys coriacea*) as the single living member, and Cheloniidae, with six species split into two sub-families, the Chelonini and the Caretteni: the hawksbill (*Eretmochelys imbricata*) the Kemp's ridley (*Lepidochelys kempii*), the olive ridley (*Lepidochelys olivacea*), the loggerhead (*Caretta caretta*), the green (*Chelonia mydas*), and the flatback (*Natator depressus*) turtles (Figure 1) (Naro-Maciel et al. 2008, Duchene et al. 2012, Jensen et al. 2013).

The seven extant sea turtle species are not quaint archaic relics. They inhabit oceanic and neritic habitats from the tropic to the subarctic regions and lay their eggs in tropical and temperate latitudes all around the globe (Wallace et al. 2010). Sea turtles are an essential component of marine ecosystems where they can play different roles as consumers, prey, competitors, hosts for parasites and pathogens, substrates for epibionts, nutrient transporters, and, finally, ecosystem engineers (Bjorndal & Jackson 2003, Heithaus 2013). Although consumption of sea turtles by humans traces back thousands of years, only few centuries ago (Frazier 2003) these species still occurred in numbers that today are difficult to imagine (Jackson 1997, Jackson et al. 2001, Spotila 2004). When Columbus discovered the Cayman Islands in 1503 he named it "Las Tortugas" because sea turtles literally filled up the ocean and ships were constantly bumping on them (Spotila 2004). Scientists estimate the overall number of sea turtles at that time in thousands of millions and believe that these organisms regulated the functioning of their ecosystem (Bjorndal & Jackson 2003). However, the expansion of maritime commercial trade led also to a drastic increase in the rate of decline of sea turtle populations around the world. Sea turtles were in fact an abundant, cheap and easy to get source of fresh meat that could be taken aboard and kept alive without any food for months during the long at-sea voyages. In few decades, the sea turtle populations in the Caribbean disappeared (Spotila 2004). The same process reiterated in other parts of the world with the further expansion of European civilization and the great improvement of navy technology, gear and equipment (Campbell 2003, Spotila 2004). For centuries, direct harvest has been the main cause of the decline in sea turtle abundances and many societies around the world still make a consumptive use of these species (Thorbjarnarson et al. 2000). However, modern threats to sea turtles are

mostly incidental and derive from them simply getting in our way (Spotila 2004). The unintended capture during commercial fisheries operations (by-catch), coastal development, habitat degradation, marine pollution and climate change are currently the main factors driving the worldwide decline of sea turtle populations (Wallace et al. 2011). The greatly reduced number of sea turtles affects also the extent to which they can fulfil their ecological roles in maintaining the structure and function of marine ecosystems (Bjorndal & Jackson 2003, Heithaus 2013).

Over the last thirty years, there has been a growing interest of environmental agencies, non-governmental organisations (NGOs) and the general public regarding the status of sea turtles and the need to protect and restore their populations (Campbell 2007). This has been matched by the concurrent increase of research attention on a wide variety of topics related to sea turtle biology and conservation (Hamann et al. 2010). Significant advances in our understanding of physiology, genetics, behaviour and health have been achieved, and conservation groups have scored several victories. However, there is still much to do. On a global scale, six of the seven sea turtle species are still categorized as vulnerable (*Caretta caretta*, *Dermochelys coriacea* and *Lepidochelys olivacea*), endangered (*Chelonia mydas*, *Lepidochelys kempii*), or critically endangered (*Eretmochelys imbricata*) by the IUCN Red List of Threatened Species as their population trends are decreasing (IUCN 2015). Despite being one of the best studied groups of marine megafauna, the lack of basic knowledge on sea turtle biology and the human-turtle-environment interactions is still one of the main causes hindering management actions (Hamann et al. 2010). This reflects the logistic challenges of studying sea turtles in the open ocean due to their solitary nature, migratory behaviour and longevity. In a recent attempt to gather and prioritise research themes to assist sea turtle conservation, 35 sea turtle researchers from 13 nations identified and assembled twenty meta-questions in five priority research categories (Table 1.1). Since population traits as well as environmental conditions vary geographically, answers to those questions must be sought at a regional level in order to provide information adequate to designing effective management strategies and conservation responses to the anthropogenic threats (Wallace et al. 2010, Wallace et al. 2011).

Table 1.1. The five priority research categories and twenty meta-questions relating to sea turtle research and conservation elaborated in 2010 by 35 international sea turtle specialists (Adapted from Hamann et al. 2010). In bold are reported the questions addressed in the present study.

1. Reproductive biology	2. Biogeography	3. Population ecology	4. Threats	5. Conservation strategies
1.1. What are the factors that underpin nest site selection and behaviour of nesting turtles?	2.1. What are the population boundaries and connections that exist among rookeries and foraging grounds?	3.1. Can we develop methods to accurately age individual turtles, determine a population's (or species') mean age at maturity, and define age-based demography?	4.1. What will be the impacts from climate change on sea turtles and how can these be mitigated?	5.1. How can we effectively determine the conservation status of sea turtle populations?
1.2. What are the primary sex ratios being produced and how do these vary within or among populations and species?	2.2. What parameters influence the biogeography of sea turtles in the oceanic realm?	3.2. What are the most reliable methods for estimating demographic parameters?	4.2. What are the major sources of fisheries bycatch and how can these be mitigated in ways that are ecologically, economically and socially practicable?	5.2. What are the most viable cultural, legal and socioeconomic frameworks for sea turtle conservation?
1.3. What factors are important for sustained hatching production?	2.3. Where are key foraging habitats?	3.3. How can we develop an understanding of sea turtle metapopulation dynamics and conservation biogeography?	4.3. How can we evaluate the effects of anthropogenic factors on sea turtle habitats?	5.3. Which conservation strategies are working (have worked) and which have failed?
		3.4. What are the past and present roles of sea turtles in the ecosystem?	4.4. What are the impacts of pollution on sea turtles and their habitats?	5.4. Under what conditions (ecological, environmental, social and political) can consumptive use of sea turtles be sustained?
		3.5. What constitutes a healthy turtle?	4.5. What are the etiology and epidemiology of fibropapillomatosis (FP), and how can this disease be managed?	

1.2. THE LOGGERHEAD TURTLE IN THE MEDITERRANEAN SEA

The loggerhead turtle is a globally distributed species that nests on sandy beaches throughout the subtropical and temperate regions. Like the other sea turtles, loggerheads are long-lived, wide-ranging animals with a complex life cycle (Figure 1.2). After leaving their natal beaches, hatchlings swim into major ocean surface currents and undergo a first developmental phase in the oceanic zone that may last a decade or more. During this phase, loggerheads possess limited swimming capacities and mostly rely on ocean currents to disperse towards suitable developmental habitats although some level of active dispersal may be also involved (Putman & Mansfield 2015). Then, the now larger juveniles recruit to neritic foraging grounds to complete their development. The transit between these two phases is complex because juveniles exhibit significant behavioural plasticity and may return to the oceanic environment on shorter time scales (McClellan & Read 2007). Moreover, recent studies suggest that a portion of individuals in some populations maintain an oceanic foraging behaviour all through their life (Hatase et al. 2002, Hawkes et al. 2006). Adults undertake periodic reproductive migrations from foraging grounds to breeding areas that may be thousands of kilometres away, with females that are philopatric to their natal nesting beach (Bowen et al. 2005).

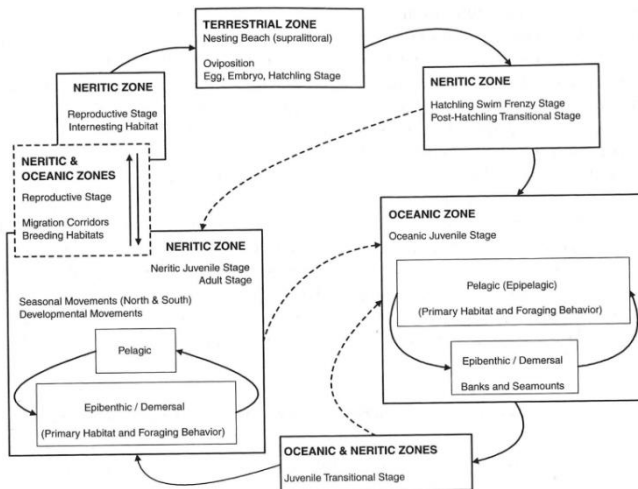


Figure 1.2. Loggerhead turtle life cycle (Adapted from Bolten 2003)

This homing behaviour enhances the formation of population structure by reducing the gene flow among geographically separated nesting beaches. In this context, Management Units (MUs) are defined as population segments that are demographically isolated with respect to female recruitment (Moritz 1994). Males undergo similar migrations although they may be less faithful to the natal site and mate opportunistically on migratory corridors or foraging grounds (Bowen et al. 2005). Generally, migratory connectivity is weak, that is loggerhead turtles from one breeding area migrate to a variety of foraging grounds where they mix with individuals from other demographically independent populations (Bolker et al. 2007) (Figure 2).

Located at the northern edge of the species' range, the Mediterranean Sea hosts a biologically and geographically discrete loggerhead turtle subpopulation (Casale 2015), or Regional Management Unit (RMU, Wallace et al. 2010) that is the result of at least two immigration events from Atlantic rookeries (Garofalo et al. 2009, Clusa et al. 2013a). The species has survived the climatic oscillations which occurred in the basin since the Pleistocene by contracting or expanding its nesting range in accordance with the migration of its thermal niche (Clusa et al. 2013a). Today, the loggerhead turtle is the most common sea turtle species in the Mediterranean Sea with a total of >7200 nests estimated per year (Casale & Margaritoulis 2010). Regular nesting occurs exclusively in the warmer eastern basin, mainly in Libya, Greece, Turkey and Cyprus (Figure 1.3, Casale & Margaritoulis 2010) although in the last two decades, the number of sporadic nests documented in the Western Mediterranean has considerably increased (Bentivegna et al. 2008, Tomas et al. 2008, Sénégas et al. 2009, Bentivegna et al. 2010).

Based on the available genetic information, at least seven different MUs have been identified within the Mediterranean RMU: Calabria (Italy), Libya, western Greece and Crete, Dalyan (western Turkey), Dalaman (western Turkey), and the Levant (central and eastern Turkey, Cyprus, Israel and Lebanon) (Garofalo et al. 2009, Yilmaz et al. 2011, Saied et al. 2012, Clusa et al. 2013a). Loggerhead turtles nesting in the Mediterranean Sea are significantly smaller than those in the Atlantic but some intraregional variability exists where the females nesting in Cyprus are the smallest within the basin (Margaritoulis et al. 2003). The vast majority of loggerhead nesting occurs between the beginning of June and early August (Margaritoulis et al. 2003). Mean incubation periods range 47.3-59.6 days. Hatching success (HS%) is similar at all nesting beaches for which this parameter is available ranging from 60-80% (Margaritoulis et al. 2003, Cardona et al. 2015). Sex ratios have been estimated at few nesting sites only, but it appears that

hatchling production in the Mediterranean Sea is female biased with the proportion of females ranging from 61% to 100% (for a review see Cardona et al. 2015).

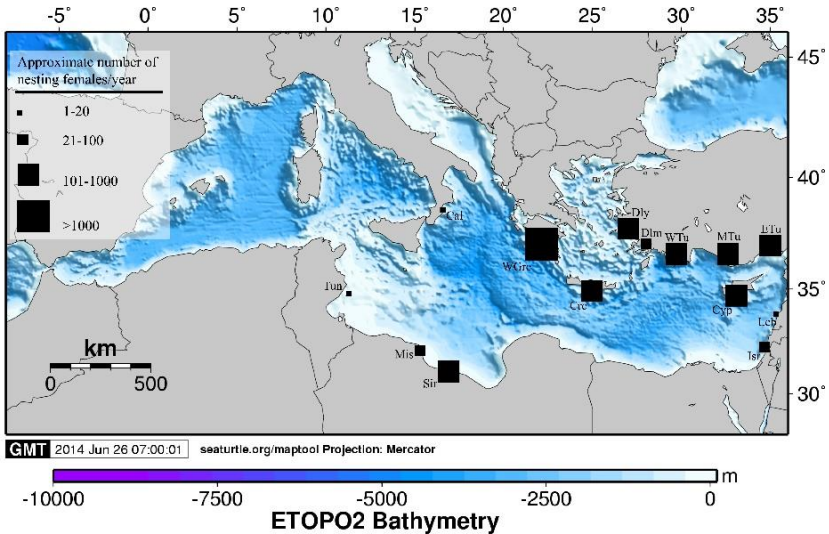


Figure 1.3. Regular nesting areas of Loggerhead turtles in the Eastern Mediterranean Sea

Loggerhead turtles practically occupy all Mediterranean marine areas (Casale & Margaritoulis 2010). According to current knowledge, the most important neritic foraging grounds for juvenile and adult individuals are located on the wide continental shelves in the north Adriatic Sea and off the southern coasts of Tunisia (Margaritoulis et al. 2003, Casale & Margaritoulis 2010). Other large continental shelf areas in the Eastern Mediterranean, such as the bays of Mersin and Iskenderun in Turkey and the Nile Delta in Egypt might also represent important neritic habitats for the species but their exact relevance must still be evaluated (Margaritoulis et al. 2003). Loggerhead adults and juveniles regularly forage also in the Western Mediterranean Sea and are commonly found in shallow sandy coasts in the south Tyrrhenian Sea (Bentivegna et al. 2001, Hochscheid et al. 2013) Oceanic developmental areas have been identified in the Alboran Sea, the Balearic Sea, the Sicily Channel and the Ionian Sea (Casale & Margaritoulis 2010). Nursery and early developmental areas are less well known (Margaritoulis et al. 2003). Based

on the number of stranded early juveniles Casale et al. (2010) suggested the importance of the south Adriatic Sea as nursery area at least for young loggerheads from the Greek MU. More recently, using hatchling drift simulation models, the Levantine zone and the Ionian-south Adriatic area have been indicated as the most important nursery areas for the Mediterranean loggerhead turtle sub-population but model results must yet be verified by field surveys (Casale & Mariani 2014).

Mixed stock analyses indicate that turtles from the different Mediterranean MUs do not distribute homogeneously within the basin (Laurent et al. 1998, Carreras et al. 2006, Maffucci et al. 2006, Clusa et al. 2013b, Garofalo et al. 2013, Maffucci et al. 2013). Moreover, the demographic composition of the oceanic foraging habitats in the Western Mediterranean Sea, Sicily Straits and Ionian Sea appears to be characterized by a high proportion of loggerhead turtles with an Atlantic origin (Laurent et al. 1998, Carreras et al. 2006, Maffucci et al. 2006, Clusa et al. 2013b, Garofalo et al. 2013, Maffucci et al. 2013). The percentage of these Atlantic travellers appears to decrease significantly at neritic foraging grounds in both the Western and Eastern side of the basin but several areas have not yet been fully characterised (Laurent et al. 1998, Carreras et al. 2006, Maffucci et al. 2006, Clusa et al. 2013b, Garofalo et al. 2013, Maffucci et al. 2013).

The Mediterranean loggerhead turtle sub-population has undergone severe exploitation in the past. Fisheries targeting directly sea turtles have been operating in several areas of the Mediterranean Sea up to early 1980s (Margaritoulis et al. 2003). Although commercial exploitation of sea turtle is currently forbidden, intentional killing and illegal consumption of turtle meat still occurs in some countries but it does not represent a significant conservation issue (Margaritoulis et al. 2003, Casale & Margaritoulis 2010, Nada & Casale 2011). Today, the major threats to loggerhead turtle survival come from fisheries by-catch and anthropogenic deterioration of both marine and coastal habitats (Margaritoulis et al. 2003). Casale (2011) estimated that over 132.000 sea turtles are accidentally caught each year in the Mediterranean Sea of which more than 44.000/year die as result of the capture. Uncontrolled coastal development and exploitation have led to the degradation of several nesting habitats around the basin with some sites known to have hosted nesting activity in the past, but which are not utilised anymore by the species (Margaritoulis et al. 2003). Finally, ingestion of plastic marine debris has a higher incidence in the Mediterranean Sea than in other regions in the Atlantic or Pacific oceans (Tomas et al. 2002, Lazar & Gracan 2011, Campani et al. 2013, Camedda et al. 2014).

Nevertheless, it is not all bad and wrong. Over the last three decades, loggerhead turtles have been afforded an increasing level of protection under several international conventions. Today, all Mediterranean countries possess national laws to protect sea turtles and many conservation programmes have been started and are currently underway, specifically to protect the major nesting beaches in the region (Casale & Margaritoulis 2010). Moreover, during the last years, several demonstrative actions have been founded to test conservation measures to reduce turtle by-catch in Mediterranean fisheries (e.g. NETCET Project, <http://www.netcet.eu/ref> or TURTLELIFE Project, <http://www.tartalife.eu/>). Thanks to these efforts, population trends are stable or even increasing and it has been possible to downgrade the Mediterranean loggerhead sub-population from “endangered” to “least concern” in the 2015 Red List Assessment of the International Union for the Conservation of Nature and Natural Resources (IUCN) (Casale 2015). However, these results are completely conservation dependent and cessation of conservation programmes around the Mediterranean Sea would most likely have significant detrimental effects (Casale 2015). The conservation status of the loggerhead turtle in the Mediterranean Sea is still far from being optimal and research will play a major role in assuring that conservation strategies and interventions are based on the best available knowledge and are promptly adjusted to possible future changes (Hamann et al. 2010, Cardona et al. 2015, Casale 2015).

This PhD thesis aims at advancing basic biological knowledge on the loggerhead turtle to foster the conservation of this charismatic and iconic species in the Mediterranean Sea by answering a number of sub-objectives related to the research priorities identified for sea turtle (Table 1.1, Hamann et al. 2010):

- Are there any anatomical features that are functionally related to stage specific aquatic habits?
- What are the juvenile and adult sex ratios at foraging grounds and how do they relate with those produced at nesting beaches?
- Which connections exist among rookeries and foraging grounds and what parameters shape them?
- Is there any evidence that climate warming is already affecting the loggerhead turtle in the western Mediterranean and what are the likely future impacts?

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2. ADAPTATIONS TO MARINE LIFE

2.1 BONE DENSITY IN THE LOGGERHEAD TURTLE: FUNCTIONAL IMPLICATIONS FOR STAGE SPECIFIC AQUATIC HABITS

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This chapter has been published in *Journal of Zoology* 291, 4: 243-248 (2013).

Abstract

Several studies investigated how changes in bone density are related to the evolution of complex buoyancy control systems in aquatic mammals. Very little is known on sea turtles, although this is one of the most ancient tetrapod groups that successfully colonized the marine environments. Here, we investigated for the first time the relationship between bone density and body size in the loggerhead turtle, *Caretta caretta*, with the aim to elucidate possible functional connections with the species' aquatic habits. Humeri were extracted from the carcasses of 72 loggerhead turtles ranging in size from 7 to 89 cm (males = 18, females = 44, unknown = 10). Whole bone density was determined by Archimedes' principle. Sexes exhibited comparable humerus densities (t-value = 0.49, $P > 0.05$). Mean humerus density (1.33 g cm^{-3}) was intermediate within the range reported for marine mammals and suggested no extreme specialization towards an either pelagic or benthic lifestyle. Turtle size and humerus density were significantly correlated (Pearson's correlation = 0.638, $P < 0.01$). Small juveniles had very light bones compared to adults in accordance with their stage specific pelagic diving and foraging behaviour.

Bone density in the loggerhead turtle: functional implications for stage specific aquatic habits

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Keywords

Caretta caretta; humerus density; marine tetrapods; bone ballast; diving behaviour.

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Editor: Mark-Oliver Rödel

Received 28 February 2013; revised 25 June 2013; accepted 26 June 2013

doi:10.1111/jzo.12060

Abstract

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Introduction

Tetrapods that secondarily invaded aquatic habitats underwent a remarkable suite of structural adaptations to overcome the mechanical constraints of locomotion and stability in water (Taylor, 2000; Gray *et al.*, 2007). The most obvious changes occurred at the gross morphological level. Animals acquired a streamlined body form and modified appendages effective for aquatic propulsion, trim control and drag reduction (Carroll, 1985; Fish & Stein, 1991; Llorente *et al.*, 2008). Equally important were the modifications of structural properties of the body that affected the ability of the animal to dive and surface easily, forage successfully and escape predators (Wall, 1983; Williams *et al.*, 2000; Houssaye, 2009, 2012). Changes in skeletal mass and density, for example, are functionally correlated to the evolution of buoyancy control mechanisms in aquatic tetrapods and have been suggested to reflect the particular habitat and foraging strategy adopted by different species (Wall, 1983; Fish & Stein, 1991; Taylor, 2000; Houssaye, 2009).

Manatees, *Trichechus manatus*, order Sirenia, have taken the use of bones as hydrostatic ballast system (i.e. bone ballast) to extreme levels (Taylor, 2000). These slow moving, herbivorous animals possess the highest bone density among aquatic mammals (humerus density = 2.0 g cm^{-3} , (Wall, 1983))

that enables them to be negatively buoyant even at very shallow depths where they normally rest and forage (Taylor, 2000; Houssaye, 2009). On the other hand, some of the most highly aquatic mammals belonging to the orders Cetacea and Pinnipedia, have evolved an extremely light skeleton that allows them to swim fast and dive deep in pelagic habitats (e.g. the elephant seal, *Mirounga angustirostris*, humerus density is 1.15 g cm^{-3}) (Wall, 1983; de Buffrenil, Sirc & Schoevaert, 1986; Gray *et al.*, 2007; Houssaye, 2009).

Testudines are unique among tetrapods primarily because of their shell, which includes girdles that lie within the rib cage, and which influences many aspects of their life (Llorente *et al.*, 2008). This group exhibits a significant ecological diversity and several taxa adapted to an aquatic existence, occupying most of the available habitats, from freshwater bodies to the open sea (Wyneken, Godfrey & Bels, 2008). The superfamily Chelonioidae, which includes both the extant families of sea turtles, the Dermochelyidae (genus *Dermochelys*) and the Cheloniidae (genera *Chelonia*, *Caretta*, *Eretmochelys* and *Lepidochelys*), is the only extant group of Testudines that adapted successfully to the marine environment (Pritchard, 1997). Sea turtles display a more streamlined body than freshwater species, and their forelimbs were modified into long wing-like rigid flippers and hindlimbs into semi-rigid paddles (Renous *et al.*, 2008). The morphology and growth pattern of

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paddles (Renous et al 2008). The morphology and growth pattern of appendicular bones have been shown to differ between hard shelled turtles, all belonging to the family Cheloniidae, and the leatherback turtle, *Dermochelys coriacea*, which has evolved a lighter skeleton as an adaptation to its nearly exclusive pelagic lifestyle (Rhodin, Ogden & Conlogue 1981, Snover & Rhodin 2008). However, little information is available on the structural properties of bone in hard shelled turtles and how these influence buoyancy control during the different phases of the life cycle.

