Population structure of *Caretta caretta* in the Mediterranean Sea: from nesting beaches to foraging grounds

Marcel Clusa Ferrand

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PhD Thesis

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Estructura poblacional de *Caretta caretta* al mar Mediterrani: de platges de posta a zones d'alimentació

Memòria presentada per **Marcel Clusa Ferrand** per optar al grau de Doctor per la Universitat de Barcelona

Marcel Clusa Ferrand

Tutor i Director:

Dr. Lluís Cardona Pascual
Departament de Biologia Animal
Universitat de Barcelona

Directora:

Dra. Marta Pascual Berniola
Departament de Genètica
Universitat de Barcelona

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Estructura poblacional de *Caretta caretta* al mar Mediterrani: de platges de posta a zones d'alimentació

RESUM

INTRODUCCIÓ GENERAL

Els oceans cobreixen una gran part del planeta Terra i acullen entre el 50 i 80% de tota la seva biodiversitat (Sala i Knowlton 2006). Amb més del 40% de la població mundial vivint a zones costaneres (IOC/UNESCO 2011), els oceans han esdevingut una font indispensable de recursos i, com a conseqüència, la biodiversitat marina està altament amenaçada no només per l'explotació directa sinó també per factors antropogènics secundaris com la pol·lució, el turisme massiu, les pràctiques agrícoles i les indústries (Gray 1997).

Una de les causes més rellevants que estan empenyent a la desaparició de diverses espècies marines és la pesca i les captures accidentals associades a aquesta. La megafauna marina (taurons, tortugues marines, aus marines i mamífers marins) és especialment vulnerable a aquesta captura accidental degut a la maduració sexual en edats avançades, la presència d'un cicle vital de llarga durada i el baix rendiment reproductiu, entre altres, de les seves espècies (Lewison et al. 2004a).

Com a conseqüència d'aquesta captura accidental, diverses espècies de grans vertebrat marins es troben actualment amenaçades. Per tal de poder aplicar plans de gestió per a la seva conservació, però, és essencial conèixer-ne el seu cicle vital, la distribució i els usos de l'hàbitat per entendre quines són les possibles amenaces que afecten les diferents poblacions i a on es localitzen. En el cas de les tortugues marines, això pot esdevenir però un fet de gran dificultat degut a les llargues migracions que aquestes recorren, a les dimensions de les seves zones de distribució i al complex cicle de vida de les seves espècies (Bayliff 1994; Croxall et al. 2005).

TORTUGUES MARINES: CICLES VITALS COMPLEXES

Les tortugues marines són rèptils adaptats a viure a l'aigua però que continuen vinculades al medi terrestre durant la reproducció ja que les femelles necessiten sortir de l'aigua per posar els ous en platges de nidificació (els mascles són 100% marins i no emergeixen durant el cicle vital; Pritchard 1997). Hi ha set espècies de tortugues marines i totes, excepte la tortuga llaüt (*Dermochelys coriacea*),

comparteixen un cicle vital similar. Aquest consisteix en una primera fase juvenil en la qual els individus són transportats passivament per corrents principals en zones oceàniques seguida d'una etapa nerítica més o menys estricta depenent de l'espècie i població. Un cop les tortugues assoleixen la maduresa sexual, aquestes recluten a zones d'alimentació des d'on, periòdicament, fan migracions cap a les zones de reproducció (generalment prop de les zones de nidificació; Bowen et al. 2005). Després de l'aparellament, els mascles tornen a les zones d'alimentació i les femelles surten a les platges a posar els ous en nius excavats a la sorra (Arendt et al. 2012a; Schofield et al. 2010).