The loggerhead turtle, *Caretta caretta*, is one of the extant members of the family Cheloniidae. This species possesses a complex life history characterized by a succession of life stages and corresponding ontogenetic habitat shifts and migrations (Bolten 2003). Emerging from the nest, hatchlings enter the sea and swim actively to reach major offshore currents where the oceanic juvenile stage begins (Bolten 2003). After a decade or more, the older juveniles recruit to coastal areas to enter a neritic developmental stage which will last at least another decade (Bolten 2003). The switch between these two phases is reversible and neritic juveniles may return to the oceanic environment on shorter time scales (McClellan & Read 2007). Only adult females venture back to the land to lay their eggs (Miller 1997).

In the present paper, we determine for the first time the density of the loggerhead turtle humerus and examine the relationship between bone density and turtle size with the aim to contribute to a better understanding of bone density patterns and the possible functional relation to the diving behaviour and foraging strategy adopted during the different phases of the species' life cycle.

Material and methods

Right humeri were sampled from the carcasses of 72 loggerhead turtles that were found either floating lifeless at the water surface or stranded dead along the coast of the south Tyrrhenian sea, central Mediterranean, between June 2009 and December 2010. No turtles were purposefully sacrificed in the course of the present study. All specimens were obtained through the local Sea Turtle Stranding and Rescue Network coordinated by the Stazione Zoologica Anton Dohrn of Naples.

The humerus bone was selected because it is easily removed from dead animals, its chondro-osseous development has already been described and it is commonly employed in similar studies (Wall 1983, Gray et al. 2007, Snover & Rhodin 2008).

Standard curved carapace length (CCL_{st}) was measured to the nearest 0.1 cm in each specimen. Sex was determined during necropsies by visual examination of the gonads and associated reproductive ducts.

After humeri were extracted from the flippers, they were flensed and boiled to remove any remaining tissue and allowed to dry outdoors for a minimum of 2 weeks. Additional removal of fat from bones was not performed because it has been shown to bias density measurements by Archimedes' principle (Keenan et al. 1992). This method provides a true whole bone density value and it is relatively inexpensive and easy to perform compared to more sophisticated alternatives, such as dual-energy X-ray absorptiometry or computer tomography (Keenan et al. 1997).

Entire humeri were hydrated by submerging them in distilled water at reduced atmospheric pressure (48 kPa). Duration of the hydration period was experimentally established using five humeri of different size. The time at cessation of bubble formation was annotated for each bone and the maximum time recorded (approximately 2 h) + 1 h was thereafter set as the standard hydration duration for the experiments. Bones were always hydrated together in groups of at least four humeri prior to each measurement trial.

Bone density was obtained using a custom made density determination kit that consisted of two platforms suspended by a wire frame that was mounted onto the scale's balance dish (Figure 2.1). The upper platform was submerged in a beaker containing distilled water that was supported by an independent structure that did not touch the balance or weighing platforms. The other platform was directly connected to the weighing plate of the analytical balance (Adventurer Pro AV812, Ohaus Europe GmbH, Nänikon, Switzerland).

The weighing procedure was carried out with each individual bone in the following steps: First, the bone was removed from the hydration chamber and held a few seconds in air to let the water drop. Thereafter, the bone was gently dabbed to remove excess surface moisture and weighed in air on the lower platform. Then it was moved to the upper platform and its under water weight was recorded. Finally, the bone was removed from the water, dabbed slightly and reweighed in air to control for effects of order of weighing (air-water vs. water-air).

All measurements were performed to the nearest 0.01 g.

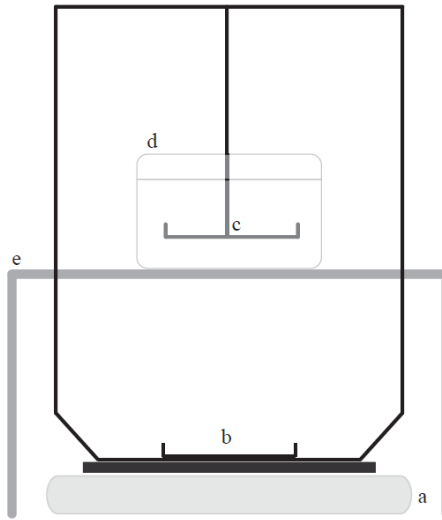


Figure 2.1. Density determination system. (a) analytical balance; (b) lower platform for in air weight measurements; (c) upper platform for in water density measurements; (d) beaker containing distilled water; (e) structure supporting the beaker.

All measurements were performed to the nearest 0.01 g. Water temperature in the beaker was measured for each weighing period by an electronic digital thermometer (Checktemp, HANNA Instrument Inc., Woonsocket, RI, USA) with $\pm 0.3^\circ\text{C}$ accuracy to correct water density values in the formula

$$\text{Humerus density} = (A A^{-1} - B) \times \rho_{\text{dw}}$$

where A is the weight of the hydrated bone in air, B is the weight of the hydrated bone submerged in water and ρ_{dw} is the density of distilled water at a given temperature.

For each bone, density was calculated twice using for A the first obtained value for weight in air, then the second value from the repeated measure in air.

Bone densities were then compared by paired t-test to detect if the order of weighing had any effect on the estimated bone density. Size distributions of the two sexes were compared using a Kolmogorov–Smirnov test in the

software package PAST (Hammer et al. 2001). The two samples t-test was employed to compare humerus density in males and females. The Pearson product moment correlation coefficient (Sokal & Rohlf 1995) between CCL and humerus density was calculated in Minitab 15 (Minitab Inc., State College, PA, USA).

A Model II regression [also known as ‘reduced major axis’ or ‘geometrical mean’ regression; (Sokal & Rohlf 1995)] was used to investigate the relationship between CCL (m) and humerus density data. This regression technique is more appropriate than standard ordinary least squares regression when both variables are measured with some error (Smith 2009). Error estimates of both the slope and intercept were calculated using bootstrapping over the cases (10 000 iterations). All calculations were performed with RMA for JAVA v. 1.21 software (Bohonak & van der Linde 2004).

Results

Loggerhead turtle sizes ranged from 7 to 89 cm (CCL_{st}, n = 72, median = 56.85 cm, first quartile = 40.77 cm, third quartile = 66.20 cm). Sex was not determined in 10 carcasses because of their advanced status of decay. The remaining sample was composed of 18 males and 44 females. Size distributions did not differ between sexes (D = 0.220, P > 0.05; Table 2.1).

Table 2.1. Size (CCL_{st}) of the loggerhead turtles analysed.

Sex	N	Median	1st quartile	3rd quartile
<i>Male</i>	18	58.10	52.95	76.75
<i>Female</i>	44	57.95	40.77	63.3
Total	72	56.85	40.77	66.2
All measurement units are in cm				

The humeri of five individuals, ranging in size from 7 to 11.1 cm (CCL_{st}), remained neutral or slightly positive buoyant after the hydration period which prevented us from determining their density. Therefore, they were removed from the subsequent analysis. The order of weighing did not affect density measurements (paired t-test value = -3.4, P < 0.01). Sexes exhibited comparable mean humerus density (t-value = 0.49, P > 0.05; Table 2.2). The overall mean humerus density was $1.33 \pm (\text{sd}) 0.05 \text{ g cm}^{-3}$.

Table 2.2. Summary statistics of volumetric density of loggerhead turtle humerus.

Sex	n	Mean	SD	Minimum	Maximum
Male	18	1.33	0.05	1.22	1.41
Female	44	1.33	0.05	1.19	1.43
Total	67	1.33	0.05	1.19	1.43

Units are g cm⁻³

Loggerhead turtle size and humerus density were significantly correlated (Pearson’s correlation = 0.638, P < 0.01). The Model II regression equation was

$$\text{Humerus density} = 1.166 + 0.296 \text{ CCL (m)}$$

(99% bootstrapped confidence intervals: 1.103 to 1.213 and 0.218 to 0.400 for the intercept and the slope respectively, Figure 2.2).

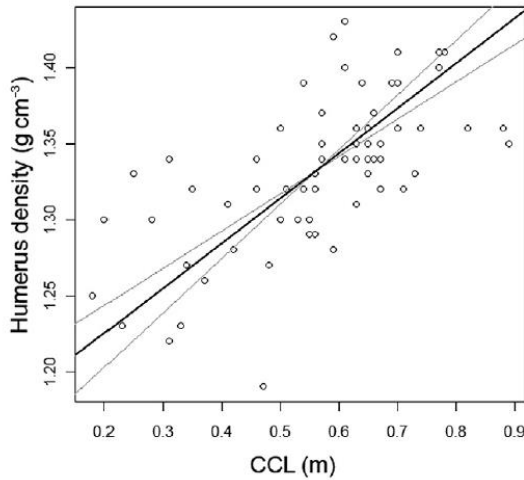


Figure 2.2. Model II regression of humerus density versus CCLst. 99% bootstrapped confidence intervals are reported in light grey.

Discussion

Tetrapods secondarily invaded the marine environment several times during their evolutionary history (Houssaye 2009). The transition from a terrestrial to an aquatic lifestyle required some striking modifications of body form and functions. Changes in skeletal mass, ranging from extremely high to extremely low density, have been suggested to be functionally related to the evolution of buoyancy control mechanisms and diving behaviours (Wall 1983, Taylor 2000, Gray et al. 2007, Houssaye 2009).

High bone density (bone ballastin) is usually associated with animals living in shallow waters and foraging on the bottom while low density is one of the aquatic specializations for deep diving and pelagic lifestyle (Taylor 2000, Gray et al. 2007).

The mean humerus density of the loggerhead turtle indicates that this species has denser bone than exclusively pelagic marine tetrapods but bone ballastin is not as extreme as in some slow swimming, shallow diving mammals (Wall 1983, Taylor 2000). No additional information is available on long bone density in sea turtles. However, the analysis of the chondro-osseous morphology supports our finding showing that loggerhead humerus is less spongy and has relatively more lamellar bone than that of the almost exclusively pelagic leatherback turtle (Rhodin et al. 1981, Snover & Rhodin 2008). The loggerhead turtle lifestyle is coherent with the evolution of such a reduced degree of bone ballastin (Taylor 2000). This species is a surfacer (Kooyman 1989, Hochscheid et al. 2010) that spends more than 90% of its time submerged and uses its lungs both as buoyancy organ and major oxygen store when diving (Hochscheid, Bentivegna & Speakman 2003). Nonetheless, both juveniles and adults have been reported occasionally to float motionless at the surface either to absorb solar radiation, to recover from anaerobic activity or simply to rest (Hochscheid et al. 2010).

Several studies indicated that this species usually forage and rest either on the sea floor or, if this is not within reach, at mid-water (Minamikawa, Naito & Uchida 1997, Hochscheid et al. 2010) where it preys mostly on slow moving or sessile species (Plotkin, Wicksten & Amos 1993, Tomas, Aznar & Raga 2001, Lazar et al. 2011). Increased bone density would offer some advantages in terms of stability and larger air volume that can be inhaled during shallow dives and benthic feeding but an extremely enlarged and dense skeleton would strongly penalise the animals during their long distance migrations especially when they stay in the open sea and perform deeper dives (Wall 1983, Taylor 2000, Bolten 2003).

The similarity in the mean humerus densities of males and females deserves further investigation. Our sample did not include a significant number of adults to allow us to observe sex-related differences of long bone densities that could be associated with the reproduction process and the development of the calcareous egg shell as reported in other turtle species (Edgren 1960). Increase in bone density with size, which is a proxy for turtle age, was expected because of the pattern of chondroosseous development in this species. The appendicular bones of the loggerhead turtle are laid down as cartilaginous anlagen with only a periosteal cuff of cortical bone while laminar compact bone is deposited later in growth (Snover & Rhodin 2008). Similar patterns have also been reported in other aquatic tetrapods (Wall 1983, de Buffrenil et al. 1990, Taylor 2000), and fit perfectly with the ontogenetic habitat shifts and changes in foraging habits of the species. Neonate loggerhead turtles have very limited diving capabilities, swim at low speeds and are largely inactive drifters that adopt a low-energy float-and-wait foraging strategy (Witherington 2002, Witherington, Hirma & Hardy 2012). The neutral or slightly positive buoyant humerus that we found in individuals with less than 11.1 cm of CCL, approximately up to 1 year old, is a clear benefit during this early surface-epipelagic stage (Witherington 2002). Later in life, turtles become more active, dive to deeper depths and after a decade or more in the oceanic province start to recruit to neritic habitats (Bolten 2003). Larger juveniles and adults spent the majority of their time in coastal areas and are important bioturbators of benthic systems (Lazar et al. 2011). These individuals feed predominantly on benthic invertebrates that they dig out from soft sediments using their front flippers and beak, a foraging method known as infaunal mining (Preen 1996; Schofield et al. 2006; Lazar et al. 2011). Therefore, they would profit from the combination of denser bones and the smaller lung volumes than juvenile individuals (Hochscheid et al. 2007) as a static ballast system (Taylor 2000, Gray et al. 2007). The results obtained in the present study suggest that bone density is functionally correlated to the marine aquatic habits of the loggerhead turtle during the various phases of its complex life cycle. Although the comparison with other tetrapod taxa provides evidence of a moderate degree of bone ballastin in this species, further investigation are required because of the lack of information on bone density in terrestrial and semiaquatic turtles. Although caution must be used because of the extent of bone remodelling reported in this species (Snover & Rhodin 2008), the relation between humerus density and turtle age deserve additional study to understand if this measure can be a valuable alternative to the enumeration of lines of arrested

growth in skeletochronological studies to infer turtle age, as demonstrated in some cetacean species (Guglielmini et al. 2002; Butti et al. 2007).

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3. SEX RATIOS

3.1. SEX RATIO OF JUVENILE LOGGERHEAD TURTLES IN THE MEDITERRANEAN SEA: IS IT REALLY 1:1?

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This chapter has been published in *Marine Biology* 160, 5: 1097-1107 (2013).

Abstract

Sex ratios are a crucial parameter for evaluating population viability. In species with complex life history patterns and temperature sex determination mechanisms, such as the loggerhead turtle (*Caretta caretta*), sex ratios may vary within a population and among populations. In the Mediterranean, juvenile sex ratios appear to not differ significantly from 1:1, although estimates for hatchling sex ratios are highly female biased. The immigration of males from the Atlantic has been suggested as a possible cause of such variation. Here, we present results of a multi-year investigation (2000–2011) on the sex ratios of loggerhead turtles foraging along the south Tyrrhenian coast, Western Mediterranean, with the aim of providing a better understanding of the potentially underlying forces that drive regional and age-dependent differences in sex ratios. Sex was determined through visual examination of the gonads in 271 dead turtles (curved carapace length range 29.5–89 cm). A fragment of the mitochondrial DNA control region was sequenced from 61 specimens to characterise the demographic composition of this foraging assemblage by applying a many-to-many mixed stock analysis approach. No significant association was found between sex ratios and years or size classes, although the largest size was male biased. Juvenile sex ratio was 1.56:1, which was different from an even sex ratio but still less female biased than hatchling sex ratios from Mediterranean beaches. Results of the mixed stock analysis indicate that juvenile sex ratios in the Mediterranean are largely unaffected by immigration of Atlantic individuals into the basin, as previously suggested. Continued long-term monitoring of juvenile sex ratios is necessary to detect biologically significant sex ratio shifts in the Mediterranean loggerhead turtle population.

Sex ratio of juvenile loggerhead turtles in the Mediterranean Sea: is it really 1:1?

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Received: 30 August 2012 / Accepted: 19 December 2012
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Introduction

Sex-determining mechanisms in gonochoristic vertebrates can be conveniently divided into two general categories: genotypic (GSD) where gender is determined by sex chromosomes at the time of fertilization and environmental (ESD) where offspring become male or female in response to environmental cues during development (Bull 1983). The sex ratio, that is, the number of males and females in a population, is a key demographic parameter crucial for evaluating population viability.

Fisher (1930) was the first to provide a theoretical explanation of why, under natural selection, the two sexes are usually produced approximately in equal numbers if parental investment is uniform. Subsequent studies emphasised several circumstances in which Fisher's assumptions do not hold and population sex ratios may depart from 1:1 (Hamilton 1967; Bull and Charnov 1989; Charnov and Bull 1989; Lovich and Gibbons 1990). Natural population sex ratios can be influenced by different demographic factors including skewed sex ratios at

Communicated by R. Lewison.

Electronic supplementary material The online version of this article (doi:10.1007/s00227-012-2160-x) contains supplementary material, which is available to authorized users.

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Published online: 01 January 2013

 Springer

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The loggerhead turtle, *Caretta caretta*, is a suitable model organism to investigate the dynamics of sex ratios in natural populations. This species possesses a complex life history pattern characterised by a succession of life stages and corresponding ontogenetic habitat shifts and migrations (Bolten 2003). Emerging from the nest, hatchlings enter the sea and swim actively to reach major offshore currents where the oceanic juvenile stage begins (Bolten 2003). After a decade or more, the now larger and older juveniles recruit to coastal areas to enter a neritic developmental stage which will last at least another decade (Bolten 2003). The switch between these two phases is reversible and neritic juveniles may return to the oceanic environment on shorter time scales (McClellan & Read 2007). Foraging assemblages using both the oceanic and the neritic developmental habitats are mixed stocks composed of individuals originating from different nesting beaches (Bowen et al. 2004, Carreras et al. 2006, Maffucci et al. 2006). Upon sexual maturation, loggerhead turtle females begin periodic reproductive migrations to nest in the vicinity of their natal beach. Males may undergo similar migrations, although they can mate opportunistically on migratory corridors or coastal foraging grounds (Bowen et al. 2005).

The loggerhead turtle, like all sea turtle species, possesses a temperature-dependent sex determination (TSD) in which incubation temperature

experienced by embryos during the thermosensitive period (TSP, middle third of the incubation) has a major influence on the hatchling sex ratio (Mrosovsky 1994). At incubation, temperatures around 29 °C 50 % of either sexes are produced; while cooler temperatures produce more males, warmer ones favour females (Carthy et al. 2003, Wibbels 2003). Female skewed hatchling sex ratios have been estimated at most nesting sites, although great variability exists in the methodology and scope of the different studies (Mrosovsky 1994, Hanson et al. 1998, Godley et al. 2001a, b, Wibbels 2003, Kaska et al. 2006, Hawkes et al. 2007, Zbinden et al. 2007). In general, very few of these studies have adequately taken into account the effect of nest location on hatchling sex ratios or the possible seasonal and yearly variations in gender production (Wibbels 2003, TEWG 2009). Sex ratios in other life stages also are of interest because they represent a condensation of many years of hatchling production and integrate a number of different life history events (Wibbels 2003, TEWG 2009). However, they are logistically more difficult to obtain because juveniles and adults must be sampled at sea, have a solitary life style and spend most of their life in places that are difficult to access (Wibbels 2003). Moreover, external morphological cues can be used exclusively for sexing adults because sexual dimorphic characters (i.e. the long tail and the strongly curved front claws on the second digit typical of males) appear only as turtles approach sexual maturity (Wibbels et al. 1987, Kamezaki 2003). Different methods have been used for sexing juveniles including serum testosterone, histology, laparoscopy or direct observation of gonadal morphology during necropsies (Wibbels et al. 2000, Wibbels 2003, Casale et al. 2006, Lazar et al. 2008). Interpretation of sex ratio data in immature and adult portions of loggerhead turtle populations requires additional information on the size/age class composition and demographic structure of the analysed aggregations (Casale et al. 2006, Delgado et al. 2010). Adult sex ratios may be biased by sex-specific differences in the breeding pattern and migratory behaviour of males versus females that are likely to be less intense in juveniles (Wibbels 2003). Moreover, the various source nesting beaches that contribute to a specific foraging assemblage may have different hatchling sex ratios (Casale et al. 2006, Delgado et al. 2010). Previous investigations suggested that female-biased sex ratios are maintained in juvenile assemblages from the Atlantic ocean where an approximately 2:1 sex ratio (F:M) has been consistently reported from both oceanic and neritic habitats (Wibbels 2003, Delgado et al. 2010). In contrast, immature sex ratios in the Mediterranean Sea appear not to differ significantly from 1:1 (Casale et al. 2006) which is surprising considering the

highly female biased hatchling sex ratios estimated for the majority of the Mediterranean nesting beaches (Table 1) (Godley et al. 2001a, b, Oz et al. 2004, Rees & Margaritoulis 2004, Margaritoulis 2005, Zbinden et al. 2007). The immigration of large numbers of males from the Atlantic population was suggested to explain such a discrepancy (Casale et al. 2002, 2006) but evidence supporting this hypothesis is limited. Moreover, it was recently found that the oceanic juvenile loggerhead turtle assemblage foraging in the eastern Atlantic, the putative source of Atlantic individuals entering the Mediterranean Sea, exhibits a female-biased sex ratio of 2:1 (Delgado et al. 2010).

In this study, we analysed a multi-year dataset on the loggerhead turtle assemblage foraging along the southwestern coasts of Italy, central Mediterranean. Information on mtDNA control region sequence diversity was employed to investigate the demographic structure of this stock and of those utilising adjoining juvenile habitats in the Mediterranean and Atlantic regions by applying the ‘many-to-many’ mixed stock analysis approach (Bolker et al. 2007). Our objectives were to: (1) understand the dynamics of loggerhead turtle sex ratio on this important Mediterranean juvenile habitat, (2) clarify the spatial variation of loggerhead turtle juvenile sex ratios in Mediterranean and Atlantic areas, and (3) verify whether immigration of Atlantic individuals can adequately explain the spatial variation of the sex ratio in loggerhead turtle juvenile assemblages from the Mediterranean Sea.

Materials and methods

Sample collection

We examined the carcasses of 271 loggerhead turtles ranging in size from 29.5 to 89 cm (curved carapace length, CCL) that were found either floating lifeless at the water’s surface or stranded dead along the coast of the south Tyrrhenian sea, central Mediterranean (from lat 41°13’ N, long 13°03’ E to lat 30°40’, long 15°48’, Figure 3.1) in the period 2000–2011. Previous studies have demonstrated that this area is regularly utilised by loggerhead turtles for foraging (Bentivegna 1997, Bentivegna & Paglialonga 1998, Bentivegna et al. 2001). Since 1983, the Stazione Zoologica of Naples (SZN) coordinates a sea turtle stranding and rescue network in the study area (Bentivegna et al. 2003). Sampling and observer efforts were uniform during the sampling period.