De totes les espècies de tortugues marines, la tortuga babaua (*Caretta caretta*) presenta un dels cicles més complexos descrits, estudiat durant dècades a través de telemetria per satèl·lit, tècniques de captura-marcatge-recaptura, isòtops estables i estudis genètics (Bolten 2003; Mansfield i Putman 2013). Aquests estudis han demostrat que les tortugues babaues són filopàtriques i que, per tant, les femelles retornen a posar els ous allà on elles van néixer, resultant en una forta fidelitat inter-anual (Miller et al. 2003). La precisió d'aquesta filopatria encara és desconeguda en moltes poblacions però els mecanismes involucrats per tal de trobar les platges d'origen han estat molt estudiats i actualment s'accepten dues hipòtesis no excloents: l'ús d'impronta geomagnètica i l'ús d'impronta química. La primera hipòtesi contempla l'ús per part de les femelles de variacions en els camps magnètics del planeta per tal d'orientar-se a gran escala i apropar-se a la zona de nidificació d'origen. La segona hipòtesi suposa que les femelles, un cop properes a la zona de nidificació, identifiquen la platja d'origen a través d'olors i elements químics dissolts a l'aigua (Lohmann et al. 2013).

La filopatria és una estratègia altament favorable per a les femelles ja que assegura la viabilitat de les platges però també ho és pels mascles, ja que incrementa la probabilitat de trobar una femella amb la que emparellar-se (Schofield et al. 2009). Tot i que està acceptat que els mascles segueixen rutes similars a les femelles (Hatase et al. 2002; Godley et al. 2008; Schofield et al. 2010), la distribució i rutes migratòries dels mascles són menys conegudes degut a la dificultat de mostreig ja que aquests no surten a les platges. Així, encara es desconeix si l'aparellament es dóna només en zones properes a les platges de nidificació (Bowen et al. 2005) o si

també succeeix al llarg de la migració cap a zones d'aparellament o en zones d'alimentació.

DISTRIBUCIÓ DE LA TORTUGA BABAUA

La tortuga babaua es troba a tots els oceans i mars tropicals i temperats del planeta. Les zones de nidificació més importants es localitzen a la costa sud-est dels Estats Units i Golf de Mèxic (32,000-56,000 femelles nidificants), Cap Verd (5,000) i Brazil (4,000). A més d'aquestes, també s'ha enregistrat una substancial activitat nidificant a l'oest d'Austràlia, Japó, Oman, Sud Àfrica i al mar Mediterrani (Miller et al. 2003). Pel què fa a les zones d'alimentació, la tortuga babaua es pot trobar alimentant-se a tots els oceans i mars temperats però la distribució de les seves poblacions no és homogènia i està altament influenciada pels patrons de corrents d'aigua i la disponibilitat de preses.

Els patrons de distribució dels nounats estan mediats pels patrons de circulació d'aigua superficial un cop surten del niu i arriben a l'aigua (Bolten 2003). Això és degut a la flotabilitat positiva de les tortugues i la seva limitada capacitat natatòria durant els estadis primerencs (Milsom 1975), fet que comporta que els individus flotin passivament a través d'oceans sencers empesos per forts corrents (Carr i Meylan 1980). Les rutes seguides durant aquesta etapa i durant els primers estadis juvenils són encara desconegudes degut a la impossibilitat de fer seguiment per satèl·lit d'individus de talla petita. Tot i així, estudis recents han pogut desenvolupar emissors de petita talla que han permès ja el seguiment de cohorts de la costa sud-est dels Estats Units a través de l'Atlàntic (Mansfield et al. e*n revisió*). Aquest i altres estudis basats en anàlisis genètiques i models de dispersió suggereixen que nounats de l'Atlàntic nord-oest arriben a les costes europees empesos pel corrent del Golf i que, un cop allà, són arrossegats fins al mar Mediterrani per un corrent d'entrada que flueix permanentment des de l'Atlàntic cap a l'interior del mar Mediterrani (Millot i Taupier-Letage 2004). Contràriament, pel què fa a la població atlàntica de Cap Verd, aquesta no es troba al mar Mediterrani (Monzón-Argüello et al. 2009) degut a la manca de corrents que uneixin aquest arxipèlag i la conca mediterrània, fet que dificulta l'arribada d'individus d'aquesta zona (Mansfield i Putman 2013).

Aquests fets demostren que la contribució de les diferents zones de nidificació a una zona d'alimentació concreta dependrà no només de la grandària poblacional sinó també dels patrons de circulació que connectin ambdues zones (Bowen i Karl 2007; Hays et al. 2010). A més, posen de manifest la necessitat de determinar els patrons de distribució i ús de l'habitat de les poblacions mundials ja que certes amenaces podrien estar afectant una mateixa població en zones molt allunyades de la seva zona de nidificació.

LA TORTUGA BABAUA AL MAR MEDITERRANI

La tortuga babaua és la tortuga marina més abundant al mar Mediterrani, amb 7,200 nius estimats a l'any (Casale i Margaritoulis 2010) i una distribució que avarca tota la conca.

Zones de nidificació

Les zones de nidificació més rellevants del mar Mediterrani (Grècia, Turquia, Xipre i Líbia) es troben a la part central i oriental del Mediterrani mentre que al Mediterrani occidental, en canvi, la nidificació és esporàdica o inexistent en moltes regions (Tomás et al. 2008).

L'anàlisi de fragments d'ADN mitocondrial (ADNm) ha demostrat prèviament l'existència d'estructuració genètica entre les zones de nidificació mediterrànies (Encalada et al. 1998; Laurent et al. 1998; Carreras et al. 2007; Garofalo et al. 2009; Yilmaz et al. 2011; Saied et al. 2012). Aquest fet és degut al caràcter filopàtric de l'espècie i a que l'ADNm és un marcador d'herència materna (Bowen i Karl 2007). Així, l'estudi més extensiu de la conca fins al moment (Carreras et al. 2007) ha definit la presència de quatre unitats de gestió gràcies a la presència d'haplotips exclusius. Aquest estudi, però, es va dur a terme a través de l'anàlisi de fragments curts d'ADNm (380pb) i, amb el recent desenvolupament de nous *primers* capaços d'amplificar llargs fragments d'ADNm (815pb), noves unitats podrien sorgir tal i com ja ha succeït en posteriors estudis a Turquia, Cap Verd i Estats Units (Yilmaz et al. 2011; Monzón-Argüello et al. 2010; Shamblin et al. 2012). Un anàlisi global de l'estructura genètica en zones de nidificació mediterrànies mitjançant l'ús de llargs fragments d'ADNm no s'ha dut a terme

encara, però potencialment permetria definir noves unitats de gestió en comparació al publicat fins al moment amb fragments curts.

Tot i que l'ADNm s'ha usat tradicionalment en estudis d'estructura genètica en zones de nidificació, aquest no considera la contribució dels mascles en aquesta estructura. Per contra, el flux gènic entre poblacions mediat tant per femelles com per mascles pot ser estudiat a través de l'ADN nuclear (ADNn). Tot i que un cert grau d'estructuració s'ha detectat prèviament al mar Mediterrani mitjançant l'anàlisi de 7 microsatèl·lits d'ADNn (Carreras et al. 2007), hi ha estudis que no ho recolzen (Yilmaz et al. 2011; Garofalo et al. 2013) i suggereixen, per tant, que el flux gènic mediat per mascles entre zones de nidificació és un fet comú a la conca. Les discrepàncies entre aquests estudis es podrien deure a la reduïda mida mostral en algunes zones però també podrien ser degudes al baix nombre de marcadors usats. Monzón-Argüello et al. (2008) ha aïllat recentment nous microsatèl·lits per aquesta espècie que podrien augmentar la resolució d'aquesta estructuració genètica però tampoc havien estat emprats en zones de nidificació mediterrànies abans d'aquesta tesi.