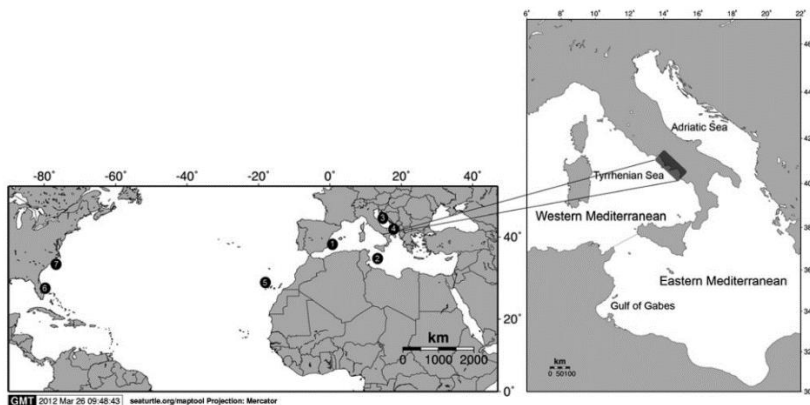


Figure 3.1. Map showing the location of the juvenile loggerhead turtle habitats considered in the present study. The sampling location in the south Tyrrhenian sea is highlighted in grey in the right panel, while black circles in the left panel represent the areas analysed in previous studies (1 north-west Mediterranean, 2 central Mediterranean, 3 north-east Adriatic, 4 south-west Adriatic, 5 Madeira Island, 6 southern USA, 7 northern USA). Maptool is a product of SEATURTLE. ORG. (Information is available at www.seaturtle.org)

Laboratory procedures

Sex was determined during necropsies by visual examination of the gonads and associated reproductive ducts. This method is commonly employed to sex dead turtles and has been proved to be one of the most reliable sexing techniques in individuals larger than 30 cm of CCL, approximately the minimum size of the turtles in our sample (Lazar et al. 2008). The state of the analysed loggerhead carcasses varied from fresh dead to moderately decomposed. Muscle or skin samples for genetic analysis were collected from 61 (F = 38, M = 23) of the sexed individuals and stored in 95 % ethanol for the subsequent genetic analysis. Automation of the genomic DNA extraction method was accomplished on the Biomek FX workstation (Beckman Coulter Inc.) equipped with the ORCA Robotic arm using NucleoSpin96 Tissue, (MACHEREY– NAGEL GmbH & Co. KG) following the manufacturer's protocol. Genomic DNA was purified by binding and eluting to a silica membrane using vacuum filtration. A fragment of mtDNA encompassing tRNAThr, tRNAPro and the control region was amplified by polymerase chain reaction (PCR) using the primers LCM15382 (50-GCT

TAACCCTAAAGCATTGG-30) and H950 (50-GTCTCG GATTTAGGGGTTTG-30) (Abreu-Grobois et al. 2006). These primers amplify a fragment of ~800 bp that completely encompasses the shorter (380 bp) region which has been used as reference to define mtDNA haplotypes in the literature (<http://accstr.ufl.edu/ccmtDNA.html>). The shorter fragment was employed in the many-to-many mixed stock analysis because only few data on the longer sequence at nesting populations and foraging grounds are available in the literature up to date. PCRs were prepared in automation and performed in 50 l volumes using the following conditions: 95° for 5 min, followed by 35 cycles of 95° for 1 min, 50° for 1 min, 72° for 1.5 min and 72° for 7 min. PCR products were purified in automation using the Millipore Multiscreen HTS PCR 96-Well Plate Kit (Millipore Corporation) and sequenced in both directions. Sequence reactions were prepared using BigDye Terminator Cycle Sequencing technology (Applied Biosystems) and purified using the Agencourt CleanSEQ Dye terminator removal Kit (Agencourt Bioscience Corporation) on the Biomek FX workstation (Beckman Coulter Inc). Products were analysed on an Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems). Forward and reverse chromatograms of each sample were analysed and assembled using the software package SeqManII (DNASTAR Inc.). Resulting sequences were aligned using the Bioedit Sequence Alignment Editor 7.0.9.0 (Hall 1999).

Data analysis

Size distributions of the two sexes were compared using a Kolmogorov–Smirnov test in the software package PAST (Hammer et al. 2001). Chi-square analysis in Minitab 15 (Minitab inc.) was employed to evaluate sex ratio differences among years across the study period, to assess possible age-/size-dependent sex ratios and to examine whether observed frequencies of males and females differed significantly from the theoretical Fisherian ratio (1:1) or from sex ratios reported from other Mediterranean and Atlantic foraging grounds. Association between size and sex ratio was assessed by dividing the sample into six arbitrary size classes based upon the CCL corresponding to <40 cm (N = 12), 40.1–50 (N = 43), 50.1–60 (N = 59), 60.1–70 (N = 104), 70.1–80 (N = 41) and >80 (N = 12). Juvenile sex ratio was obtained by excluding individuals with a CCL greater than 70 cm. This value was chosen after considering the mean CCL of nesting females in the Mediterranean Sea (Margaritoulis et al. 2003). Adult sex ratio was calculated considering only those individuals with a CCL >75 cm.

Genetic differentiation between the two genders was verified with the exact test of population differentiation, computed with 100,000 steps in the Markov Chain with 10,000 dememorization steps, and pair wise genetic distance (Ust), computed with 10,000 random permutations. In all tests that required the estimates of sequence divergence, we used the Tamura–Nei model of nucleotide substitutions which was designed for control region sequences (Tamura & Nei 1993). All statistical analyses were carried out using the statistical software package ARLEQUIN v.3.5.1.2 (Excoffier & Lischer 2010). The different nesting populations contributing to the foraging area were defined employing a Bayesian mixed stock analysis (MSA) based on the many-to-many approach (Bolker et al. 2007). This method, contrary to the most common many to one allows the analysis of a meta-population made up of multiple sources and multiple mixed stocks, thus providing the opportunity to compare directly the demographic composition of several foraging assemblages (Bolker et al. 2007). Six additional foraging grounds were considered in the analysis corresponding to, or in the close proximity of areas for which sex ratio data were available: central Mediterranean Sea (Casale et al. 2008), north central Adriatic Sea (Giovannotti et al. 2010), north-eastern Spain (Carreras et al. 2006), Madeira (Bolten et al. 1998), southern USA (Bowen et al. 2004) and northern USA (Bowen et al. 2004). Fourteen nesting populations were used as possible sources for the MSA: (1) Libya (N = 49); (2) Greece (N = 60); (3) Mediterranean Islands comprising Cyprus and Crete (N = 54), (4) Dalyan (N = 40), (5) Dalaman (N = 20), (6) western Turkey (N = 76), middle Turkey (N = 48), eastern Turkey (N = 72), (7) Cape Verde (N = 187), (8) Florida coast/northern Gulf of Mexico (N = 49), (9) south Florida (N = 109), (10) northeast Florida to North Carolina (N = 105), (11) Dry Tortugas (N = 58) and (12) Quintana Roo, Yucatan (N = 20) (sources of haplotype frequencies: Bowen et al. 2005, Carreras et al. 2007, Encalada et al. 1998, Monzon-Arguello et al. 2010, Yilmaz et al. 2011, Saied et al. 2012). As required by the mixed stock analysis, both the source population and the mixed stock datasets exhibited significant spatial structure (Ust = 0.81, p value < 0.01 and Ust = 0.27, p value < 0.01, respectively). The average annual number of nests was used as a proxy of rookery sizes and incorporated into the analysis as an ecological covariate assuming that the overall contribution of a rookery is proportional to its size (Okuyama & Bolker 2005). Estimates of rookery size were derived from the literature (Ehrhart et al. 2003, TEWG 2009, Casale & Margaritoulis 2010, Monzon-Arguello et al. 2010). To distinguish larger from smaller contributions, an

arbitrary cut-off value of 5 % was utilised for the “foraging centric” estimates (Bolker et al. 2007).

Results

Due to the small number of animals sexed in 2005 and 2006 (4 and 8 turtles, respectively), these year cohorts were pooled together for the statistical analysis. The sex ratio across years was consistently biased towards females with only 2001 and 2002 exhibiting equal proportions of the two genders (Figure 3.2). No statistical association between year cohorts and sex ratio was found ($\chi^2 = 5.33$, $df = 10$, p value >0.05); therefore, all individuals were pooled together for further analysis.

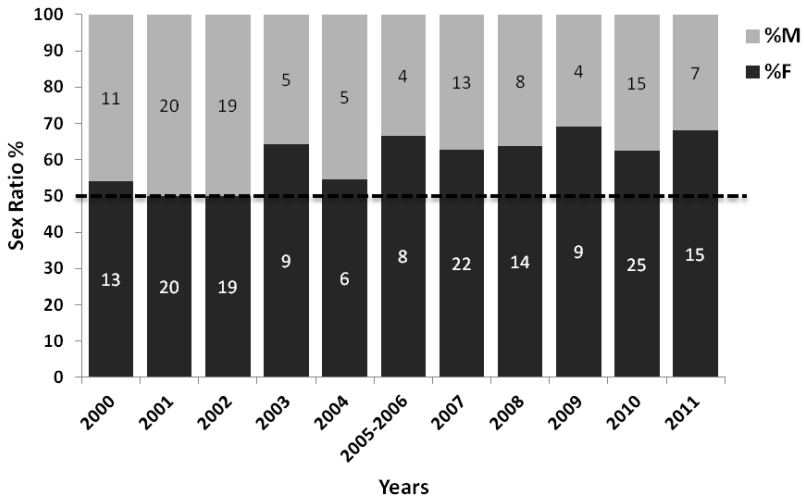


Figure 3.2. Observed sex ratios between 2000 and 2011; bars are labelled with the absolute numbers of males and females found each year (2005 and 2006 were pooled together because of low numbers). The dashed line indicates even sex ratio.

The overall sex ratio was 1.44:1 (F:M, $N_{\text{female}} = 160$, $N_{\text{male}} = 111$) which differed significantly from both an even sex ratio ($\chi^2 = 8.86$, $df = 1$, p value <0.01) and the 2:1 sex ratio ($\chi^2 = 7.76$, $df = 1$, p value <0.01).

Genders exhibited comparable size distributions ($D = 0.11$, p value >0.05) with a mean CCL \pm SD of 61.9 ± 12.5 and 61.0 ± 11.0 cm for males and females, respectively (Figure 3.3).

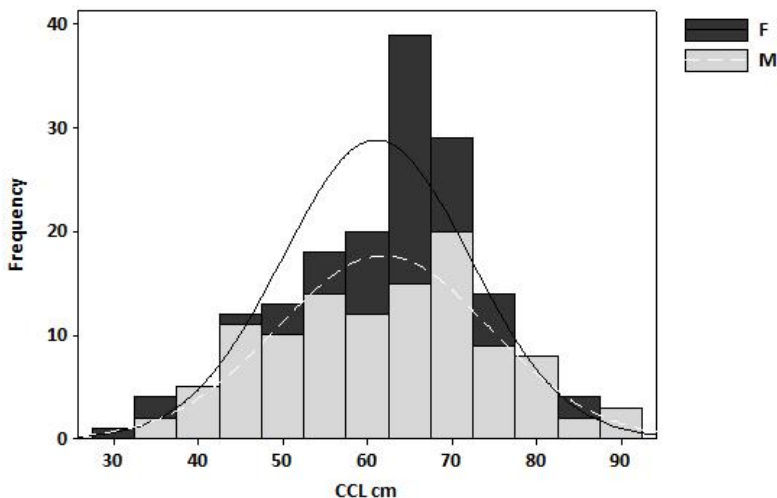


Figure 3.3 Size–frequency distribution of male and female loggerhead turtles analysed in this study

All but the largest size class was dominated by females (Figure 3.4). However, no significant association between size and sex ratio was found ($\chi^2 = 3.88$, $df = 5$, p value >0.05). When considering only sexually mature animals ($N = 26$, data not shown), males were more frequent than females constituting 61.5 % of the sample (1:1.45 sex ratio). Sex ratio in the juvenile portion was estimated to be 1.56:1 ($N = 218$) which was significantly different from 1:1 ($\chi^2 = 10.57$, $df = 1$, p value <0.01) but not from 2:1 ($\chi^2 = 3.53$, $df = 1$, p value >0.05). Results of statistical comparison between our observed juvenile sex ratio and those reported from different Mediterranean and Atlantic juvenile foraging grounds are reported in Table 3.1.

No genetic differentiation was found between the two sexes ($\Phi_{st} = -0.01787$, p value >0.05 ; exact p value $= 0.27420 \pm 0.0210$ SD); therefore, all samples were pooled together for the subsequent analysis. The Markov chain Monte Carlo (MCMC) method was used to obtain the posterior distributions of the parameters of interest in the manyto- many MSA. Three chains of 20,000 iterations were run in each analysis from over-dispersed, randomly selected starting points. The Gelman–Rubin diagnostic criterion was <1.2 for all variables indicating the convergence of MCMC. When considering

Mediterranean foraging habitats, South Florida and Dry Tortugas were the only Atlantic source nesting populations exhibiting contributions above the arbitrary cutoff value of 5 % in 3 and 1 Mediterranean foraging areas, respectively (Table 3.2). However, only in north-eastern Spain, did Atlantic juveniles seem to constitute a significant portion of the juvenile stock (Table 3.2). On the contrary, all contributions from the Mediterranean source populations to the Atlantic foraging areas were estimated well below the arbitrary cut-off value (Table 3.2).

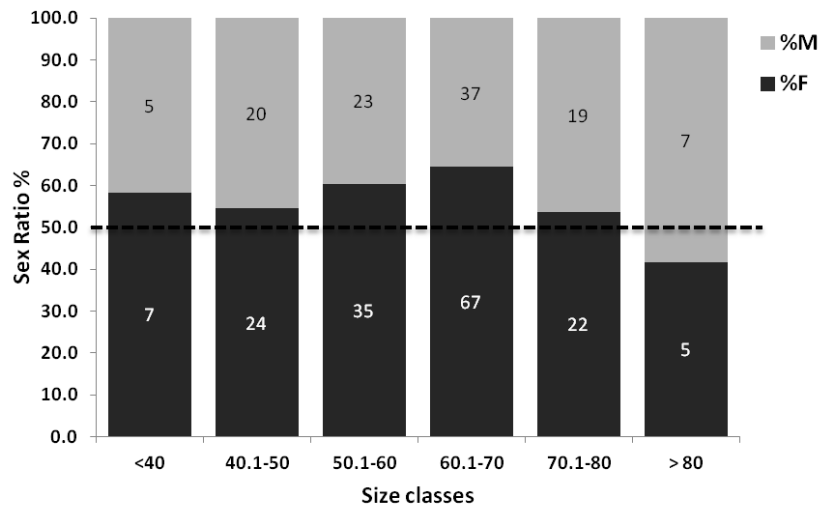


Figure 3.4. Observed sex ratios for each size cohort; bars are labelled with the absolute numbers of males and females found in each category. The dashed line indicates even sex ratio

Table 3.1 Sex ratios reported from different juvenile foraging grounds in the Mediterranean Sea (in italics) and Atlantic Ocean and estimated from Mediterranean nesting beaches. Significance values compared to the observed juvenile sex ratio in the study area are shown (* indicates significant results at p value\0.05, ** indicates significant results at p value\0.01)

Region	N	Sex ratio%	F	M	P-value	Reference
<i>south Tyrrhenian sea</i>	<i>218</i>	<i>61</i>	<i>133</i>	<i>85</i>		<i>present study</i>
<i>north west Mediterranean</i>	104	53.8	56	48	0.034*	<i>Casale et al. 2006</i>
<i>central Mediterranean</i>	66	54.5	36	30	0.055	<i>Casale et al. 2006</i>
<i>north east Adriatic</i>	57	57.9	33	24	0.352	<i>Casale et al. 2006</i>
<i>south west Adriatic</i>	83	51.8	43	40	0.007**	<i>Casale et al. 2006</i>
Madeira Island	207	66.7	138	69	0.076	<i>Delgado et al 2010</i>
S.USA (Florida)	256	66	169	87	0.119	<i>Wibbels et al. 1987</i>
N.USA (North Carolina)	89	67.4	60	29	0.044*	<i>Braun-McNeill et al. 2007</i>
Foraging grounds						
Fethiye, Turkey	65				0.217	<i>Kaska et al. 2006</i>
Patara, Turkey	70.5				0.002**	<i>Oz et al. 2004</i>
Zakynthos, Greece (2002)	68				0.027*	<i>Zbinden et al. 2007</i>
Zakynthos, Greece (2003)	75				0.000**	<i>Zbinden et al. 2007</i>
Kyparissia, Greece	79				0.000**	<i>Rees and Margaritoulis 2004</i>
Alagaði, Cyprus	89-99				0.000**	<i>Godley et al. 2001</i>
Nesting beaches						

Table 3.2. Foraging-centric estimated contributions (mean \pm standard deviation) of the different juvenile habitats considered in this study. Contributions above the cut-off value of 5 % are reported in bold

Legend

Source populations: LIB Lybia, GRE Greece, MISL Mediterranean Islands, DALY Dalyan, DALA Dalaman, WTR western Turkey, MTR middle Turkey, ETR eastern Turkey, CV Cape Verde, YUC Yucatan, DT Dry Tortugas, NWFL Florida coasts/northern Gulf of Mexico, NEF-NC north-east Florida to north Carolina, SFL south Florida

Foraging habitats: TYR south Tyrrhenian Sea, CM central Mediterranean Sea, NES north-eastern Spain, NCA north central Adriatic, MAD Madeira, S USA southern USA, N USA northern USA

Foraging habitats									
Mediterranean					Atlantic				
	TYR	CM	NES	NCA	MAD	SUSA	NUSA		
	<i>mean ±sd</i>	<i>mean ±sd</i>	<i>mean ±sd</i>	<i>mean ±sd</i>	<i>mean ±sd</i>	<i>mean ±sd</i>	<i>mean ±sd</i>		
LIB	12±6	9±5	11±6	4±4	0±1	1±1	1±1		
GRE	31±13	29±15	16±11	46±12	1±2	2±2	2±2		
MISL	7±6	11±9	10±7	8±6	1±1	1±1	1±1		
DALY	2±2	3±2	3±2	3±3	0±0	1±1	0±0		
DALA	1±1	1±1	1±1	1±1	0±0	0±0	0±0		
WTR	5±4	7±5	7±6	8±6	0±0	1±1	1±1		
MTR	11±8	10±8	8±7	7±6	1±1	2±2	1±1		
ETR	8±6	7±6	8±6	8±6	1±1	1±1	1±1		
Mediterranean									
CV	3±3	3±2	2±2	2±2	12±7	2±2	1±1		
YUC	3±3	3±3	3±3	3±3	1±2	4±3	2±1		
DT	4±4	5±5	6±5	4±4	1±1	1±1	1±1		
NWHL	3±3	3±3	5±4	2±2	1±2	2±2	2±2		
NEF:NC	3±3	3±3	4±3	2±2	5±6	5±5	23±8		
SFL	7±6	7±6	17±9	3±3	76±14	77±13	64±12		
Atlantic									

Discussion

Information on sex ratios of endangered species with temperature-dependent sex determination such as the loggerhead turtle is needed to understand their demography and mating system, to identify possible shifts in population vigour and to recognise the impact of climate change or other environmental alterations (Mrosovsky et al. 2009, TEWG 2009). While direct observation of the sex of adults is possible because they have sexual dimorphism (Wibbels et al. 1987, Kamezaki 2003), it is not feasible for juveniles. Different methods have been used to obtain sex ratios in immature loggerhead turtles including direct observation of gonadal morphology during necropsies (Casale et al. 2006, Lazar et al. 2008, TEWG 2009). The examination of the sex ratio in the juvenile portion of the population has been suggested as one of the most effective ways to study loggerhead population sex ratio because it represents the condensation of many years of hatchling production and circumvents some of the logistical problems associated with the study of adult and hatchling sex ratios (e.g. intraseasonal and inter-annual variation in hatchling sex ratio production and sex-specific adult migratory behaviour, Wibbels et al. 1987). However, long-term assessments are necessary to mitigate the effect of inter-annual variability and get an estimate of the actual population sex ratio (Wibbels 2003, Wyneken et al. 2006). This was evident when looking at our 11-year dataset. The overall sex ratio was female biased (1.44:1, F:M) with no significant association between year cohorts and sex ratios, although with some annual variation (Figure 3.2). However, the analysis of the first 3 years provided nearly equal sex ratios (1.04:1, F:M, $N = 102$), which were very close to those reported from other Mediterranean foraging habitats (Casale et al. 2006). In contrast, during the successive 2003–2011 time window ($N = 169$), the sex ratio was significantly female biased (1.48:1 F:M). Although this difference could be related to a change in the hatchling sex ratio or to a variation in the nesting populations contributing to the south Tyrrhenian foraging assemblage, it was more likely the result of the intrinsic yearly fluctuation of sex ratios estimated from stranded turtles (Wibbels 2003). Several sources of bias are known to influence stranding data such as weather conditions, fishing effort and human presence along the coast, (Epperly et al. 1996, TEWG 2009). Therefore, only long-term datasets and the most conservative approaches should be used in the interpretation of such information.

Small and large neritic juveniles (corresponding to Stages III and IV following the definition in TEWG (2009)) represented the larger proportion of both sexes (Figure 3.3). Post-hatchlings and oceanic juveniles

(corresponding to Stages I and II following the definition in TEWG (2009)) were absent probably because there are no relevant nesting areas in the western Mediterranean (Casale & Margaritoulis 2010) and hatchlings from major rookeries are not expected to passively drift to the west coast of Italy (Hays et al. 2010a). Adults were only occasionally found in the study area ($N = 26$). Several studies have shown that adult loggerhead females are philopatric to their natal nesting areas from where they migrate to specific foraging grounds also located in the eastern Mediterranean such as the Adriatic Sea and the Gulf of Gabès (Tunisia) (Godley et al. 2003, Broderick et al. 2007, Hays et al. 2010a). Males may be less tied than females in terms of destination and amplitude of post-reproduction movements since they can mate opportunistically on migratory corridors or on common coastal foraging grounds (Bowen et al. 2005). This may explain the inversion of the sex ratio observed in the adult portion of our sample. Stage-specific sex ratio shifts in stranded turtles have been already documented along the Atlantic coast of the United States (Wibbels et al. 1987, Wibbels 2003, TEWG 2009) and, besides differences in migratory patterns and behaviour, they may have a number of explanations which are not mutually exclusive such as, for example, hatchling sex ratio shifts across time, mortality rates and/or probability of stranding that is not uniform for both sexes by size classes. Our results differed significantly from the female-biased sex ratio previously estimated for adults inhabiting central Mediterranean waters (1.55:1, F:M, $N = 69$) (Casale et al. 2005). This discrepancy was probably related to the different sampling areas, which included in Casale et al.'s (2005) study also migratory corridors and areas nearby nesting grounds, and hence reflects the known problems associated with estimating adult sex ratios in loggerhead turtle populations (TEWG 2009).

In contrast to adults, juveniles exhibited a female-biased sex ratio with only minor variation among size classes (Figure 3.4) which may indicate that the hatchling sex ratio of the populations contributing to the loggerhead turtle stock foraging along the southern Tyrrhenian coasts was stable across the last few decades. This is the first time that a female-biased sex ratio was reported for Mediterranean juveniles, although statistical comparison suggests that our results were not significantly different from sex ratios found in the central Mediterranean and the north-east Adriatic regions (Casale et al. 2006) (Table 1). Probably, the relative small sample size and the study period in Casale et al.'s study (2006) influenced appreciably their possibility to detect such a slight departure from an even sex ratio.

Considering all information together (Table 3.1), it appears that loggerhead turtle juvenile sex ratios in the Mediterranean Sea are female biased but less than the approximate 2:1 ratio commonly reported from Atlantic juvenile habitats (Wibbels 2003, TEWG 2009, Delgado et al. 2010) and also significantly lower than hatchling sex ratios estimated for the majority of the Mediterranean nesting beaches (Table 1; Godley et al. 2001a, b, Oz et al. 2004, Rees and Margaritoulis 2004, Kaska et al. 2006, Zbinden et al. 2007). Several causes can explain these differences. It was hypothesised that a strong male-biased immigration of Atlantic juveniles could account for the juvenile sex ratio observed in the Mediterranean Sea (Casale et al. 2002, 2006), but our estimates from the many-to-many MSA analysis cast some doubts on this. Despite the intrinsic caveats of the statistical approach employed, which allow only qualitative, not quantitative interpretation of the results (Bolker et al. 2007), and the partial overlap between the areas sampled for sex ratios assessment and those utilised in the MSA program, the estimated Atlantic contributions were incongruent with the male-biased immigration hypothesis. In fact, the north central Adriatic was the only Mediterranean area with literally no Atlantic contribution (i.e. always below the arbitrary cut-off value of 5 %, Table 3.2) but the nearby south-west Adriatic region has the highest proportion of males reported in the Mediterranean Sea (Table 3.1). At the same time, the north-west Mediterranean presented a sex ratio comparable to those found in the central Mediterranean and the south-west Adriatic, although the estimated Atlantic contribution was considerably higher (Table 3.1). Differences in sex-specific death rates, behaviour and spatial distribution may help create the observed sex ratios in the juvenile loggerhead turtle assemblages from the Mediterranean Sea, but more data are necessary to investigate these aspects. However, the consistency in juvenile sex ratios from both oceanic and neritic developmental grounds across the wide Atlantic–Mediterranean region (Table 3.1) suggests that the actual number of females produced at the different nesting beaches may be significantly overestimated (Delgado et al. 2010). Nest locations, timing of nesting and weather conditions are known to have a profound influence on hatchling sex ratio (Wibbels 2003) and wide variation in sex ratios within a beach or region across years have been reported (Wyneken et al. 2006). Moreover, the mechanism for TSD also remains unclear (Wibbels 2003). It has been shown that steroids can enhance or override the effects of incubation temperature in reptiles with TSD (Wibbels et al. 1991) and previous work on painted turtles (*Chrysemys picta*) suggested a seasonal shift in the sex ratio caused by seasonal variation in yolk steroid levels (Bowden et al. 2000). This means

that in species in which females lay two or more clutches per nesting season, sequential clutches from the same individual may respond differently to the same thermal regime (Bowden et al. 2000). If this mother influence would also be present in the loggerhead turtle, then the existence of similar seasonal shifts would tend to lower the annual production of individual females, thus helping to explain, in combination with inter-annual fluctuation in the environmental condition at the nesting sites, the discrepancy between estimated hatchling sex ratio and those found on juvenile habitats.

If we take the peculiar breeding pattern of loggerhead turtles into account, where females lay their eggs at intervals of approximately two years while males reproduce each season (Hays et al. 2010b), a juvenile sex ratio close to 2:1 (F:M), as the one observed for the wide Atlantic–Mediterranean region, may turn into an operational sex ratio very close to the theoretical optimal Fisherian sex ratio of 1:1 (Hays et al. 2010b, Wright et al. 2012). In this context, the lower value found in the Mediterranean Sea may derive from the combination of two factors, the evolutionary recent colonization of this basin (Encalada et al. 1998), meaning that the population is not yet at the equilibrium, and the more temperate thermal regime compared to the most important Atlantic nesting beaches.

In conclusion, our data demonstrated that, in contrast to what was previously known, juvenile sex ratios in the Mediterranean Sea are female biased, although to a lesser extent than the Atlantic stock. Immigration of Atlantic individuals into the Mediterranean Sea does not explain the observed spatial variation in the proportion of the two genders in this region. The discrepancy to hatchling sex ratios estimated from Mediterranean nesting beaches warrant further investigation but it may be the result of a systematic overestimate of the actual numbers of females produced. Juvenile sex ratios are the integration of multiple years of hatchling sex ratio production and may present a more viable estimate of the population's functional sex ratio provided that long time series, large sample sizes and the most rigorous criteria for sex identification are used. It is of the utmost importance to continue to monitor sex ratios in areas such as the south Tyrrhenian foraging ground where multiple-year datasets are available, in order to detect biologically significant sex ratio shifts in the Mediterranean loggerhead turtle population.

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3.2. SEX RATIOS OF ADULT LOGGERHEAD TURTLES IN THE MEDITERRANEAN FORAGING HABITATS

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An extended version of this chapter has been published by Casale et al. in *Scientia Marina* 78, 2: 303-309 (2014).



Adult sex ratios of loggerhead sea turtles (*Caretta caretta*) in two Mediterranean foraging grounds

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Summary: Sea turtles show temperature-dependent sex determination (TSD) and information on sex ratios at different life stages is necessary both for population dynamics models for conservation and to shed light on the possible adaptive value of TSD. Adults represent the less abundant class of sea turtle populations and adult sex ratios at foraging grounds are very difficult to obtain. We first analysed biometric data of 460 juvenile and adult loggerhead sea turtles ranging from 60 to 97.5 cm curved carapace length (CCL), in which a clear bimodal distribution of tail length (the main secondary sexual character of adult males) was observed in the size class >75 cm CCL. We then sexed 142 adult turtles in this size class collected from the Tunisian shelf and from the southwestern Tyrrhenian Sea, observing a proportion of females of 51.5% (95% CI: 41.2-61.8%; n=97) and 40.0% (95% CI: 25.7-55.7%; n=45) respectively. Our results complement previous studies and support their findings of similar and more balanced sex ratios in adult and juvenile loggerhead turtles in the Mediterranean, in contrast with highly female-biased sex ratios of hatchlings.

Keywords: *Caretta caretta*; sea turtle; adult; sex ratio; maturity; temperature-dependent sex determination; Mediterranean.

Proporción de sexos para adultos de tortugas marinas (*Caretta caretta*) en dos áreas de alimentación mediterráneas

Resumen: El sexo de las tortugas marinas viene determinado por la temperatura (TSD) y la información sobre la proporción de sexos en las diferentes etapas de la vida es necesaria, tanto para los modelos de dinámica de poblaciones para su conservación como para conocer el posible valor adaptativo a TSD. Los adultos representan la clase menos abundante de las poblaciones de tortugas marinas y la proporción de sexos para adultos en las áreas de alimentación son muy difíciles de obtener. En primer lugar, analizamos los datos biométricos de 460 jóvenes y adultos de tortugas bobas marinas en que la longitud del caparazón curvado (LCC) oscilaba entre 60 y 97.5 cm, donde se observó una distribución bimodal clara de la longitud de la cola (el carácter sexual secundario principal del macho adulto) en la clase de tamaño >75 cm de CCL. A continuación, sexamos 142 tortugas adultas en esta clase de tamaño obtenida en la zona de plataforma de Túnez y desde el suroeste del mar Tirreno, observando una proporción de hembras del 51.5% (IC del 95%: 41.2 a 61.8%, n=97) y 40.0% (IC del 95%: 25.7 a 55.7%, n=45), respectivamente. Nuestros resultados complementan estudios previos y apoyan una proporción de sexos similares y más equilibradas en tortugas bobas adultas y juveniles en el Mediterráneo, en contraposición con la proporción de sexos altamente sesgadas de hembras de las crías.

Palabras clave: *Caretta caretta*; tortuga marina; adulto; proporción de sexos; madurez; determinación de sexo determinado por la temperatura; Mediterráneo.

Citation/Como citar este artículo: Casale P., Freggi D., Maffucci F., Hochscheid S. 2014. Adult sex ratios of loggerhead sea turtles (*Caretta caretta*) in two Mediterranean foraging grounds. Sci. Mar. 78(2): 303-309. doi: <http://dx.doi.org/10.3989/scimar.03988.30E>

Editor: D. Oro.

Received: December 5, 2013. **Accepted:** February 21, 2014. **Published:** May 30, 2014.

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INTRODUCTION

The sex of sea turtles is determined by the temperature to which an embryo is exposed during its develop-

ment (Wibbels 2003), as in many other reptiles (Janzen and Paulkistis 1991). In general, skewed sex ratios are more common in species with temperature-dependent sex determination (TSD) than in species with a geno-

Loggerhead turtle adult sex ratios are very difficult to obtain. Despite adults showing clear external sexual dimorphism, notably an elongated tail in males, that make them relatively easy to sex compared to hatchling or juvenile turtles (Wibbels, 2003; Casale et al. 2005), they represent by far the less abundant class in the population. They aggregate at mating sites near nesting beaches during the breeding season (e.g. Schofield et al. 2009) but sex ratios observed at these sites may not represent the actual adult sex ratio of their population because of possible different breeding periodicity of males and females (Miller 1997). More reliable adult sea turtle sex ratios can be obtained at foraging grounds where, however, they are very difficult to collect because of their low abundance.

I obtained biometric data of adult loggerhead turtles incidentally captured by fishing gear, or found stranded or floating at sea along the south-eastern Tyrrhenian Sea (between 41°13'N, 13°03' E and 30°40'N, 15°48'E) during the period 2000-2013 (N=45). Only turtles of the size class where the external sexual dimorphism was well developed (> 75 cm CCL) were considered. In order to obtain adult sex ratios, individual turtles were sexed according to the following two criteria: Alive turtles (N=12) were sexed based on the distance from the posterior margin of the carapace to the tip of the tail (carapace-tail) or the distance from the posterior margin of the carapace to the cloaca (carapace-cloaca), depending on the individual cases. These measurements were previously proposed as the best indicators of sex in adult individuals (Casale et al. 2005). In four cases, photographs were used to determine whether carapace-cloaca was >0 (the sexing threshold), i.e. whether the cloaca was internal or external with respect to the margin of carapace, which can be easily determined by eye. Dead turtles (N=33) were sexed through visual examination of the gonads during necropsies.

In order to improve the significance of the study and to provide a better description of loggerhead turtle adult sex ratio in the Mediterranean Sea, data from the south-eastern Tyrrhenian Sea were collated with those obtained by Dr. Casale from the Department of Biology and Biotechnologies “Charles Darwin”, University of Rome “La Sapienza”, and his colleagues in the waters around Lampedusa island, Italy (42°40'N; 16°50'E) in the period 1991-2012 (Figure 3.5).

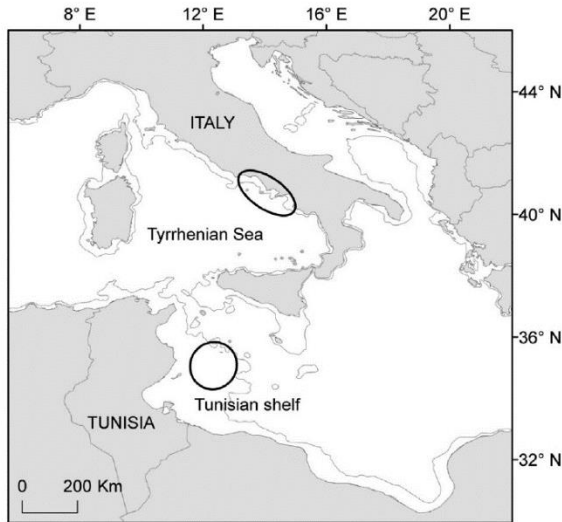


Figure 3.5. Central Mediterranean. The two neritic foraging areas where juvenile and adult loggerhead turtles were collected are approximately shown by ellipses: the southeastern Tyrrhenian and the Tunisian shelf. The 200-m isobath, conventionally indicating the continental shelf, is also shown (Adapted from Casale et al. 2014)

95% confidence intervals of sex ratios were calculated according to the method for binomial distributions (Zar 1999). Pair-wise statistical tests among known loggerhead sex ratios in the Mediterranean were conducted by Fisher exact test on 2x2 contingency tables with real numbers of individuals. Females represented 40.0% (95% CI: 25.7 – 55.7%; $n = 45$) of the adults in southeastern Tyrrhenian Sea, a proportion that was not significantly different from the 51.5% (95% CI: 41.2 - 61.8%; $n = 97$) obtained in the Lampedusa sample (Fisher exact test; $p = 0.17$; $n = 142$).

The two sex ratios observed in this study were not significantly different from the only other adult sex ratios available from Mediterranean foraging ground (Ionian Greece,) or from juvenile sex ratios in different areas, except for the adult vs. juvenile sex ratios in the Tyrrhenian Sea (Table 3.3). There is therefore a clear discrepancy between hatchling sex ratio which is female biased at most of the major nesting sites of the Mediterranean Sea and juvenile and adult sex ratio at foraging habitat that appear to be more balanced

with the latter sometimes even male biased (Table 3.3). A similar discrepancy has also been observed in the north-west Atlantic (Wibbels 2003). There are three possible explanations; 1) post-hatchling/juvenile females experience a higher mortality than males; 2) the current juvenile sex ratios are not representative of the entire population and higher female-biased sex ratios occur in foraging areas not yet investigated; 3) the current hatchling sex ratios are not representative of the entire population.

In conclusion, our results complement previous studies on loggerhead turtles in the Mediterranean and support similar and more balanced sex ratios in adults and juveniles than in hatchlings. In order to allow a better understanding of the dynamics of loggerhead turtle sex ratios in the Mediterranean Sea, it is necessary to assess juvenile and adult sex ratios at those major foraging grounds not yet investigated, such as the neritic areas in Libya, Egypt, Turkey and eastern Greece.

Table 3.3. Known sex ratios of different life stages (hatchlings, juveniles and adults) of loggerhead sea turtles in the Mediterranean. Hatchling sex ratios are from nesting beaches while juvenile and adult sex ratios are from foraging areas. Sex ratios where real numbers of males and females are available (i.e. where n is shown) were compared (Fisher exact test), and those significantly different are indicated as follows: * this sex ratio resulted different (P<0.01) in all the pairwise tests with the other values; five pairs of sex ratio values which differed with p<0.05 are indicated by five pairs of the symbols \diamond , \square , $+$, $\$$, \circ (Adapted from Casale et al. 2014)

Category	Area	Method	Proportion of females (%) (95%CI; n)	Source
Adults	Central Mediterranean	Tail length	51.5 (41.2-61.8; 97)	Casale et al. 2014
	South-East Tyrrhenian	Gonads (gross morphology)	40.0 (25.7-55.7; 45) \square	Data from the present PhD thesis, Casale et al. 2014
	Anavriklos Gulf	Tail length	43.9 (34.3-53.9; 107) $+\$$ \circ	Rees et al. (2013)
Juveniles	Italy (all marine areas)	Tail length	60.9 (48.4-72.4; 69) \circ	Casale et al. (2005)
	Central Mediterranean	Blood hormones	55.6 (41.4-69.1; 54)	Casale et al. (1998)
		Gonads (gross morphology)	54.5 (41.8-66.9; 66)	Casale et al. (2006)
	North-West Mediterranean	Gonads (gross morphology)	53.8 (43.8-63.7; 104)	Casale et al. (2006)
	South-West Adriatic	Gonads (gross morphology)	51.8 (40.6-62.9; 83)	Casale et al. (2006)
Hatchlings	North-East Adriatic	Gonads (gross morphology)	57.9 (44.1-70.9; 57)	Casale et al. (2006)
	South-East Tyrrhenian	Gonads (gross morphology)	61.0 (54.2-67.5; 218) $+\diamond$	Mafrucci et al. (2013)
	Zakynthos, Greece	Incubation duration	68-75	Zbinden et al. (2007)
	Kyparissia, Greece	Incubation duration	73.2-80.6	Katselidis et al. (2012)
	Anamur, Turkey	Incubation duration	70	Rees and Margaritoulis (2004)
		Gonads (histology)	85.2	Uyar et al. (2012)
		Gonads (histology)	75 (71.5-78.4; 637)*	Uyar et al. (2012)
	Fethiye, Turkey	Incubation duration	60.8	Kaska et al. (2006)
		Gonads (histology)	60	Kaska et al. (2006)
	Patara, Turkey	Nest temperature	64.7 (53.6-74.8; 85) $\$$ \square	Oz et al. (2004)
	Alagadi, Cyprus	Incubation duration	70.5	Godley et al. (2001)
			89-99	

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4. DEMOGRAPHIC COMPOSITION OF FORAGING AGGREGATIONS

4.1. DISTRIBUTION OF LOGGERHEAD JUVENILES IN MEDITERRANEAN FORAGING GROUNDS

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An extended version of this chapter has been published by Clusa et al. in *Marine Biology* 161: 509-519 (2013)

Fine-scale distribution of juvenile Atlantic and Mediterranean loggerhead turtles (*Caretta caretta*) in the Mediterranean Sea

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Received: 25 July 2013 / Accepted: 26 October 2013
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Abstract Loggerhead turtles nesting in the Mediterranean Sea exhibit remarkable genetic structuring. This paper tests the hypothesis that young loggerhead turtles from different rookeries do not distribute homogeneously among the major Mediterranean foraging grounds, due to a complex pattern of surface currents. We extracted long fragments of mitochondrial DNA from 275 stranded or bycaught juvenile turtles from six foraging grounds (Catalano-Balearic Sea, Algerian basin, Tyrrhenian Sea, Adriatic Sea, northern Ionian Sea and southern Levantine Sea). We used a Bayesian mixed-stock analysis to estimate the contributions from rookeries in the Mediterranean, the North-west Atlantic and Cape Verde to the studied foraging grounds. Differences were found in the relative

contribution of juvenile turtles of Atlantic and Mediterranean origin to each foraging ground. A decreasing proportion of Atlantic juveniles was detected along the main surface current entering the Mediterranean, with a high prevalence of turtles from eastern Florida in the Algerian basin and lower numbers elsewhere. In regard to the turtles of Mediterranean origin, juveniles from Libya prevailed in central and western Mediterranean foraging grounds other than the Algerian basin. Conversely, the Adriatic Sea was characterised by a large presence of individuals from western Greece, while the southern Levantine Sea was inhabited by a heterogeneous mix of turtles from the eastern Mediterranean rookeries (Turkey, Lebanon and Israel). Overall, the distribution of juveniles may be related to surface circulation patterns in the Mediterranean and suggests that fisheries might have differential effects on each population depending on the overlap degree between foraging and fishing grounds.

Communicated by T. Reusch.

Electronic supplementary material The online version of this article (doi:10.1007/s00227-013-2353-y) contains supplementary material, which is available to authorized users.

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Published online: 06 November 2013

 Springer

Sea turtles are highly migratory species that regularly undertake long distance migrations between foraging and nesting habitats that may span entire ocean basins (Bolten 2003, Plotkin 2003). Individuals from different rookeries coexist on common foraging grounds. Several factors such as rookery size, distance between rookeries and foraging grounds, juvenile natal homing behaviour, and ocean currents may play a role in shaping the mixture of turtles in these foraging aggregations (for a review see Jensen et al. 2013). Unveiling the link between nesting rookeries and foraging grounds is challenging, but it is also a fundamental component of effective management and conservation.

The loggerhead turtle (*Caretta caretta*) is the most abundant sea turtle species in the Mediterranean Sea, where juveniles of Atlantic and Mediterranean origin share common foraging habitats (Laurent et al. 1993, 1998, Bowen et al. 2003, Carreras et al. 2006). Previous studies have shown that small Atlantic individuals enter through the Strait of Gibraltar during their oceanic developmental migration and remain in the Mediterranean Sea for several years, dispersing widely within the basin (Revelles et al. 2007a, Eckert et al. 2008). Eventually, they traverse back across the Atlantic to complete their development and breed in areas closer to their site of origin, thus maintaining isolated these two genetically distinct regional management units (RMUs, Wallace et al. 2010, Carreras et al. 2011). Juvenile loggerhead turtles from the Mediterranean rookeries also disperse within the whole Mediterranean Sea but their differential contributions to the foraging aggregates off Tunisia, Libya, Egypt, Turkey, Italy or Spain are still not well known (Margaritoulis et al. 2003).

Due to the natal Phylopatry of nesting females, loggerhead turtle rookeries exhibit significant mitochondrial DNA (mtDNA) haplotype frequency shifts that can be used to infer the natal origin of turtles in feeding habitats by using Mixed Stock Analysis (MSA, Grant et al. 1980). The vast majority of previous research in the Mediterranean Sea has been based on the analysis of a ~380bp fragment of mtDNA (Laurent et al. 1998, Maffucci et al. 2006, Carreras et al. 2007, Casale et al. 2008, Carreras et al. 2011, Saied et al. 2012). However, the limited assignation power of these markers in addition to the incomplete sampling of some foraging aggregations has precluded the assessment of the contributions of these rookeries to the different Mediterranean foraging grounds. Abreu-Grobois et al. (2006) developed a new set of primers that amplify longer segments of mtDNA increasing the resolution of genetic structuring (Monzón-Argüello et al. 2010, Shamblin et al. 2012, Clusa et al. 2013). Thus, origin assignment of juveniles from

Mediterranean foraging grounds is expected to improve at both regional and fine-scale level.