Zones d'alimentació

La distribució de juvenils i adults en zones d'alimentació ha estat extensament estudiada al mar Mediterrani a través del seguiment d'individus mitjançant telemetria per satèl·lit (Cardona et al. 2005; Bentivegna et al. 2007; Revelles et al. 2007b; Cardona et al. 2009; Casale et al. 2013), tècniques de captura-marcatge-recaptura (Margaritoulis et al. 2003; Casale et al. 2007; Revelles et al. 2008) i estudis genètics (Carreras et al. 2006; Maffucci et al. 2006; Casale et al. 2008b; Saied et al. 2012; Garofalo et al. 2013).

Els individus juvenils atlàntics i mediterranis comparteixen zones d'alimentació al mar Mediterrani i la contribució de cada regió d'origen a aquestes zones pot ser estudiada genèticament mitjançant l'anàlisi d'ADNm i l'aplicació d'un *mixed stock analisis* (MSA, Grant et al. 1980; Pella i Masuda 2005). El MSA permet estimar la proporció de tortugues de cada zona de nidificació present en una zona d'alimentació i ha permès descriure la distribució de tortugues al Mediterrani occidental en estudis previs. Carreras et al. (2006) van estimar que les tortugues juvenils atlàntiques es concentraven a la conca algeriana, properes a les costes

africanes, mentre que els juvenils d'origen mediterrani s'alimentaven majoritàriament al llarg de les costes europees al Mediterrani occidental. Tot i així, el coneixement sobre la distribució dels individus atlàntics a la resta del mar Mediterrani és encara limitat. Pel què fa als juvenils d'origen mediterrani, els estudis basats en l'ús de MSA han estimat una rellevant presència d'individus d'origen grec a zones d'alimentació del mar Tirrè, al mar Adriàtic i a la resta del Mediterrani central (Saied et al. 2012; Maffucci et al. 2013). Aquests estudis, però, es basaven en fragments curts d'ADNm i, juntament amb la manca de mostres d'algunes zones de nidificació rellevants, no va fer possible una estima acurada de les contribucions de les zones de nidificació mediterrànies a les majors zones d'alimentació de la conca.

A més dels estudis genètics, altres metodologies han permès també l'estudi de la distribució de tortugues d'origen mediterrani per la conca. Els més freqüents són estudis basats en telemetria per satèl·lit i captura-marcatge-recaptura (Margaritoulis et al. 2003) però aquests comporten elevats costos econòmics i requereixen estudis a llarg termini per tal de poder extreure'n dades concloents. Degut a aquestes limitacions, estudis recents han aplicat l'anàlisi d'isòtops estables en teixits de tortugues babaues (Hatase et al. 2002; Revelles et al. 2007a; McClellan et al. 2010; Zbinden et al. 2011), ja que informen de la dieta dels individus i de la zona on aquests s'han alimentat. Això es deu a que el senyal isotòpic dels teixits reflecteix el senyal isotòpic de la xarxa tròfica present a una àrea o regió determinada, fet que permet identificar la zona d'alimentació usada (Hobson 1999; Fry 2006).

Els elements més usats en ecologia tròfica tant terrestre com marina són el carboni i el nitrogen (Newton 2010). En el cas del carboni, la proporció de ^{13}C a ^{12}C (expressat com a $\delta^{13}C$) informa sobre la font de carboni que entra a la cadena alimentària i, per tant, el que permet distingir entre les xarxes tròfiques alimentàries costaneres i oceàniques. Quant a la relació de ^{15}N a ^{14}N ($\delta^{15}N$), aquesta experimenta un enriquiment progressiu en cada nivell tròfic a causa de l'excreció preferencial de l'isòtop més lleuger (Peter i Fry 1987). Així, $\delta^{15}N$ es pot utilitzar per definir la posició tròfica de qualsevol individu dins de la xarxa tròfica d'una àrea determinada. No obstant això, les zones d'alimentació poden diferir en el *baseline*

per al nitrogen, i per tant, les diferències individuals en $\delta^{15}N$ poden sorgir a causa de diferències en les zones d'alimentació utilitzades (Hobson i Wassenaar 2008).