In this study I sampled 29 loggerhead turtle juveniles (curved carapace length, CCL < 69 cm) foraging along the South Tyrrhenian coasts (Figure 4.1).

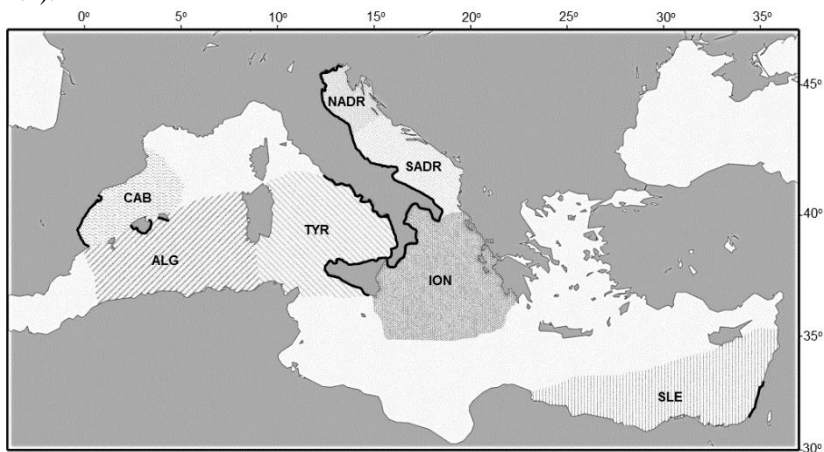


Figure 4.1. Mediterranean foraging grounds for loggerhead turtles sampled in this study: CAB (the Catalanian-Balearic basin), ALG (the Algerian basin), TYR (the Tyrrhenian basin), NADR (the northern Adriatic Sea), SADR (the southern Adriatic Sea), ION (Ionian Sea) and SLE (the southern Levantine Sea). Black lines represent surveyed coastlines (Adapted from Clusa et al. 2013)

DNA was extracted with the QIAamp extraction kit (QIAGEN®) following manufacturer's instructions. An 815 bp fragment of the mtDNA control region was amplified by polymerase chain reaction (PCR) using the primer pair LCM15382 (5'-GCTTAACCCTAAAGCATTGG-3') and H950 (5'-GTCTCGGATTTAGGGGTTT-3') (Abreu-Grobois et al. 2006) following the protocols described in Clusa et al. (2013). All samples were sequenced in both forward and reverse directions on an ABI 3730 automated DNA Analyser at the Molecular Biology Service of the Stazione Zoologica Anton Dohrn to confirm variable sites on both strands of DNA. The resulting 815 bp fragment contains the 380 bp fragment traditionally used in molecular studies on loggerhead turtles (Norman et al. 1994).

Sequences were aligned with BioEdit v7.1.6 (Hall 1999) and compared to haplotypes previously described for this species and compiled by the Archie

Carr Center for Sea Turtle Research of the University of Florida (ACCSTR; <http://accstr.ufl.edu>).

Long mtDNA haplotype frequencies of juvenile loggerhead turtles that forage in the South Tyrrhenian Sea were shared with Dr Clusa from the Department of Animal Biology and IRBio of the University of Barcelona, to be collated to those obtained, by him and his colleagues, from 7 different foraging grounds in the Mediterranean Sea (Table 4.1, Figure 4.1). The aim of this collaboration was to better understand the distribution of Atlantic and Mediterranean juveniles among different Mediterranean foraging habitats. For details on the analytical procedure see Clusa et al. (2013b).

A total of 17 different haplotypes were found in the analysed Mediterranean foraging grounds (Table 4.1), all of them described in previous studies. Overall, haplotype and nucleotide diversities in foraging areas were highly variable (h range: 0.668-0.095; π range: 0.0248-0.0001) with the Tyrrhenian basin presenting intermediate values of haplotype (0.409 ± 0.084) and nucleotide (0.0109 ± 0.0057) diversities (Table 4.1).

Highly significant genetic structuring was found between the studied foraging grounds (Global $F_{st} = 0.201$, $p < 0.001$). Because F_{st} differentiation tests showed no statistical differences between the northern and southern Adriatic Sea ($F_{st} = -0.037$, $p = 0.936$), these two foraging grounds were pooled as Adriatic Sea (ADR) for further analyses. The PCoA ordination reflected the deeper differentiation between the Algerian basin and the rest of foraging grounds. The analysis also separated the Catalano-Balearic basin and the Tyrrhenian Sea from the rest (Figure 4.2).

The stock composition of the different foraging grounds, revealed by Bayesian MSA, showed that only in the Algerian basin the Atlantic contribution was dominant ($58.4 \pm 11.2\%$). Nonetheless, Atlantic individuals could be detected in all the foraging grounds, although, at much lower proportions (Figure 4.3). The contribution of Mediterranean rookeries varied among the analysed foraging grounds (Figure 4.3). The Tyrrhenian Sea hosted mainly individuals from Libya (47.4 ± 31.3) that represented also the vast majority of juveniles foraging along the south Ionian coasts ($70.4 \pm 34.9\%$). The Adriatic Sea was inhabited mainly by western Greece individuals ($57.8 \pm 33.3\%$). Juvenile turtles from Misurata (38.6 ± 29.1) and western Greece (31.3 ± 23.7) contributed to the Catalano-Balearic foraging ground..

Table 4.1. Absolute mtDNA haplotype frequencies found in the Mediterranean foraging grounds for juvenile loggerhead turtles: CAB (the Catinian-Balearic basin), ALG (the Algerian basin), TYR (the Tyrrhenian basin), NADR (the northern Adriatic Sea), SADR (the southern Adriatic Sea), ION (Ionian Sea) and SLE (the southern Levantine Sea). Total number of haplotypes (n), haplotype diversity (h) and nucleotide diversity (π) found in each foraging ground included at the bottom of the table. Mean standard deviations included (\pm) (Adapted from Clusa et al. 2013).

	CAB	ALG	TYR	NADR	SADR	ION	SLE
CC-A1.1	2	21	5				
CC-A1.3	1	2	1				1
CC-A2.1	30	31	39	26	20	21	28
CC-A2.8						1	
CC-A2.9	2	4	1			5	
CC-A3.1	2	4	2	2	1	5	3
CC-A5.1	1						
CC-A6.1				1			
CC-A10.3						1	
CC-A10.4							1
CC-A14.1	1	3					
CC-A20.1			2				
CC-A28.1						1	
CC-A29.1							1
CC-A31.1			1				
CC-A32.1	1						
CC-A55.1							
n	40	65	51	29	21	35	34
h	0.439 \pm 0.098	0.668 \pm 0.041	0.409 \pm 0.084	0.197 \pm 0.095	0.095 \pm 0.084	0.613 \pm 0.083	0.321 \pm 0.101
π	0.0095 \pm 0.0050	0.0248 \pm 0.0123	0.0109 \pm 0.0057	0.0002 \pm 0.0004	0.0001 \pm 0.0002	0.0010 \pm 0.0008	0.0033 \pm 0.0020

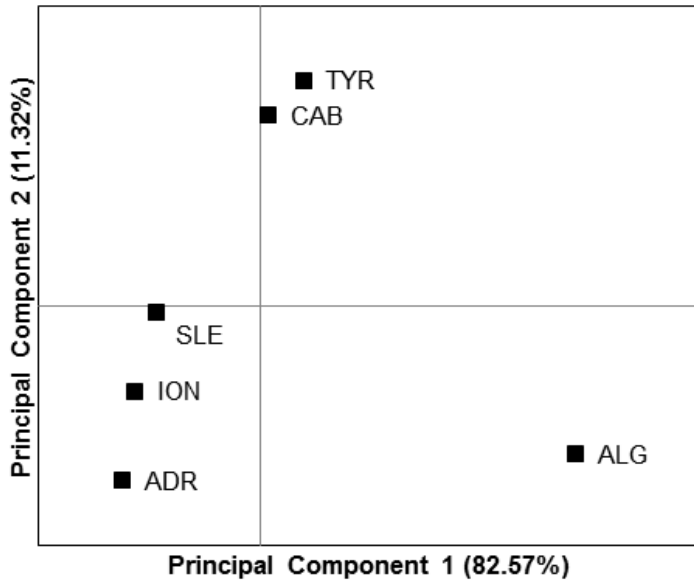


Figure 4.2. Principal Coordinate Analysis based on genetic distances (FST) between Mediterranean foraging grounds for juvenile loggerhead turtles. Percentage of variation explained by each coordinate included in brackets in the figure axes. CAB (the Catalanian-Balearic basin), ALG (the Algerian basin), TYR (the Tyrrhenian basin), ADR (Adriatic Sea), ION (Ionian Sea) and SLE (the southern Levantine Sea) (Adapted from Clusa et al. 2013).

Finally, the southern Levantine Sea showed a high proportion of individuals from the easternmost rookeries in the Mediterranean Sea: Israel, Lebanon and Turkey (Figure 4.3).

Overall, our results provided a better understanding of the distributions of Atlantic and Mediterranean juvenile turtles within the Mediterranean Sea both at a regional and fine-scale level and showed the complexity of loggerhead turtle migratory patterns with marked differences among populations belonging to the same RMU.

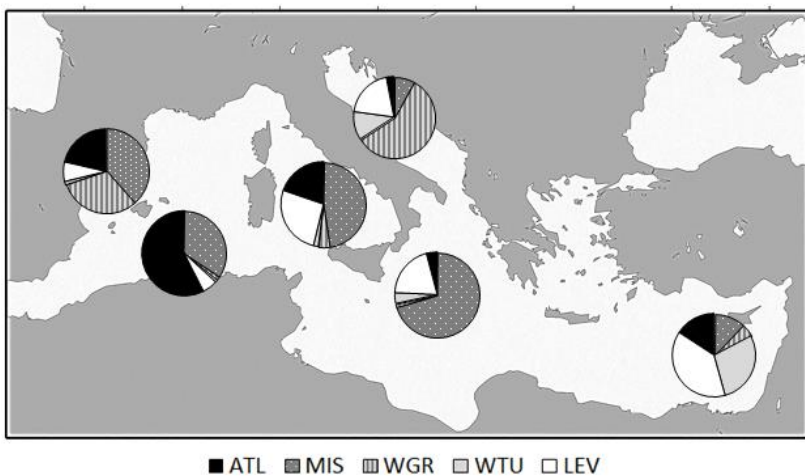


Figure 4.3. Relevant fine-scale rookery contributions (%) to Mediterranean foraging grounds estimated by MSA. Rookeries: ATL (Atlantic rookeries), MIS (Misurata), WGR (western Greece), WTU (western Turkey), LEV (other Levantine rookeries) (Adapted from Clusa et al. 2013).

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4.2. CONNECTIVITY AND STOCK COMPOSITION OF LOGGERHEAD TURTLES FORAGING ON THE NORTH AFRICAN CONTINENTAL SHELF (CENTRAL MEDITERRANEAN): IMPLICATIONS FOR CONSERVATION AND MANAGEMENT.

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This chapter has been accepted for publication in *Marine Ecology* (acceptance letter received on the 29 December 2015).

Abstract

The loggerhead turtle is a highly migratory species with a complex life cycle that involves a series of ontogenetic habitat shifts and migrations. Understanding the linking among nesting populations and foraging habitats is essential for the effective management of the species. Here we used mixed stock analysis to examine the natal origin of loggerhead turtles foraging on the North African continental shelf off Tunisia, one of the most important Mediterranean neritic habitats. An 815bp fragment of the mitochondrial DNA control region was sequenced from 107 individuals sampled from 2007 to 2009. No temporal variation in haplotype frequencies was detected. Juveniles (N = 87) and adults (N = 23) exhibited weak but significant genetic differentiation that resulted in different stock compositions. Libya was the main source population but the proportion of turtles from this rookery was higher in adults (median = 80%) than in juveniles (median = 35%). Western Greece was the second most important contributing population. Juvenile stock composition derived from mixed stock analysis and the estimates produced by numerical simulation of hatchling dispersion in the Mediterranean Sea were significantly correlated, supporting the recent theory that loggerheads imprint on possible future neritic habitats during the initial phase of their life. This association was not significant for adults suggesting that other factors contribute to shape their distribution. Overall, our results showed that human activities on the south Tunisian continental shelf pose an immediate threat to the survival of the Libyan rookery.



Connectivity and stock composition of loggerhead turtles foraging on the North African continental shelf (central Mediterranean): implications for conservation and management.

Journal:	<i>Marine Ecology</i>
Manuscript ID	MAE-2223.R1
Manuscript Type:	Regular Paper
Date Submitted by the Author:	12-Jan-2016
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Keywords:	connectivity, genetic diversity, mtDNA, mixed stock analysis, feeding ground, loggerhead turtle

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Introduction

Protecting highly migratory species presents a unique conservation challenge because populations are typically comprised of a complex system of interconnected subpopulations that use widely separated and ecologically disparate habitats (Webster et al. 2002, Martin et al. 2007, Taylor & Norris 2010). Stress placed at one location detrimentally affect population levels at many distant areas (Watson et al. 2011). Recognising the natal origin of individuals at non breeding habitats and understanding the factors that shape population connectivity, i.e. the geographic linking of individuals or populations between stages of an animal's life cycle, is crucial for our ability to manage these species effectively (Martin et al. 2007, Bjørndal & Bolten 2008, Taylor & Norris 2010). Despite its importance, connectivity in marine systems is still not completely understood, particularly in those species with elusive behaviours and complex life cycles that comprise ontogenetic shifts in habitat utilization (Thomas & Bell 2013).

The endangered loggerhead turtle, *Caretta caretta* (Linnaeus, 1758), is one of the best known examples of such species. After leaving their nesting beach, post-hatchling turtles drift passively with ocean currents dispersing over vast oceanic areas where they grow for several years before recruiting to neritic foraging habitats to complete their development (Bowen et al. 2004). The now larger juveniles are skilled swimmers that may show strong fidelity to a chosen foraging area or move among different habitats and even return to the oceanic environment on shorter time scales (McClellan & Read 2007, Casale et al. 2008a). Upon reaching maturity, adults undertake periodic reproductive migrations from foraging grounds to breeding areas that are often hundreds or even thousands of kilometres away, with females that exhibit strong phylopatriy to their natal beach (Bowen et al. 2005). Post-breeding turtles migrate back to their foraging habitat to which they show a high level of fidelity (Broderick et al. 2007).

Understanding how juveniles and adults select a specific neritic foraging habitat is complex and several factors have been suggested to affect this process such as juvenile natal homing, distance between rookeries and foraging sites, and ocean currents (for a review, see Jensen et al 2013). Recently it has been proposed that juveniles and adults recruit to favourable neritic areas that they experienced as drifting hatchlings during the initial phase of their oceanic developmental stage (Hays et al. 2010, Scott et al. 2014). Evidence to support this hypothesis can be provided comparing information from numerical simulation of hatchling dispersal with data on the demographic composition of juvenile and adult neritic habitats obtained

through molecular techniques (Jensen et al. 2013). In particular, maternally inherited mitochondrial DNA (mtDNA) has been widely employed to elucidate links between loggerhead turtle nesting populations and distant foraging grounds (for a review see Jensen et al. 2013). The Phylopatry of females enhances in fact the formation of population structure by reducing the gene flow among geographically separated nesting beaches. These distinct Management Units (MUs, *sensu* Moritz 1994) are characterized by significant differences in mtDNA haplotype frequencies that can be used in mixed stock analysis (MSA) to estimate the demographic composition of foraging aggregations (Pella & Masuda 2001, Bolker et al. 2007). The ability of MSA to assign individuals to their rookery of origin has long been limited by the low resolution power of the 380-bp mtDNA fragment commonly employed as genetic marker (for a review, see Jensen et al. 2013). Recently the analysis of an expanded region of the mtDNA resulted into a significant increase of the genetic structure among loggerhead turtle rookeries (Saied et al. 2012, Clusa et al. 2013a, Garofalo et al. 2013, Garofalo et al. 2009, Shamblin et al. 2012, Yilmaz et al. 2011) and thereby of the resolution power of MSA (Clusa et al. 2013b, LaCasella et al. 2013).

In the Mediterranean Sea, the wide African continental shelf off Tunisia is one of the most important neritic foraging habitats for loggerhead turtle juveniles and adults (Casale & Margaritoulis 2010, Margaritoulis et al. 2003). This is the second widest continental shelf area in the region and is characterized by favorable geomorphological, climatic and oceanographic conditions that support one of the most productive ecosystems in the Mediterranean Sea (Hattab et al. 2013). The area has been intensively exploited since the early 1980s (Hattab et al. 2013) and currently supports 60% of the Tunisian fleet operations and contribute 42% of the national annual fish production (DPGA 2007). Due to its high productivity, the region attracts also fleets from other countries predominated by industrial and semi industrial scale Italian bottom trawlers (Papaconstantinou & Farrugio 2000). More than 10.000 loggerhead turtles are accidentally caught each year (Casale et al. 2007, Jribi et al. 2007, Jribi et al. 2008, Echwikhi et al. 2012) which is indicative of a strong conservation issue for the species even if we possess only rough estimates of the actual fisheries induced mortality (Casale 2011). Knowledge of the demographic composition of this aggregation is thus essential for quantitative predictions of population level impacts of the anthropogenic threats, as well as for designing effective conservations strategies of the species in the Mediterranean Sea (Hamann et al. 2010).

Recently, Casale and Mariani (2014) using numerical simulation of hatchling dispersal, proposed a general distribution model of Mediterranean loggerhead turtles that predicted a significant difference in the demographic composition of neritic foraging habitats in the eastern basin. Under this model the continental shelf off the south Tunisian coasts would be inhabited mainly by individuals from the Libyan MU with lower contributions from western Greece and Turkey (Casale & Mariani 2014). Moreover, stock composition of this foraging aggregation was predicted to be clearly differentiated from that of the Adriatic Sea, the only other major neritic habitat in the eastern Mediterranean for which haplotype frequencies of the expanded mtDNA sequence are already available in the literature (Clusa et al. 2013b).

In this study, we assessed the natal origin of loggerhead turtles that forage on the continental shelf off the south coasts of Tunisia using sequence data of the expanded mtDNA fragment and hierarchical Bayesian mixed stock analysis (Bolker et al. 2007). Juveniles and adults were analysed separately in order to evaluate potential differences in the demographic composition of these foraging cohorts. Stock compositions derived by the MSAs were compared to previous estimates from numerical simulation of hatchling dispersion in the Mediterranean Sea in order to evaluate the potential role of hatchling “experience” in shaping the demographic structure of loggerhead turtle neritic foraging aggregations.

Materials and Methods

Sample collection.

Tissue samples from 107 loggerhead turtles stranded dead ($n = 94$) or accidentally caught by bottom trawler ($n = 13$) on the African continental shelf off south Tunisia (hereafter STUN, Figure 4.4) were collected between 2007 and 2009 by biologists of the National Stranding Network of cetaceans and marine turtles as part of the ongoing monitoring project of the loggerhead turtle presence in Tunisia (Bradai et al. 2008). Each individual was measured using a flexible meter to the nearest 0.5 cm (standard curved carapace length, CCL_{st}) and a sample of skin or pectoral muscle was taken and stored in 95% ethanol.

Laboratory procedures.

Details of DNA extraction, amplification and sequencing are available in Saied et al. (2012). In brief, a 815 bp fragment of the mitochondrial DNA was amplified using primers LCM15382 and H950 (Abreu-Grobois et al. 2006). This sequence completely encompasses the 380-bp fragment previously used

as reference to define mtDNA haplotypes in this species (<http://accstr.ufl.edu/ccmtdna.html>). PCR products were sequenced in both directions on an Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Thermo fisher/Applied Biosystems) with BigDye Terminator Direct Cycle Sequencing Kit and POP-7™ polymer. For each individual, forward and reverse chromatograms were assembled using the software package SeqManII (DNASTAR Inc.) in order to confirm variable positions. Resulting sequences were aligned using the Bioedit Sequence Alignment Editor 7.0.9.0 (Hall 1999) and classified according to the online haplotype registry maintained by the Archie Carr Center for Sea Turtle Research of the University of Florida (ACCSTR, <http://accstr.ufl.edu/cclongmtdna.html>).

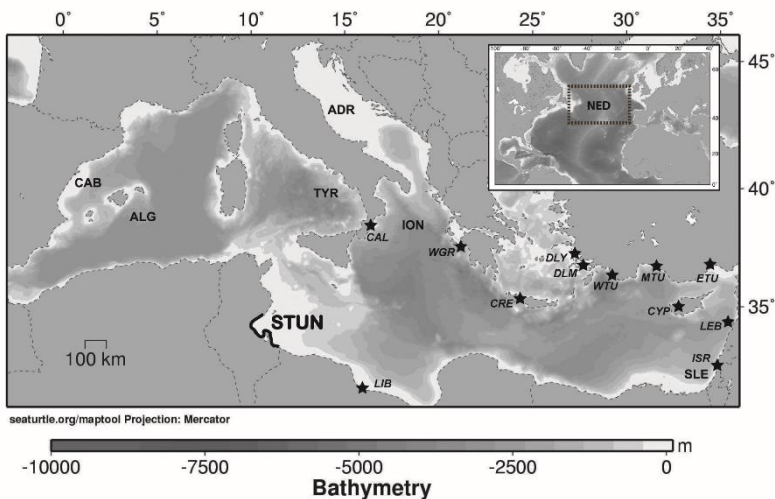


Figure 4.4. Locations of the loggerhead turtle juvenile foraging grounds considered in the present study. Bathymetry is shown in grayscale. Solid black line indicates approximately the sampling area. Foraging ground abbreviations are: south Tunisia (STUN), Catalano-Baleaeric Sea (CAB), Algerian basin (ALG), Tyrrhenian Sea (TYR), Adriatic Sea (ADR), northern Ionian Sea (ION), southern Levantine Sea (SLE) and North Atlantic Northeast Distant region (NED). Stars indicate the Mediterranean nesting populations considered in the many-to many mixed stock analysis: Libya (LIB), Calabria (CAL), western Greece (WGR), Crete (CRE), Dalaman (DLM), Dalyan (DLY), western Turkey (WTU), middle Turkey (MTU), eastern Turkey (ETU), Cyprus (CYP), Lebanon (LEB), Israel (ISR).

Data analysis.

Average turtle sizes in the three sampling years were compared using a one-way ANOVA in Minitab 15 (Minitab inc.).

Temporal variation in haplotype frequencies was verified using haplotype frequency-based AMOVA with significance values of fixation indices obtained with 10000 permutations and the exact test of population differentiation (Raymond & Rousset 1995), computed with 100000 steps in the Markov Chain with 10000 dememorization steps.

The sample was divided in two groups, juveniles (STUN_{juv}) and adults (STUN_{ad}), using the threshold of 69 cm CCL_{st}, corresponding to the average minimum size of nesting females in the Mediterranean Sea, which has been recently adopted by Clusa et al. (2013b) to investigate the stock structure of loggerhead turtle juvenile foraging grounds in the Mediterranean Sea. Genetic differentiation between the two groups was verified by exact test of population differentiation (Raymond & Rousset 1995), computed with 100000 steps in the Markov Chain with 10000 dememorization steps, and pairwise F_{st} (Excoffier et al. 1992). Haplotype (h) and nucleotide (π) diversities were calculated for each group and for the entire dataset.

Demographic composition of adult turtles

The demographic composition of STUN_{ad} was estimated by using a Bayesian many-to-one MSA as implemented in the computer program BAYES (Pella & Masuda 2001) because, to our knowledge, there are no information on the 815 mtDNA haplotype frequencies of loggerhead adult foraging aggregations. Published data from 12 Mediterranean rookeries (Garofalo et al. 2009, Yilmaz et al. 2011, Saied et al. 2012, Clusa et al. 2013a) and 9 Atlantic rookeries (Monzon-Arguello et al. 2010a, Shamblin et al. 2012) that have been analysed in previous studies were used as baseline for the MSA: Libya, Calabria, western Greece, Crete, Cyprus, Lebanon, Israel, Dalyan, Dalam, western Turkey, middle Turkey, eastern Turkey, northern USA, central eastern Florida, southeastern Florida, Dry Tortugas, Mexico, southwestern Florida, central western Florida, northwestern Florida, Cape Verde. The average annual nest counts derived from the literature was used as a proxy of rookery sizes to weight priors assuming that the overall contribution of a rookery is proportional to its size (Bass et al. 2004). The incorporation of such ecological covariates have been shown to strengthen sea turtle mixed stock analysis results (Okuyama & Bolker 2005). A total of 20000 Markov Chain Monte Carlo steps were run for 21 chains started with 95% of the mixed sample initially contributed by each source population and

the remaining 5% was divided equally among the remaining populations. Convergence of chains was considered reached when the Gelman-Rubin criterion was < 1.2 .

Demographic composition of juvenile turtles

Haplotype frequencies for STUN_{juv} were compiled with published data from 6 Mediterranean and 1 Atlantic juvenile foraging grounds for which information on the longer sequence were available at the present (Clusa et al. 2013b, LaCasella et al. 2013): the Catalano-Balearic Sea, the Algerian basin, the Tyrrhenian Sea, the Adriatic Sea, the northern Ionian Sea, the southern Levantine Sea and the North Atlantic Northeast Distant region (Figure 4.4). Genetic partitioning among juvenile foraging grounds was examined using haplotype frequency-based AMOVA with significance values of fixation indices obtained with 10,000 permutations. Genetic differentiation between pairs of juvenile foraging grounds was verified with the exact test of population differentiation, computed with 100000 steps in the Markov Chain with 10000 dememorization steps, and F_{st} (Excoffier et al. 1992), computed with 10,000 permutations. Multiple comparison was accounted for by applying the modified false discovery rate (FDR) threshold (Narum 2006). All statistical analyses were carried out using the statistical software package ARLEQUIN v.3.5.1.2 (Excoffier & Lischer 2010).

Relationships among juveniles foraging grounds were projected in two dimensions by nonmetric multidimensional scaling (MDS) based on pairwise F_{st} values, performed using the *metaMDS* function in the *vegan* package of R v. 3.0.3 (R core Development Team). The MDS is an ordination procedure that depicts complex relationships among statistical properties of samples, such as their genetic relationships (Lessa 1990). The degree of correspondence between the distances among samples implied by MDS and the genetic distance matrix input is measured inversely by a stress function. Stress values range from 0 to 1 where 0 means that the MDS map perfectly reproduces the input data.

A Bayesian many-to-many MSA (Bolker et al. 2007) was used to assess the demographic composition of each juvenile foraging ground. This method allows the analysis of a meta-population made up of multiple sources and multiple mixed stocks, thus providing the opportunity to compare directly the demographic composition of several foraging assemblages (Bolker et al. 2007). The baseline sample was the same utilised in the MSAs for the adults. The Markov chain Monte Carlo (MCMC) method was used to obtain the posterior distributions of the parameters of interest. Four chains of 20000

iterations were run in each analysis from over-dispersed, randomly selected starting points with a burn-in of 10,000 runs. The Gelman-Rubin criterion was < 1.2 for all variables indicating the convergence of MCMC. These MSAs were performed using the `mm.wbugs` function in the `mixstock` package of R v. 3.0.3 (R core Development Team).

Correlation between stock compositions from mixed stock analysis and hatchling drift model.

To test the recently proposed paradigm that hatchling dispersal patterns affect the future distribution of juveniles and adults we compared the demographic composition of foraging aggregations obtained via MSA with patterns of particle distribution produced by hatchling drifting model in the eastern Mediterranean Sea (details of the methods in Casale & Mariani 2014). For the juvenile cohort the stock composition determined via many-to-many MSA for the Adriatic Sea was incorporated into the analysis to test possible difference between the two most important neritic foraging grounds in the region. Only MSA estimates for the potential source populations utilised also in the hatchling drifting model were considered and adjusted consequently in order to reach a total contribution of 100%. Relationship between genetic and particle profiles were investigated with major axis regression in the `lmodel2` package of R v. 3.0.3 (R core Development Team), regressing arcsine transformed proportions, with a p-value based on 1000 permutations.

Results

Genetic diversity

There was no significant variation in mean turtle size among the three sampling years (mean \pm SD; CCLst₂₀₀₇ = 61 ± 11 cm; CCLst₂₀₀₈ = 61.5 ± 10 cm; CCLst₂₀₀₉ = 58.5 ± 8 cm; $F = 0.93$, $df = 2$, $p > 0.05$).

Haplotype frequencies did not vary significantly among years (AMOVA $F_{st} = 0.0065$, $df = 2$, $p > 0.05$, exact test $p > 0.05$ in all pairwise comparisons); therefore all samples were pooled for the subsequent analysis.

Overall seven distinct haplotypes were detected based on the long sequence alignment which corresponded to five variants of the shorter fragment previously used as reference to define mtDNA haplotypes in this species. The increased reading frame allowed to split the most frequent haplotype CCA-2 (83%) into two additional variants, CCA-2.1 (58%) and CCA-2.9 (25%) and to further differentiate CC-A1 (5%) to CC-A1.1 (3%), CCA1.4 (2%). Only one variant was observed for the remaining three haplotypes: CC-A3.1 (8%), CC-A26.1 (3%) and CC-A32.1 (1%) (Table 4.2).

Table 4.2. MtDNA haplotype compositions, haplotype (h) and nucleotide (π) diversities detected in South Tunisia for the whole group (STUN_{tot}), juveniles (STUN_{juv}) and adults (STUN_{ad}) are shown. Results considering only the 380 bp fragment are reported in italics.

	STUN _{juv}	STUN _{ad}	STUN _{tot}
<i>Short hapl.</i>			
<i>CC-A1</i>	<i>4</i>	<i>1</i>	<i>5</i>
CC-A1.1	3	0	3
CC-A1.4	1	1	2
<i>CC-A2</i>	<i>69</i>	<i>20</i>	<i>89</i>
CC-A2.1	52	10	62
CC-A2.9	17	10	27
<i>CC-A3</i>	<i>8</i>	<i>1</i>	<i>9</i>
CC-A3.1	8	1	9
<i>CC-A26</i>	<i>3</i>	<i>0</i>	<i>3</i>
CC-A26.1	3	0	3
<i>CC-A32</i>	<i>0</i>	<i>1</i>	<i>1</i>
CC-A32.1	0	1	1
<i>h_{short}</i>	<i>0.316±0.063</i>	<i>0.245±0.112</i>	<i>0.301± 0.056</i>
<i>π_{short}</i>	<i>0.007±0.004</i>	<i>0.006±0.004</i>	<i>0.006±0.004</i>
h_{long}	0.571±0.052	0.644±0.062	0.597±0.041
π_{long}	0.005±0.003	0.005±0.003	0.005±0.003

All haplotypes have been found on loggerhead turtle nesting beaches described in previous studies. Three haplotypes are exclusive to Mediterranean rookeries (CC-A2.9, CC-A26.1 and CC-A32.1), two are

found only in Atlantic rookeries (CC-A1.1 and CC-A1.4) and two are geographically widespread and shared between Atlantic and Mediterranean rookeries (CC-A2.1 and CC-A3.1). The absence of orphan haplotypes, i.e. sequences observed in mixed stocks but not at nesting beaches, was indicative of the adequate sampling of the potential source populations contributing to the study area.

The sample was composed of 84 juveniles (mean $CCL_{st} \pm SD = 57 \pm 7$ cm) and 23 adults (mean $CCL_{st} \pm SD = 75 \pm 4$ cm). F_{st} test revealed significant genetic differentiation between the two groups ($F_{st} = 0.051$, $p < 0.05$) that was not detect by the exact test of population differentiation (exact $p > 0.05$). Genetic diversity indices were comparable between adults and juveniles (Table 4.2) and among the highest reported for Mediterranean loggerhead turtle foraging grounds.

Table 4.3. Genetic differentiation between juvenile loggerhead turtle foraging grounds obtained with the long mtDNA sequence. Above the diagonal are the Exact test of population differentiation values, below the diagonal are the F_{st} values (in bold are significant values after FDR correction, $\alpha = 0.013$). Juvenile foraging aggregation abbreviations are given in Fig 1

	STUN _{juv}	CAB	ALG	TYR	ADR	ION	SLE	NED
		0.031±	0.000±	0.001±	0.000±	0.000±	0.003±	0.000±
STUN _{juv}		0.004	0.000	0.000	0.000	0.000	0.001	0.000
			0.008±	0.683±	0.064±	0.008±	0.636±	0.000±
CAB	0.02493		0.001	0.006	0.004	0.002	0.005	0.000
				0.003±	0.000±	0.000±	0.000±	0.038±
ALG	0.08337	0.09901		0.001	0.000	0.000	0.000	0.006
					0.035±	0.000±	0.180±	0.000±
TYR	0.04608	-0.01374	0.09801		0.003	0.000	0.006	0.000
						0.000±	0.242±	0.000±
ADR	0.12854	0.04174	0.24744	0.04413		0.000	0.009	0.000
							0.001±	0.000±
ION	0.01331	0.03206	0.08987	0.05361	0.16395		0.000	0.000
								0.000±
SLE	0.06323	-0.00711	0.15924	0.00169	0.00499	0.07601		0.000
NED	0.16021	0.17244	0.01318	0.16318	0.27623	0.1616	0.22273	

Demographic composition of adult turtles

MSA indicated that the adults sample was composed primarily of turtles from the Libyan rookery (median = 80%, Q1 = 51%, Q3 = 97%). Only one additional source population, western Greece, exhibited a contribution that departed significantly from 0 (median = 8%, Q1 = 0%, Q3 = 36%). The relative contribution of each potential source population is reported in Table 4.4.

Table 4.4. Stock composition of on the North African continental shelf off south Tunisia estimated by Bayesian MSA. Source populations are : Libya (LIB), Calabria (CAL), western Greece (WGR), Crete (CRE), Cyprus (CYP), Lebanon (LEB), Israel (ISR), Dalyan (DLY), Dalam (DLM), western Turkey (WTU), middle Turkey (MTU), eastern Turkey (ETU), northern US (NUS), central eastern Florida (CEFL), southeastern Florida (SEFL), Dry Tortugas (DRT), Mexico (MEX), southwestern Florida (SWFL), central western Florida (CWFL), Cape Verde (CV).

		STUN_{juv}		STUN_{ad}	
		mean±sd	median(2.5%-97.5%)	mean±SD	median(2.5%-97.5%)
Mediterranean Source Populations	LIB	35.3±7.0	34.8 (22.3-49.4)	78.6±12.1	80.1(51.3- 96.9)
	CAL	0.2±0.2	0.1 (0.0-0.6)	0.0±0.1	0.0(0.0- 0.0)
	WGR	8.5±7.0	6.7 (0.2-26.8)	10.7±10.1	8.1(0.0- 35.7)
	CRE	2.4±2.4	1.6 (0.1-8.7)	0.1±1.0	0.0(0.0- 0.9)
	CYP	5.7±4.9	4.3 (0.2-17.6)	0.2±1.4	0.0(0.0- 1.7)
	LEB	0.8±0.8	0.6 (0.0-3.1)	0.0±0.6	0.0(0.0- 0.0)
	ISR	0.7±0.6	0.5 (0.0-2.5)	0.0±0.8	0.0(0.0- 0.0)
	DLY	3.0±2.5	2.3 (0.1-9.3)	0.1±1.1	0.0(0.0- 0.1)
	DLM	1.4±1.2	1.1 (0.0-4.5)	0.0±0.4	0.0(0.0- 0.0)
	WTU	7.6±6.1	6.2 (0.3-22.6)	0.3±1.9	0.0(0.0- 3.0)
	MTU	5.9±5.2	4.5 (0.1-19.4)	0.3±1.8	0.0(0.0- 2.7)
	ETU	6.7±5.7	5.2 (0.3-21.1)	0.3±1.8	0.0(0.0- 3.0)
Atlantic Source Populations	NUS	2.0±1.8	1.5 (0.0-6.8)	0.1±0.6	0.0(0.0- 1.0)
	CEFL	3.1±2.9	2.4 (0.1-10.5)	2.8±4.3	0.9(0.0- 15.2)
	SEFL	5.5±5.3	4.1 (0.2-18.7)	2.2±5.1	0.0(0.0- 18.2)
	DRT	1.8±1.6	1.4 (0.1-6.0)	0.0±0.7	0.0(0.0- 0.0)
	MEX	2.7±2.5	1.9 (0.1-9.7)	0.9±2.8	0.0(0.0- 9.8)
	SWFL	1.3±1.2	0.9 (0.0-4.5)	0.0±0.3	0.0(0.0- 0.0)
	CWFL	2.7±2.4	2.1 (0.1-8.8)	0.1±0.9	0.0(0.0- 0.8)
	NWFL	1.3±1.2	0.9 (0.0-4.4)	0.0±0.4	0.0(0.0- 0.0)
	CV	1.4±1.3	1.0 (0.0-5.4)	3.2±4.1	1.6(0.0- 14.6)

Demographic composition of juvenile turtles

Results of AMOVA showed significant overall differentiation among the eight juvenile aggregations considered in the analysis ($F_{st} = 0.152$, $df = 7$, $p < 0.001$). After FDR correction, exact tests of population differentiation between juvenile foraging aggregations were significant for 20 out of 28 pairwise comparisons (Table 4.3). Using the same correction method 17 F_{st} comparisons were significant (Table 4.3).

The MDS two dimensional plotting of the F_{st} values showed a tight association between the North Atlantic Northeast Distant region and Algerian samples that were clearly separated from all other foraging grounds (Figure 4.5). Within this second cluster, $STUN_{juv}$ was markedly distant from the Adriatic Sea (Figure 4.5). The low stress value (0.02) indicates a good fit of the data.

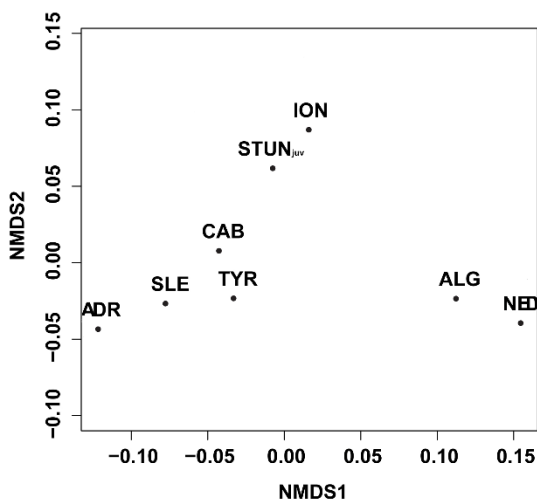


Figure 4.5. Nonmetric Multidimensional Scaling (NMDS) based on the genetic distances (F_{st}) between loggerhead turtle juvenile foraging grounds.

Many-to-many MSA results confirmed the differentiation between the $STUN_{juv}$ and the Adriatic foraging aggregations. Both were composed almost exclusively by juveniles of a Mediterranean origin (overall Mediterranean median contribution $> 80\%$) but $STUN_{juv}$ was frequented mostly by juveniles

from Libya (median = 35%, Q1 = 22%, Q3 = 49%) while the Adriatic Sea hosted individuals mainly from western Greece (median = 41%, Q1 = 16%, Q3 = 63%).

Correlation between stock compositions from mixed stock analysis and hatchling drift model.

Association between hatchling proportions obtained via ocean modelling and stock composition estimated by MSA was significant in both juvenile cohorts (STUN_{juv} $p < 0.01$; Adriatic Sea $p < 0.05$) but not in the adult group ($p > 0.05$).

Discussion

Haplotype frequencies: temporal stability and effect of turtle size

Loggerhead turtle foraging grounds are dynamic systems whose composition is influenced by the combined effect of recruitment, mortality and emigration of individuals from different rookeries (Bjorndal & Bolten 2008, Jensen et al. 2013). The stability of mean turtle size across years suggests that turtles move into and out of the study area at relatively consistent sizes although there is very limited information on the residence time of individuals at this neritic foraging habitat (Casale et al. 2012). Despite the increase in gene diversity (Table 1) with respect to previous studies based on the analyses of the 380-bp mtDNA fragment (Chaieb et al. 2012), we also found no significant temporal structuring in haplotype frequency. This is probably the result of the integration of many years of hatchling production that occurs at this foraging location. Based on the size range of the loggerhead turtles analysed (CCL_{st} 36–81.5 cm) and the growth rates recently estimated for the Mediterranean population (Piovano et al. 2011), the STUN sample is composed by at least 25 year classes which obviously reduces the potential effect of the genetic heterogeneity of a single year cohort (Bjorndal & Bolten 2008, Jensen et al. 2013). So far, temporal variation has been detected only for immature green turtles foraging in southern Bahamas over a twelve years period (Bjorndal & Bolten 2008). Rapid turnover of the foraging individuals and long-time series may be therefore essential to identify temporal variation in the stock structure, given the resolution power of the genetic marker currently employed (Bjorndal & Bolten 2008, Jensen et al. 2013).

The wide size range of the loggerhead turtles analysed here confirm also that the continental shelf off the South Tunisian coasts is an important neritic habitat for both juveniles and adults. The co-occurrence of these different life stages should always be considered in the analysis of loggerhead turtle stock

composition because their distribution may be influenced by different combinations of intrinsic and extrinsic forces such as sea surface currents, natal homing and reproductive migratory behaviour (Bowen et al. 2004, Carreras et al. 2006, Clusa et al. 2013b, Jensen et al. 2013). Due to the absence of information on maturity or breeding status of the sampled individuals, juveniles were identified using as size based threshold following Clusa et al. (2013b). This method has obvious limitations since large immature individuals may be incorrectly classified as adults, an error that is more likely to be committed with loggerhead turtles of Atlantic origin which attain sexual maturity at a much bigger size than Mediterranean individuals (Piovano et al. 2011). This may be the case of the single loggerhead turtle (77.5 cm CCL_{st}) carrying the endemic Atlantic haplotype CC-A1.4 found in the adult group. Despite this caveat, we reported evidence of weak but significant difference in haplotype frequencies between the adult and juvenile cohorts driven mainly by the shift in the proportion of the Mediterranean haplotype CC-A2.9 (respectively, 43% and 20%) which is endemic of Libya and Israel (Saied et al. 2012, Clusa et al. 2013a). It is essential to continue monitoring the genetic profile of this foraging aggregation in order to better understand age class variation in haplotype frequencies.

Demographic compositions of adult and juvenile cohorts.

To the best of the authors' knowledge, this is the first attempt to investigate the natal origin of foraging adults in the Mediterranean Sea. Sample size was small but similar numbers have been recently utilised also in studies on the demographic composition of loggerhead turtle juveniles in the Mediterranean Sea (Clusa et al. 2013b, Garofalo et al. 2013). Moreover, adults are by far the smallest portion of the population, which make their sampling at sea extremely difficult.

Adults foraging on the continental shelf off the south Tunisian coasts were almost exclusively of a Mediterranean origin. None of the Atlantic rookeries contributed significantly above zero (Table 4.4) which is coherent with the strong natal homing behaviour previously suggested for Atlantic juveniles that leave the Mediterranean Sea as they approach adulthood to reproduce in areas closer to their natal region (Encalada et al. 1998, Carreras et al. 2007, Carreras et al. 2011, Clusa et al. 2013a).

The vast majority of the individuals was assigned to the nearby Libyan population with Western Greece being the only other Mediterranean MU contributing significantly to the adult cohort (Table 4.4). This strong connectivity with the Libyan population is also supported by results of stable

isotope analyses (Cardona et al. 2014) and satellite tracking of post-nesting females from Libya showing that all individuals migrate toward this neritic foraging habitat at the end of the nesting season, swimming along the coasts (Hochscheid et al. 2012). The null contribution from the other major Mediterranean MUs in Turkey, Cyprus and Crete deserve further investigation. Previous studies showed that although adults from these rookeries exhibit a disparate dispersal pattern and may preferentially forage in other areas, a portion of them migrates also to the south Tunisian continental shelf (Margaritoulis & Rees 2011, Cardona et al. 2014, Luschi & Casale 2014). Probably the limited sample size affected the resolution power of the many-to-one MSA reducing its ability to distinguish relative small contributions.

The juvenile group was composed mostly by individuals above 40 cm of CCL_{st} (96%) that have already performed the transition to the neritic stage although they may occasionally return to an oceanic life style on shorter time scales (Casale et al. 2008a, TEWG 2009). The NMDS analysis based on the F_{st} distance depicted a clear pattern of genetic structuring among the juvenile foraging grounds for which data on the expanded mtDNA sequence were available in the literature (Figure 4.5). The North Atlantic Northeast Distant region clustered with the Algerian Sea and both were widely differentiated from all other analysed locations (Table 4.3, Figure 4.5). Within this second cluster the continental shelf off the South Tunisian coasts and the Adriatic Sea, the only two neritic habitats in the eastern Mediterranean sharing similar geomorphology, trophic structure and functioning (Hattab et al. 2013), occupied opposite positions (Table 4.3, Figure 4.5).