En el cas de les tortugues marines, els estudis basats en l'anàlisi d'isòtops estables han permès identificar diferències en l'ús de l'hàbitat i tipus d'alimentació al llarg dels diferents estadis del cicle vital (Arthur et al. 2008), entre individus dins una mateix població (Reich et al. 2007) i entre diferents poblacions (Wallace et al. 2006). Així, els individus enriquits en ^{13}C i ^{15}N són generalment considerats nerítics, mentre que les tortugues empobrides en ^{13}C i ^{15}N es classifiquen com a oceàniques (McClellan et al. 2010; Eder et al. 2013). Això permet identificar les zones d'alimentació utilitzades per tortugues de diferents mides (o estadis) i diferents poblacions, fet que pot ser altament rellevant a l'hora de dissenyar plans de gestió per a aquesta espècie. Degut a diferències en l'abundància de preses i productivitat de l'hàbitat, les diferents zones d'alimentació utilitzades poden afectar, a més, certs trets de la biologia dels individus com ara la seva taxa de creixement, la durada dels estadis del cicle vital, l'edat de maduració sexual o la seva supervivència (Snover et al. 2007b; Snover 2008). Això, per tant, pot portar a diferents requeriments de conservació i posa de manifest la necessitat de tenir un bon coneixement de la distribució i ús de l'habitat de les poblacions de tortuga babaua.

Impacte de l'activitat humana

Amenaces naturals com l'erosió de platges o la depredació afecten les poblacions de tortuga babaua però l'impacte de l'activitat humana és el què ha portat a aquesta espècie a un declivi generalitzat al mar Mediterrani (Casale i Margaritoulis 2010). Les amenaces derivades de l'activitat humana presents a les zones de nidificació inclouen el desenvolupament massiu d'infraestructures costaneres, la reestructuració i modificació de les platges i el consum d'ous de tortuga a més de l'explotació directa, inexistent a pràcticament tota la conca excepte a Egipte i Grècia (Casale i Margaritoulis 2010). Pel què fa a les zones d'alimentació, les amenaces deriven del risc de col·lisió amb vaixells o la contaminació però cap amenaça és tant rellevant com ho poden ser les captures accidentals en arts de pesca.

S'estima que més de 132.000 tortugues marines (majoritàriament tortugues babaues) són capturades accidentalment al mar Mediterrani cada any; 44.000 de les

quals acaben morint (Casale 2011). De tots els arts de pesca usats a la conca, el palangre de superfície és el que comporta una major amenaça ja que 60.000 tortugues són accidentalment capturades cada any (Lewison et al. 2004b; Casale 2011) i un 35% moren degut a aquesta interacció (Álvarez de Quevedo et al. 2013). Les tortugues queden típicament enganxades als hams en intentar capturar l'esquer usat per pescar tonyines o peixos espasa o bé es queden embolicades per les aletes als fils del palangre. Un cop capturades, la seva taxa de mortalitat dependrà principalment del temps d'immersió, la profunditat dels hams en la columna d'aigua i el tipus de dany causat en la tortuga (Lewison i Crowder 2007).

Tot i que les captures accidentals derivades de la pesca d'arrossegament i tremall s'han considerat menys importants degut al menor nombre de captures en comparació al palangre de superfície, aquestes poden arribar a 39.000 captures per any (Casale 2011). Per tant, aquests arts no haurien de ser ignorats ja que, a més, les taxes de mortalitat associades a aquests tipus d'arts (generalment lligades a l'ofegament dels individus en quedar atrapats a les xarxes a una gran profunditat) són significativament més elevades que no pas en els palangres (Wallace et al. 2013). No obstant això, l'impacte d'aquests arts de pesca sobre les poblacions de diferent origen dins de cada zona d'alimentació mediterrània encara ens és desconegut.

Laurent et al. (1998) van descriure una composició diferent, en relació a les poblacions d'origen dels individus, en les captures accidentals derivades del palangre de superfície i les xarxes d'arrossegament. Així, es va suggerir que mentre la pesca pelàgica (palangre de superfície) capturava una barreja d'individus d'origen atlàntic i mediterrani, la pesca nerítica (les xarxes d'arrossegament) només capturava individus d'origen mediterrani degut a diferències en l'ús de l'hàbitat. En aquest estudi, però, es va assumir que no hi havia diferències regionals en la distribució d'animals d'origen atlàntic i mediterrani i es va comparar la composició de captures derivades de palangre al Mediterrani central i occidental amb la composició de les captures derivades de la pesca d'arrossegament al Mediterrani central i oriental. Estudis posteriors, però, han demostrat que la distribució al mar Mediterrani no és homogènia (Carreras et al. 2006, 2011; Maffucci et al. 2006) i, per tant, les diferències detectades per Laurent et al. (1998) podrien ser degudes a diferències en la distribució de tortugues d'origen atlàntic i mediterrani més que no

pas a diferències en l'ús de l'habitat d'ambdues unitats, tal i com suggerien. Tot i així, la manca d'informació referent a la distribució d'individus de diferent origen a escala local no ha permès estimar amb precisió l'impacte de les captures accidentals en les poblacions afectades fins al moment.

OBJECTIUS

L'objectiu principal d'aquesta tesi és descriure l'estructura poblacional de la tortuga babaua a les zones de nidificació i alimentació del mar Mediterrani, entendre les causes d'aquesta estructuració i avaluar-ne les seves conseqüències per a la conservació de l'espècie.

La tesi s'organitza al voltant de quatre temes principals: l'estructura de la població en les zones de nidificació (*Capítol 1*), l'estructura poblacional en zones d'alimentació (*Capítol 2*), les conseqüències biològiques dels diferents usos de l'habitat (*Capítol 3*) i l'avaluació de la pesca accidental en les poblacions que habiten al mar Mediterrani (*Capítol 4*). Els objectius concrets de cada capítol són:

ESTRUCTURA POBLACIONAL EN ZONES DE NIDIFICACIÓ

- Definir el context històric de la tortuga babaua dins la conca mediterrània tot estudiant, mitjançant l'anàlisi d'ADNm, els processos de colonització que van dur a l'actual estructura genètica present a les zones de nidificació.

- Definir les unitats genètiques presents en les colònies mediterrànies actuals mitjançant l'anàlisi de diferenciació genètica entre diferents àrees de nidificació amb marcadors d'ADNm i ADNn.

- Avaluar el flux gènic mediat per femelles i mascles entre les zones de nidificació combinant ADNm i ADNn. Aquesta informació és rellevant des del punt de vista de la conservació i gestió de l'espècie ja que permetrà comprendre els nivells d'aïllament presents a cada zona.

ESTRUCTURA POBLACIONAL EN ZONES D'ALIMENTACIÓ

- Avaluar la contribució de les colònies atlàntiques i mediterrànies de tortuga babaua en set zones d'alimentació mediterrànies mitjançant MSA amb marcadors d'ADNm.

- Inferir els mecanismes que defineixen la distribució de juvenils i relacionar-los amb la biologia de l'espècie.

CONSEQÜÈNCIES BIOLÒGIQUES DELS DIFERENTS USOS DE L'HABITAT

- Analitzar les diferències en la taxa de creixement entre les tortugues atlàntiques i mediterrànies presents al mar Mediterrani mitjançant esqueletocronologia i anàlisis genètiques.

- Estimar l'edat de maduresa sexual de les tortugues d'origen atlàntic i mediterrani per entendre l'ús de l'hàbitat i la dinàmica de les poblacions al mar Mediterrani.

- Investigar, a través d'anàlisis d'isòtops estables, la presència de diferències en el rendiment reproductiu (entès com a nombre d'ous per niu) com a possible conseqüència de l'ús de zones d'alimentació de productivitat diferent.