Results of the many-to-many MSA suggested that, contrary to the adult cohort, a proportion of juveniles derived from Atlantic rookeries (Table 4.4). These individuals probably are exploiting the nearby oceanic foraging habitat in the Ionian Sea and occasionally enter the continental shelf off south Tunisia where they may also actively forage but without fully recruiting to the neritic stage (Chaieb et al. 2012). Libya and western Greece were still the major Mediterranean contributors but with significant decrease in the proportion of loggerhead turtles assigned to the Libyan MU (median = 35%) respect to the adult cohort. Similar age class variation in stock structure have been recently reported in green turtles foraging in northern Australia (Jensen 2010). The most likely explanation is that turtles move to a different foraging habitat upon reaching sexual maturity, particularly those originating from more distant rookeries (Bowen et al. 2004). However, alternative processes may also result in the observed difference. Shifts in the demographic

compositions of juvenile and adult cohorts may derive from major reduction of the reproductive output or changes in the genetic profiles of the Libyan population that occurred in the last decades. Unfortunately, very little information exists on the historic nesting effort in Libya where scientific monitoring of loggerhead turtle nesting activity has only recently began (Hamza 2010).

Overall our MSA results showed a strong connectivity between the nesting population in Libya and the neritic habitat on the continental shelf off the south Tunisian coasts. The high proportion of adults from Libya foraging in the study area is particularly significant since human induced mortality at this location has the potential to drastically reduce the Libyan nesting population in relative short time. Due to the reproductive isolation of this MU (Saied et al. 2012, Clusa et al 2013a), immigration from other Mediterranean nesting rookeries is unlikely to counterbalance the loss of reproductive individuals. Efforts should be made for immediate experimentation of conservation measures such as Turtle Excluder Devices (TED) for trawl nets and adoption of on-board best practice that are currently tested in other Mediterranean areas (e.g. NETCET Project, <http://www.netcet.eu/ref> or TURTLELIFE Project, <http://www.tartalife.eu/>).

Comparison of the hatchling drift model with mixed stock analysis

The strong relationship between juvenile stock compositions derived from MSA and results of a hatchling dispersal model in the eastern Mediterranean supports the recently proposed hypothesis that hatchling experience affects also the distribution of neritic juveniles (Casale & Mariani 2014, Scott et al. 2014). During the initial phase of their life, hatchlings cross several potential neritic foraging sites along their drifting trajectories and they may imprint on those most suitable and productive to which they will return later in life (e.g. Scott et al. 2014). This mechanism is probably more effective in those regions, like the Mediterranean Sea, where distances between neritic foraging habitats and nesting locations are contained (Hays & Scott 2013) and the mesoscale surface circulation pattern promotes the geographical retention of hatchlings in specific areas (Casale & Mariani 2014). The dispersal model employed by Casale & Mariani (2014) did not consider mortality along drifting trajectory as a result of unfavourable environmental conditions which may affect the perceived pattern of distribution (Putman et al. 2012). Hatchlings drifting to the northern part of the Adriatic Sea within the first six months of life may experience higher mortality rates than those drifting in the south and eastern part of the Mediterranean Sea, due to the extremely low

temperatures that are reached in the area ($\leq 10^{\circ}\text{C}$). This may explain the lower significance of the relationship among stock estimates from the two approaches obtained for the Adriatic juvenile aggregation with respect to STUN_{juv} .

Adult stock composition appeared to be influenced by hatchling drifting scenario to a lesser extent respect to the juvenile cohorts. Distance from breeding areas, sex biased migrations, habitat features and prey availability along migratory corridors that connect nesting and foraging habitats may be all important parameters in shaping the distribution of adults but further studies are required to elucidate their relative importance. In particular, an effort should be made to sample adult foraging cohorts from several Mediterranean neritic habitats in order to perform an integrative analysis of adult distribution.

Conclusions

The loggerhead turtle is a highly migratory species comprised of a complex series of interconnected breeding and non breeding populations that inhabit widely separated habitats. Understanding the pattern of connectivity among the different ontogenetic habitats is thus essential to manage the species effectively. In this study we demonstrated the existence of a strong connection between the Libyan Management Unit and the neritic foraging ground off the southern coasts of Tunisia which has relevant conservation implications. Each year thousands of loggerheads are accidentally caught in fishing gears and habitat quality in the southern Mediterranean Sea has significantly deteriorated in the last decades due to increased anthropogenic pressure. These threats affect disproportionately the Libyan rookery, particularly the adult cohort, and may led to a drastic decrease in the number of nesting individuals in relatively few years. Moreover, the current instable political and economic situation in the region poses additional pressure on the survival of this important Mediterranean nesting rookery.

Overall, our results support the recently proposed model of the dispersal and distribution of loggerhead turtles in the Mediterranean Sea (Casale & Mariani 2014). Locations of juvenile and, to a lesser extent, adult foraging habitats appear to be influenced by the diversity in sites experienced by drifting hatchlings (Hays et al. 2010, Casale & Mariani 2014, Scott et al. 2014). This implies that any future modification in the surface circulation driven by the climate change will also affect the recruitment pattern to neritic foraging habitats.

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5.- CLIMATE CHANGE

5.1. SEASONAL HETEROGENEITY OF OCEAN WARMING: A MORTALITY SINK FOR ECTOTHERM COLONIZERS.

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This chapter is currently in the second round of review in *Scientific Reports* (first response from the editor received on the 10 November 2015).

Abstract

Distribution shifts are a common adaptive response of marine ectotherms to climate change but the pace of redistribution depends on species-specific traits that may promote or hamper expansion to northern habitats. Here we show that recently, the loggerhead turtle (*Caretta caretta*) has begun to nest steadily beyond the northern edge of the species' range in the Mediterranean basin. This range expansion is associated with a significant warming of spring and summer sea surface temperature (SST) that offers a wider thermal window suitable for nesting. However, we found that post-hatchlings departing from this location experience low winter SST that may affect their survival and thus hamper the stabilization of the site by self-recruitment. The inspection of the Intergovernmental Panel on Climate Change model projections and observational data on SST trends shows that, despite the annual warming for this century, winter SST show little or no trends. Therefore, thermal constraints during the early developmental phase may limit the chance of population growth at this location also in the near future, despite increasingly favourable conditions at the nesting sites. Quantifying and understanding the interplay between dispersal and environmental changes at all life stages is critical for predicting ectotherm range expansion with climate warming.



Detailed Status Information

Manuscript #	SREP-15-26828A
Current Revision #	1
Other Version	SREP-15-26828-T
Submission Date	15th December 15
Current Stage	Quality Check Started
Title	Seasonal heterogeneity of ocean warming: a mortality sink for ectotherm colonizers
Manuscript Type	Original Research
Corresponding Author	Dr. Sandra Hochscheid (sandra.hochscheid@szn.it) (Stazione Zoologica Anton Dohrn)
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Authorship	Yes
Abstract	Distribution shifts are a common adaptive response of marine ectotherms to climate change but the pace of redistribution depends on species-specific traits that may promote or hamper expansion to northern habitats. Here we show that recently, the loggerhead turtle (<i>Caretta caretta</i>) has begun to nest steadily beyond the northern edge of the species' range in the Mediterranean basin. This range expansion is associated with a significant warming of spring and summer sea surface temperature (SST) that offers a wider thermal window suitable for nesting. However, we found that post-hatchlings departing from this location experience low winter SST that may affect their survival and thus hamper the stabilization of the site by self-recruitment. The inspection of the Intergovernmental Panel on Climate Change model projections and observational data on SST trends shows that, despite the annual warming for this century, winter SST show little or no trends. Therefore, thermal constraints during the early developmental phase may limit the chance of population growth at this location also in the near future, despite increasingly favourable conditions at the nesting sites. Quantifying and understanding the interplay between dispersal and environmental changes at all life stages is critical for predicting ectotherm range expansion with climate warming.
Subject Categories	Biological sciences, Climate science, Ecology, Oceanography
Techniques	Physical sciences techniques [Remote sensing]; Life sciences techniques, Genomic analysis [DNA sequencing];
Subject Terms	Biological sciences/Ecology/Animal migration Biological sciences/Zoology/Animal behaviour Earth and environmental sciences/Climate sciences/Climate change/Projection and prediction Earth and environmental sciences/Ecology/Biooceanography
Competing Financial Interest	There is NO Competing Interest.
Applicable Funding Source	No Applicable Funding

Stage	Start Date	End Date	Approximate Duration
Manuscript Assigned to Editor	16th December 15		
Submission Not Complete	15th December 15		

Anthropogenic induced climate warming is projected to be a major challenge to Earth's biota in the 21st century (Bellard et al. 2012). Despite slower ocean warming over the last 50 years, the average speed of isotherm migrations at the ocean surface has been as fast as or even faster than the terrestrial counterpart (Burrows et al. 2011). Shifts in species' distributions have been observed in many different marine taxa and are considered as one of the key adaptive mechanisms to endure changes in ambient temperature (Parmesan 2006, Poloczanska et al. 2013). In particular, marine ectotherms, being thermal range conformers, are predicted to expand poleward with ongoing warming as new locations that were previously too cold for survival will become suitable for colonists (Sunday et al. 2012). However, the pace of redistribution depends on the cumulative effects of climate warming on all ontogenetic life stages that may occupy different habitats, differ in behavioural traits and/or thermal sensitivity (Huey et al. 2012).

In this respect, the endangered loggerhead turtle is an interesting example (Hawkes et al. 2007). This species possesses the widest nesting range among marine reptiles spanning from tropical to temperate latitudes. As all extant sea turtle species, the loggerhead turtle has survived major climate changes in the past by altering the equilibrium between colonization and local extinction, but how and whether it is responding to present day climate warming is still under debate (Hawkes et al. 2009, Poloczanska et al. 2009, Fuentes et al. 2011). Loggerhead turtles, as well as salmon and many other marine and terrestrial animals, exhibit Phylopatry, i.e. they return to their natal sites, a strategy that increases reproductive success but sets certain limits to genetic diversity and adaptability of populations (Cury 1994, Hamann & Kennedy 2012). Philopatric animals rely on imprinting mechanisms during the early life stage to recognize site specific environmental features of their birth area that guide them home. Poleward expansion of the nesting range must therefore occur through self-recruitment, which means that the occasional nesting of straying turtles at higher latitudes is associated with the production of female offspring that survive to nest decades later at the same location (Bowen & Karl 2007, Hamann & Kennedy 2012, Bates et al. 2014). Given the influence of sand temperatures on embryonic development, it is not surprising that the majority of studies on climate induced range shifts have focused on the nesting beaches (Hawkes et al. 2009, Witt et al. 2010, Pike 2014). However, other factors might create a barrier towards the colonization of northern habitats. During the first year of their life, sea turtles possess limited swimming capacities and mostly rely on ocean currents to disperse towards suitable developmental habitats (Putman

et al. 2010). Therefore, if thermal conditions encountered along their dispersal pathways are not favourable for post-hatchling survival, nesting range expansion may be impeded, especially at more poleward sites. Located at the northern edge of the species' range, the Mediterranean population is the result of at least two independent immigration events from Atlantic rookeries and it has endured climatic oscillations since the Pleistocene by shifting its nesting range in accordance with the migration of its thermal niche (Clusa et al. 2013a). Until now regular nesting has occurred exclusively in the warmer eastern basin, mainly in Libya, Greece, Turkey and Cyprus (Casale & Margaritoulis 2010). However, in the last two decades, an increasing number of sporadic nests have been documented in the Western Mediterranean in the proximity of foraging habitats along the Spanish or Italian coasts (Figure 5.1). Sporadic nests are usually scattered in time and space and may represent a mechanism by which the species explores new locations and expands the nesting range if environmental conditions have become conducive for embryonic development (Bowen & Karl 2007).

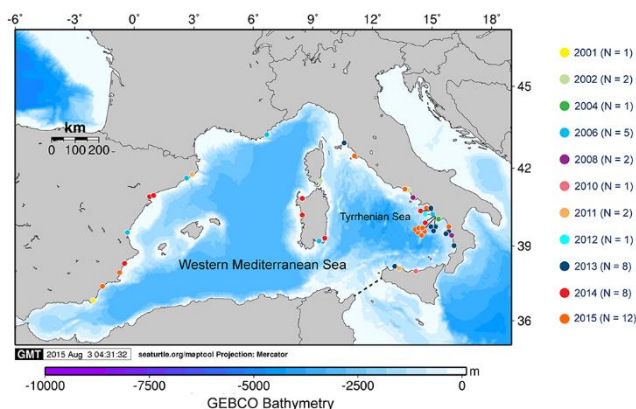


Figure 5.1. Occasional nesting by loggerhead turtles in the western Mediterranean since 2001. Only confirmed nests for which either nest chamber or egg remains were found are reported. Each dot represents a single nest with the different colour indicating the year of deposition. Our work focussed on the South Tyrrhenian coasts, the only area where nesting regularly occurs since 2012. For more information see Table 1. This map was created using the free Maptool program available at www.seaturtle.org.

Here we concentrate on the Campanian coast (SW Italy), an important foraging habitat for loggerhead turtles of Atlantic and Mediterranean origin (Maffucci et al. 2013) and the only region in the Western Mediterranean where nesting is now being recorded each year. The first nest was discovered in 2002 and since then single nests were found every 2–4 years. However, since 2012 nesting became more regular with 1–7 nests laid each year ($N = 16$), all of which were located along a 60 km strip of coastline at the southern portion of the study area (Figure 5.1). Mean hatching success of the first discovered nests ($N = 4$) was 62.6% and increased to 85.1% during the last three years ($N = 8$, excluding one inundated nest, see Table 5.1). We also found that, for clutches laid in the same week of July, incubation durations decreased by 8–16 days compared to nests before 2013 (Table 5.1). This coincided with generally higher incubation temperatures during the most recent years. In fact, mean temperatures of the middle third of incubation ranged between 28.4 and 31.1°C, and were thus within the optimal range of hatchling production as determined in controlled laboratory experiments (Fisher et al. 2014). Considering the pivotal incubation temperatures, 28.9°C (Kaska et al. 1998) and 29.3°C (Mrosovsky et al. 2002), and durations, 59.9 (Kaska et al. 1998) and 56.6 (Mrosovsky et al. 2002) days (i.e. the values producing 50% of each sex) for the Mediterranean populations, it appears that conditions have been conducive to female production with some nests approaching even a balanced sex ratio (Table 5.1). Taken together, these results support the predictions of good habitat suitability for nesting in the study area (Pike 2014) where incubation conditions have improved substantially during the monitoring period even though the low number of nests precludes any robust statistical analysis. The mean straight carapace length ($SCL = 40.5 \pm 3$ mm, range 28 – 49 mm, $N = 682$) and mean body mass ($Mb = 15.1 \pm 1.1$ g, range 11.2 – 20 g, $N = 569$) of the hatchlings from eleven Campanian nests were comparable to those recorded at eastern Mediterranean nesting beaches (Reece et al. 2002, Margaritoulis et al. 2003, Adam et al. 2007), while hatchlings were smaller and lighter than those from Atlantic rookeries (Abella et al. 2007). This may suggest that the females were indeed of Mediterranean origin, which is also consistent with previous results of mixed stock analysis of loggerhead turtles foraging along these coasts showing that the vast majority of the individuals belongs to local rookeries (Clusa et al. 2013b). However, the contribution of Atlantic females could not be definitively excluded based on results of the genetic analysis. In fact, samples from 12 out of 13 nests exhibited mtDNA haplotypes that are widely shared between Mediterranean and Atlantic rookeries (CC-A2.1 and

CC-A3.1, Table 5.1) (Shamblin et al. 2014). Moreover, although the other haplotype found at this site (CC-A10.4) has been reported exclusively from Atlantic nesting beaches (Shamblin et al. 2014), its shorter sequence (CC-A10) has been found at low frequency in Greece (Laurent et al. 1998) which suggests its probable presence also in the Mediterranean Sea. Because of the opportunistic sampling of genetic materials from the sporadic nests, pseudo-replication (i.e. the repeated sampling of the same female during a single nesting season or in different years) may be an issue. However, using photo identification it was possible to ascertain that at least six different females nested over the last eight years, four of which were observed only in 2015. Therefore, the occurrence of several nesting females in the study area indicates multiple explorations outside the regular nesting areas.

The SST trend from 1854 to 2014 shows that the south Tyrrhenian Sea has steadily warmed up after the last minimum in 1978 whereby the last three years were amongst the warmest on record (Figure 5.2). Contemporaneously the number of nests in the study area have increased, particularly during the last three years, a phenomenon that could be associated to the changes in SST.

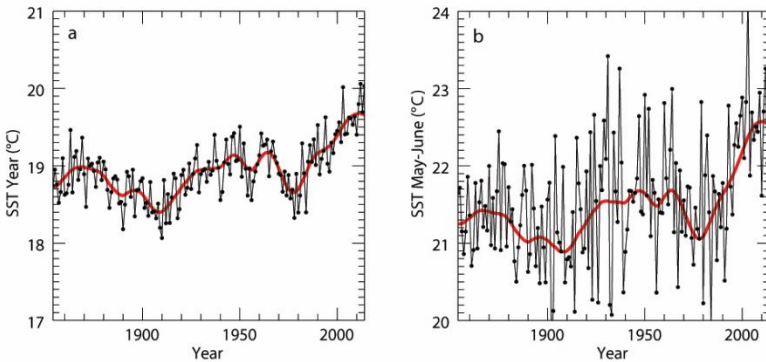


Figure 5.2. Sea surface Temperature (SST) trends in the south Tyrrhenian Sea from 1854-2014. Annual (a) and spring (b) (May-June) SST values. Singular Spectral Analysis combined with Maximum Entropy Method was used to estimate the oscillatory components in the time series.

Table 5.1. Summary data of loggerhead turtles nests in the Campania Region, SW Italy, between 2002 and 2015. Either the date of egg laying or first hatching emergence are given, depending on which occasion the nest was detected. Incubation duration is the time that elapsed between the date of egg laying and the first hatching emergence. Also given are straight carapace length (SCL) and body mass (Mb) of hatchlings, mean incubation temperature (T) during the middle third of the incubation period and mtDNA haplotypes for an 800 bp sequence (see Methods for further details on how measures and samples were collected).

Site	Date of egg laying	Date of 1 st emergence	Incubation duration	Clutch size	Mean SCL		Mean Mb		Emergence success	Hatching success	Mean middle third T (°C)	Note	mtDNA haplotype
					±s.d. (cm)	±s.d. (g)	±s.d. (g)	±s.d. (g)					
Baa Domizita	11/07/2002	-	64	92	-	-	-	-	-	49.4	27.4	Relocated	CC-A10.4
Marina di Camerota	-	17/10/2004	-	-	-	-	-	-	-	-	-	Egg remains	-
Ogliastro	25/07/2006	-	73	93	4.1 ± 0.1	14.7 ± 1	28	33.3	33.3	27.1	27.1	Relocated	-
Lucrino ^g	15/07/2008	-	46	115	4 ± 1.2	15.2 ± 0.5	88.7	92.2	92.2	34.5	34.5	Incubated on a geothermal beach	-
Ogliastro	-	28/08/2012	-	73	4 ± 0.7	15.5 ± 1.9	61.6	75.3	75.3	-	-	-	-
Palinuro	-	19/08/2013	-	132	4 ± 0.1	14.9 ± 0.7	40.9	62.1	62.1	-	-	Excavated	CC-A2.1
Palinuro ^b	15/07/2013	-	56	96	3.9 ± 0.1	14 ± 0.7	99	99	99	28.9	28.9	-	CC-A2.1
Battipaglia	-	12/10/2013	-	110	-	-	-	-	-	-	-	Excavated	CC-A2.1
Palinuro	-	06/12/2013*	-	48 [#]	-	-	-	-	-	-	-	Predated	CC-A2.1
Acciaroli ^b	30/07/2014	-	60	118	4.3 ± 0.4	-	94.9	95.8	95.8	28.4	28.4	-	-
Capaccio	-	25/08/2014	-	117	4.1 ± 0.1	14.8 ± 0.7	88.9	91.5	91.5	-	-	-	CC-A2.1
Marina di Camerota ^c	19/06/2015	-	50	99	3.9 ± 0.3	15.3 ± 0.6	83	83	83	31.1	31.1	-	CC-A2.1
Marina di Camerota	07/07/2015	-	58	60	-	-	80	80	80	-	-	-	CC-A2.1
Ascea Marina ^d	18/07/2015	-	56	87	3.9 ± 0.3	14.6 ± 0.5	80.5	80.5	80.5	29	29	Relocated	CC-A3.1
Eboli	-	28/08/2015	-	-	-	-	-	-	-	-	-	Predated	CC-A2.1
Marina di Camerota ^e	29/07/2015	-	58	56	4.1 ± 0.1	16.1 ± 0.1	87.5	89.3	89.3	29.9	29.9	-	CC-A2.1
Ascea Marina ^f	29/07/2015	-	57	55	4 ± 0.1	16.2 ± 0.5	78.2	78.2	78.2	29.1	29.1	-	CC-A2.1
Ascea Marina	30/07/2015	-	62	58	3.8 ± 0.1	13.2 ± 0.8	15.5	25.9	25.9	29.2	29.2	Inundated, relocated	CC-A2.1

Estimated from remains of predated nest

* Date nest was discovered, hatching date is unknown

Indeed, this parameter strongly influences loggerhead nesting phenology and sets the width of the optimal thermal window for nesting (Mazaris et al. 2009). In particular, the months between April and July exhibited the highest rates of warming with values ranging from $0.035^{\circ}\text{C}/\text{year}$ to $0.050^{\circ}\text{C}/\text{year}$ (Figure 5.3a). Although water temperature is not the exclusive factor that triggers the start of the reproductive season, it is known that higher SSTs during spring provoke earlier nesting (Mazaris et al. 2008). Thus, mature adult turtles foraging at these latitudes could encounter appropriate conditions for mating and nesting opportunistically (Mazaris et al. 2009) already at the beginning of the summer season. This is supported by a recent observation of two mating loggerhead turtles in the Gulf of Naples during May 2015, which is the first time that this behaviour has been reported in the Western Mediterranean.

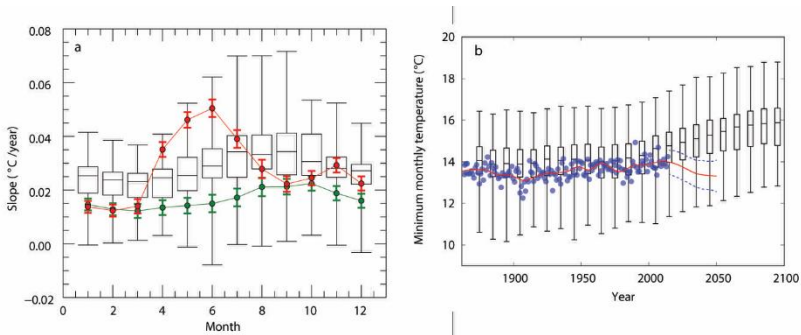


Figure 5.3. Seasonal variability of the SST trends. Monthly SST trends during the 1982-2014 period (a). Box plots represent the graphical summary obtained from all CMIP5 simulations. In red: trends in observed SSTs in the study area; in green: monthly trends for the AMO (without detrending). The latter is representative of the observed SST in the Atlantic north of the equator. (b) Minimum monthly temperature calculated from measured and reconstructed SST time series (blue circles) with superimposed Singular Spectral Analysis reconstructed signal plus the statistical prediction (red line) with the corresponding error (blue dotted lines). The black box plot refers to the minimum monthly temperature calculated from the different GCM of the CMIP5 ensemble. They indicate the minimum, the 75% percentile, the median, the 25% percentile and the maximum value inside the model ensemble.

Moreover, the combination of rapid warming in spring and only moderate warming in summer results in a wider temporal window of suitable thermal conditions that may benefit embryonic development and female offspring production. This implies that individual loggerhead turtles possess sufficient plasticity in their phylopatric behaviour to exploit nesting beaches that have become suitable with climate warming (Carreras et al. 2006). In conclusion, given that the conditions for nesting are already good and considering that they are predicted to further improve in our study area (Pike 2014), we may indeed be witnessing a colonisation event that is initiating right now.

However, the increased frequency of occasional nesting due to climate warming is not sufficient to stabilize the nesting site in the future if self-recruitment rates are very low (Heppell et al. 2003). Survival of post-hatchlings during the surface pelagic phase is necessary to sustain nesting range expansion. Using numerical simulation of post-hatchling dispersal, we found a wide spreading with most of the individuals accumulating in the south Tyrrhenian Sea during the first year of their life. The drifting pattern was consistent among all eight simulation years. In addition, we found two previously undescribed southward conveyors connecting the south Tyrrhenian Sea with the Strait of Sicily and thus with the favourable Eastern Mediterranean developmental habitat (Figure 5.4). These transport mechanisms cannot be inferred from the mere knowledge of the mean (climatological) current patterns since they are the result of nonlinear dynamic processes that are potentially relevant for the inter-basin connectivity (Palatella et al. 2014).

We then estimated hatchling survival along drifting trajectories using a published mortality function based on SST, which resulted in a mean survival index of 4.4%. This is because only those individuals that crossed to the Eastern Mediterranean through the southward channels survived in all simulation years, which highlights the unsuitability of the Western Mediterranean as a nursery area. Indeed, survival indices in the south Tyrrhenian Sea were about 17 times lower than those obtained for hatchlings departing from Libya (mean SI = 68%), and 7 times lower than SI of hatchlings from Greece (mean SI = 26%). The latter had lower SI because substantial, albeit variable proportions of hatchlings enter the nearby Adriatic Sea and reach the northern part, which is also characteristic for cold winter temperatures. Despite significant improvement of the conditions during the breeding period in the last decades, winter is currently the key determinant of species range expansion with climate warming because it drastically reduces

the number of individuals that may potentially self-recruit to this nesting location in the future.

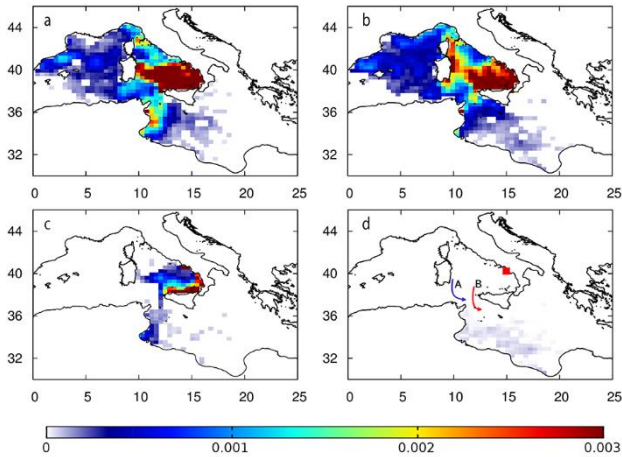


Figure 5.4. Modelled dispersion of hatchlings departing from the study area. Relative density (%) after one year of dispersal in 2007 (a, c) and in 2013 (b, d), obtained either without (a, b) and with (c, d) the mortality function based on SST. The winter (blue) and summer (red) southward conveyors connecting the south Tyrrhenian Sea with the Eastern Mediterranean are shown in Panel d. The maps were created using the free online software Gnuplot (<http://www.gnuplot.info/>) and assembled with Gimp (<http://www.gimp.org/>).

We then addressed the question of the future of the nursery area suitability in a climate change context, while duly considering the importance of the winter season. First, by rerunning the simulations adding a bias to the winter temperatures, a temperature increment of 1.5°C to 2.25°C during the colder months is required to obtain an SI comparable to the 26% and 68% benchmark values obtained for Greece and Libya, respectively. Considering the future estimates provided by the Coupled Model Intercomparison Project 5 (CMIP5, van Oldenborgh G.J. et al. 2009), RCP 4.5, for the SST in the south Tyrrhenian Sea, the 15°C degrees threshold in monthly SST minima is expected to be reached relatively early in this century (median value: 2030). However, as a whole the historical CMIP5 simulations show similar warming trends for winter and spring temperatures, in contrast with the observed seasonal trends (Figure 5.3a). Since the CMIP5 monthly trends for the region

have a phase similar to the Atlantic Multidecadal Oscillation (AMO) intensity (i.e., the SST anomaly over the North Atlantic; Figure 5.3a), the difference to the observations is possibly due to limitations in the Mediterranean basin physics in CMIP5, which basically projects the Atlantic tendencies into the Mediterranean. This tendency is maintained also in the CMIP5 forecasts for the 21st century (Shaltout & Omstedt 2014). On the contrary, the observed winter SSTs do not show a significant increase during the last decades and a statistical forecast of winter SST does not highlight any positive trend for the future (Figure 5.3b). Given the mismatch between the CMIP5 description of the recent state of the system and the observational data, we conclude that it is not possible to forecast when the thermal conditions at this post-hatchling developmental habitat will become sustainable. While we acknowledge that most of this difference is known and well explained (Schmidt G.A. et al. 2014), here we emphasise the need to further understand the limitations to the capability of capturing the seasonal response for regional seas. This is particularly important because these simulations are used to force regional models which are then used to predict local impacts of climate change.

Our study showed data in support of the existence of a Mediterranean hot spot for poleward expansion of loggerhead turtles. Through the multi-disciplinary study on the past, present and future thermal conditions in the potential expansion area, we found that inspecting the climate impact on the nesting sites is not enough to evaluate the potential for stable expansion. As demonstrated here, a complete assessment should consider the interplay between the physiological capacities of a species, like temperature sensitivity, and the environmental conditions of all major habitats utilized by the species, especially during the first life stage.

Furthermore, the winter season as a key factor has been overlooked in the past. The physics of the seasonal response to climate change per se are still unclear. Indeed, the marked seasonality we found is coherent with the large mixed layer differences (thermal inertia) between winter and spring in the region. The different dynamics of winter and spring SST responses to climate change may lead to a conundrum where the number of exploratory females and hatching success will increase with climate warming but the stabilization of this northern nesting site will still be hampered by high mortality rates of post-hatchlings due to the persistence of low winter temperatures. Clearly, this seasonal response to climate change might have implications for other marine organisms with a complex life cycle and further investigations on the occurrence of seasonally dependent trends should be conducted in other basins as well as on other marine organisms.

Methods

Nest sites, incubation temperatures and hatchling measures.

The Sea Turtle Stranding Network in the Campania Region (SW Italy) has been operating since 1983, including the collection of all data on loggerhead turtle nests in the study area (41.22°N, 13.76°E – 40.04°N, 15.64°E). These were reported by private citizens who observed either the female during nesting or the hatchlings during their emergence from the nest. Incubation temperatures were monitored at 1-h intervals in the former nests by placing data storage tags (Cefas Technology Ltd, Lowestoft, UK; and i-Button DS1923-F5, Maxim Integrated Products, Dallas Semiconductor, Sunnyvale, CA) directly at the top of the egg chamber (Table 1). After emergence from the nest hatchlings were weighed to the nearest 0.1 g with a digital balance (model GF-300, A&D Engineering, San Jose, CA, 0.01 g accuracy). Straight carapace length (SCL) was measured using callipers (0.1 mm accuracy).

MtDNA analysis.

Tissue samples for genetic analysis were obtained from 13 out of 18 nests laid since 2002. Genomic DNA was extracted using NucleoSpin Tissue kit (Macherey-Nagel, Duren, Germany) following the manufacturers' protocols and purified by binding and eluting to a silica membrane using vacuum filtration. A 850 bp fragment of the mtDNA, encompassing tRNAThr, tRNAPro and the control region, was amplified and sequenced following (Saied et al. 2012). The resulting sequences were compared to the online haplotype registry that is maintained by the Archie Carr Center for Sea Turtle Research of the University of Florida (ACCSTR; <http://accstr.ufl.edu/ccmtDNA.html>). The mtDNA haplotype of the 2002 nest was determined by Dr. Carlos Carreras.

Photo identification of nesting females.

As stated above, all except one (the female that nested on 29 July 2015 in Marina di Camerota, see Table 5.1) nesting turtles were observed by private citizens who never reported the presence of flipper tags which could aid to identify the turtles. However, they took photos or made videos of the nesting turtles (N = 9). As with other sea turtle species, loggerhead turtles have a cluster of scales on the dorsal and lateral surfaces of the head that form unique scale patterns. Photos (N = 4) or still frames extracted from videos (N = 3) of the nesting females' left side of the head, which was the most available view, were aligned to compare individual scale patterns using Adobe Photoshop® CS6 imaging software (version 13.0 x64, Adobe Systems Inc.). Two out of

nine turtles had to be excluded from this analysis because no images of the left side were available. The images were visibly assessed for clear marks of differences to identify different turtles.

Lagrangian simulations.

Numerical simulations of Lagrangian transport in the marine upper layer have been performed using the Mediterranean Forecasting System (MFS) analysis fields (velocities, temperature) for the years from 2006 to 2013. The MFS provides the basin-scale circulation field at 6.5 x 6.5 km spatial, and 1 day temporal resolution. Mesoscale two-dimensional turbulent dispersion has been parameterized with an established state-of-the-art kinematic Lagrangian model (KLM), suitable to simulate any kind of two-particle dispersion regime. In our case, the KLM has been calibrated on Mediterranean drifter trajectory data as in Lacorata et al. (2014). Another 3D KLM is added in order to simulate the small scale three dimensional turbulence on the scale of the mixed layer as in Palatella et al. (2014). The initial conditions of 25600 trajectories have been distributed off-shore (between 10 and 50 km from the coast) along a 100 km line in the vicinity of the study area, at 3 m depth. The starting dispersion is intended to simulate the position of the turtle hatchlings after the first one/two days of swimming activity. After this period the hatchlings are considered as passive buoyant tracers that follow constant depth currents, at least for the first year of life. A mortality rate is defined depending on the sea surface temperature along the Lagrangian trajectory: turtles experiencing mean SST < 15°C for more than 10 days had a 50% chance of survival for each extra day at the same conditions while those individuals that encountered SST < 10°C died instantly (Putman et al. 2012). The fraction of hatchlings that survived after a year of transport is defined as the survival index (SI). The SI, and so the stability of the release site, depends ultimately on the interaction between Lagrangian transport and SST fields, at given climate conditions. To compare these results to hatchling dispersions from regular nesting sites in the Eastern Mediterranean we repeated these simulations for two further sites, one in Zakynthos, Greece, which hosts the largest loggerhead rookery in the Mediterranean, and one in Sirte, Libya, which hosts one of the founder rookeries for the Mediterranean nesting population (Casale & Margaritoulis 2010, Clusa et al. 2013a).

Sea surface temperature analysis.

Sea surface temperature time series of the south Tyrrhenian Sea have been obtained from three different sources spanning over different time windows

and with different spatial resolutions: Extended Reconstructed Sea Surface Temperature (ERSST) from the NOAA National Climatic Data Center (1854 to the present), Hadley Centre Sea Ice and Sea Surface Temperature data set (HadISST) from Met office Hadley Centre (1870 to the present) and satellite reconstructed (interpolated through an Optimal Interpolation algorithm) AVHRR Pathfinder daily SST data from MyOCEAN-Copernicus (from 1982 to 2012). The three time series follow quite closely each other. They basically describe the same history both in terms of trends and of multi-decadal oscillations and can consequently be used interchangeably to study the long-term variation of the Tyrrhenian Sea SST field. For this reason, rather than choosing one of the three time series, we decided to adjust both monthly ERSST and HadISST using a simple slope and bias to monthly pathfinder SSTs and then we averaged the three time series to produce a single monthly time series running from January 1854 to December 2014.

Singular Spectral Analysis (SSA) was used to separate trends and oscillatory modes (red curve in Figures 2a and 2b) from noise in the SST time series.

CMIP5 data usage.

From 34 Atmosphere-Ocean General Circulation Model (AOGCM) and ESM model's output, all prepared for CMIP5, RCP 4.5, we extract monthly mean SSTs for South Tyrrhenian Sea ($12^{\circ} < \text{Longitude} < 16^{\circ} \text{ E}$, $38^{\circ} < \text{Latitude} < 41^{\circ} \text{ N}$) since January 1861 to December 2100. Data were in form of time series, then we determined, for each model's output, when monthly SST minimum exceeds the 15°C degrees threshold for post-hatchling survival (see Lagrangian simulations). Since we were interested in SST trends only, our analysis does not consider any bias correction.

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6. CONCLUSIONS

6.1. Adaptations to marine life

Tetrapods successfully invaded the marine environment several times during their evolution developing striking modifications of body form and functions (Houssaye 2009). Changes in skeletal mass and density have been suggested to be functionally related to the buoyancy control mechanisms and the diving behaviours adopted by different species (Wall 1983, Fish & Stein 1991, Taylor 2000, Houssaye 2009). For example, the leatherback turtle has evolved a light skeleton as an adaptation to its nearly exclusive pelagic lifestyle (Rhodin et al. 1981, Snover & Rhodin 2008). Very little is known on the structural properties of bone in the hard shelled sea turtles and how these may influence buoyancy during the different phases of their life cycle.

This study showed that the loggerhead turtle possess denser bones than the pelagic leatherback turtle but bone ballastin is not as extreme as in some slow swimming, shallow diving marine tetrapods (Rhodin et al. 1981, Wall 1983, Taylor 2000). Bone density increases significantly with turtle size which is coherent with the pattern of chondroosseous development described for the species (Snover & Rhodin 2008). This relation appears to be functionally correlated to the ontogenetic changes in the marine aquatic habits. During the very first years of their life, loggerhead turtles have very limited diving capabilities, swim at low speeds and are largely inactive drifters that clearly would benefit from having extremely light bones (Witherington 2002, Witherington et al. 2012). Later in life, turtles become more active, dive to deeper depths and after a decade or more in the oceanic province start to recruit to neritic habitats where they become important bioturbators of benthic systems (Lazar et al. 2011). These individuals would profit from denser bones as a static ballast system (Taylor 2000, Gray et al. 2007).

The relation between bone density and turtle size highlighted by the present study, deserves additional study to understand if this measure can be a valuable alternative to the enumeration of lines of arrested growth in skeletochronological studies to infer turtle age, as demonstrated in some cetacean species (Guglielmini et al. 2002, Butti et al. 2007).

6.2. Sex ratios

The loggerhead turtle, like all sea turtle species, possesses a temperature-dependent sex determination (TSD) (Mrosovsky 1994). Highly female skewed hatchling sex ratios have been estimated at most Mediterranean nesting sites which has raised some concerns regarding the resilience of this sub-population to climate warming (Godley et al. 2001a, Godley et al. 2001b, Rees & Margaritoulis 2004, Kaska et al. 2006, Zbinden et al. 2007, Witt et

al. 2010, Jribi et al. 2013). However, knowing the ratio of males to females in juveniles and adults is essential to understand population dynamics and assess vulnerability to climate change (Wibbels 2003).

This study reports for the first time information on the sex ratios of the juvenile and adult cohorts foraging in the South Tyrrhenian Sea. It showed that a female biased sex ratio is maintained in Mediterranean juveniles although less pronounced than in hatchlings (Godley et al. 2001a, Godley et al. 2001b, Rees & Margaritoulis 2004, Kaska et al. 2006, Zbinden et al. 2007, Witt et al. 2010, Jribi et al. 2013). The proportion of females decreases further in the adult portion of the sub-population where the ratio of males to females appear to be balanced although sampling bias problems associated with adult breeding migrations and homing behaviour may have affected the analysis and could not be definitively ruled out.

Overall this study demonstrates a clear discrepancy between the highly females skewed hatchling sex ratios and those in the juvenile and adult portions of the Mediterranean sub-population. A similar sex ratio dynamic has been observed in the north-west Atlantic (Wibbels 2003) and may have several non mutually exclusive explanations. Adult and juvenile sex ratios are a condensation of many years of hatchling production at several nesting sites and integrate a number of different life history events (Wibbels 2003). Therefore, they incorporate possible hatchling sex ratio shifts across time, male production at nesting sites not yet analysed, sex-specific mortality during development or as adults (Wibbels 2003). Sampling bias problems associated with adult breeding migrations do not affect juvenile sex ratios, which may offer a more viable estimate of the population's functional sex ratio provided that long time series, large sample sizes and the most rigorous criteria for sex identification are used.

It is of the utmost importance to continue monitoring sex ratios in the south Tyrrhenian foraging ground where the availability of long time series allows the detection of possible biologically significant sex ratio shifts in the Mediterranean sub-population. Moreover, in order to get a better understanding of the dynamics of loggerhead turtle sex ratios in the region, it is necessary to assess juvenile and adult sex ratios at those major foraging grounds not yet investigated, such as the neritic areas in Libya, Egypt, Turkey and eastern Greece.

6.3. Demographic composition of foraging aggregations

The loggerhead turtle is a highly migratory species that use widely separated and ecologically disparate habitats throughout its life (Bolten 2003).

Individuals from reproductively independent Management Units (MUs) usually mix in foraging habitats (Jensen et al. 2013). The capacity to link these turtles back to their rookery of origin is crucial for our ability to manage the species effectively (Jensen et al. 2013).

This study contributed to advance our understanding of the fine scale distribution of Atlantic and Mediterranean loggerhead turtles in the Mediterranean Sea. Juveniles of Atlantic origin were found to be significantly more abundant in the Tyrrhenian Sea than on the African continental shelf off south Tunisia. Differences in the relative contribution of Mediterranean MUs to these juvenile foraging aggregations were also found. Surface circulation pattern and hatchling imprinting on possible suitable neritic habitats during the early oceanic developmental stage were suggested as important factors shaping the composition of juvenile foraging aggregations in the Mediterranean Sea (Hays et al. 2010, Jensen et al. 2013, Casale & Mariani 2014, Scott et al. 2014).

This study provided also the first data on the demographic composition of adult foraging stocks in the Mediterranean Sea, although the sample size was relatively small due to the logistic difficulties in sampling this life stage. The analysis evidenced weak but significant differentiation between the adults and the juveniles foraging on the African continental shelf, driven mainly by the relevant increase of the Libyan contribution. This may be related to the strong homing behaviour of adult turtles that move to foraging habitats closer to the site of origin upon reaching sexual maturity (Bowen et al. 2004). However, alternative processes such as shifts in the reproductive output of the Libyan population, sex biased migrations, habitat features and prey availability along migratory corridors may be all important in shaping the distribution of adults (Jensen et al. 2013). An effort should be made to extensively sample adult foraging cohorts from several Mediterranean neritic habitats in order to perform an integrative analysis of adult distribution pattern within the basin.

Results from this study have relevant conservation implications. Each year thousands of loggerheads are accidentally caught in fishing gears and habitat quality in the Mediterranean Sea has significantly deteriorated in the last few decades due to increased anthropogenic pressure. On the African continental shelf these threats affect almost exclusively the Mediterranean Regional Management Unit (RMU) and in particular the Libyan rookery. In the Tyrrhenian Sea, instead anthropogenic pressures threat both the Mediterranean and the Atlantic RMUs. This information should be taken into

account when developing future management plans of the species in the Mediterranean Sea

6.4 Climate change

Climate warming is a major challenge to Earth' biota that forces species to adapt in situ or move to a different location in order to follow shifts in their thermal niche (Sunday et al. 2012). Many different marine taxa have already shifted their distributions to endure changes in ambient temperature (Poloczanska et al. 2013). In particular, thermal range conformers such as marine ectotherms, are expected to colonise northern habitats as they become suitable for colonists due to the ongoing climate warming (Sunday et al. 2010). However, the pace of redistribution depends on species-specific traits that may promote or hamper expansion to northern habitats (Bates et al. 2014).

This study showed that recently, the loggerhead turtle (*Caretta caretta*) has begun to nest steadily beyond the northern edge of the species' range in the Mediterranean basin. This range expansion was associated with a significant warming of spring and summer sea surface temperature (SST) that offers a wider thermal window suitable for nesting (Mazaris et al. 2009). Moreover, the analysis of incubation and nest parameters indicated that environmental conditions have improved lately supporting the predictions of good habitat suitability for nesting in this area under a climate warming scenario (Pike 2014). However, we found that post-hatchlings departing from this location experience low winter SST that may affect their survival (Davenport 1997) and thus hamper the stabilization of the site by self-recruitment. Indeed, survival indices (SIs) in the south Tyrrhenian Sea were about 17 times lower than those obtained for hatchlings departing from Libya and 7 times lower than SI of hatchlings from Greece, which highlighted the unsuitability of the Western Mediterranean as a nursery area for the loggerhead turtle. The inspection of the Intergovernmental Panel on Climate Change model projections and observational data on SST trends showed that, despite the annual warming for this century, winter SSTs show little or no trends. The different dynamics of winter and spring SST responses to climate change may lead to a conundrum where the number of exploratory females and hatching success will increase with climate warming but the stabilization of this northern nesting site will still be hampered by high mortality rates of post-hatchlings due to the persistence of low winter temperatures.

This study highlighted the importance of quantifying and understanding the interplay between dispersal and environmental changes at all life stages for predicting ectotherm range expansion with climate warming.

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APPENDIX I. COMPLETE LIST OF THE PUBLICATIONS 2013-2016

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