CAPTURA ACCIDENTAL DERIVADA D'ACTIVITATS PESQUERES

- Caracteritzar la composició poblacional i usos de l'habitat de les tortugues capturades accidentalment per arts de pesca oceànics (palangres de superfície) i nerítics (pesca d'arrossegament i tremall) en zones d'alimentació mediterrànies.

- Analitzar si diferents arts de pesca capturen juvenils de poblacions diferents a les zones estudiades.

RESULTATS I DISCUSSIÓ GENERAL

Els resultats presentats en aquesta tesi han demostrat una estructuració poblacional en zones de nidificació i alimentació més forta del què es creia fins al moment per a la tortuga babaua. S'ha datat una colonització plistocènica del mar Mediterrani, s'han estimat les contribucions de cada zona de nidificació a diferents zones d'alimentació mediterrànies, s'han analitzat els usos de l'habitat de diferents poblacions i noves unitats de gestió han estat definides. També s'ha analitzat l'efecte dels usos de l'habitat sobre la biologia de la tortuga babaua i diferències en les taxes de creixement i mides de posta s'han detectat entre individus alimentant-se en zones diferents. Aquest fet, a la vegada, s'ha vist que podria estar influenciat amb els patrons de circulació d'aigua de la conca i la trajectòria seguida pels

nounats i juvenils durant la seva migració primerenca. Finalment, aquesta tesi ha remarcat la importància de realitzar estudis a escala regional per entendre les conseqüències de la captura accidental ja que aquestes dependran de l'origen de les tortugues capturades.

L'ESTRUCTURACIÓ EN ZONES DE NIDIFICACIÓ DEL MAR MEDITERRANI DEPÈN DELS MARCADORS EMPRATS

Tot i que certs graus d'estructuració genètica ja havien estat descrits anteriorment al mar Mediterrani (Encalada et al. 1998; Laurent et al. 1998; Carreras et al. 2007; Garofalo et al. 2009; Yilmaz et al. 2011; Saied et al. 2012), l'ús de mostres provinents de zones poc explorades fins al moment (Líbia o Líban) han permès un anàlisi global de tota la conca al *Capítol 1.1* i al *Capítol 1.2*.

L'ús d'anàlisis genètiques com a eina de conservació és molt exitós però els estudis aquí descrits han demostrat que és altament dependent del nombre i tipus de marcadors emprats. Gràcies a l'anàlisi de fragments llargs d'ADNm (*Capítol 1.1*) i un elevat nombre de marcadors microsatèl·lits per ADNn (*Capítol 1.2*) nova informació referent a l'estructuració genètica en zones de nidificació ha estat descrita. Quatre unitats de gestió s'han pogut definir gràcies a l'ús de fragments llargs d'ADNm: Dalyan i Dalaman (Turquia), Líbia, Calàbria (Itàlia), i la resta de zones de nidificació orientals (Israel, Líban, Xipre, la resta de Turquia, Creta i l'oest de Grècia). Els marcadors microsatèl·lits, a més, presenten una major resolució de diferenciació a petita escala i milloren remarcablement la capacitat d'assignació de les tortugues al seu origen, tal i com s'ha vist al *Capítol 3.1* i *4.1*. Així, anàlisis d'ADNn han detectat 5 unitats prèviament no descrites: Líbia i Xipre, Israel, Líban, l'oest de Turquia i Grècia. La present tesis doctoral corrobora doncs la necessitat d'usar un elevat nombre de marcadors genètiques en estudis futurs sobre estructuració poblacional de la tortuga babaua.

DIFERENCIACIÓ GENÈTICA ENTRE ZONES DE NIDIFICACIÓ I COMPORTAMENT REPRODUCTIU

La combinació d'anàlisis d'ADN mitocondrial i nuclear (*Capítol 1.2*) ha demostrat una forta filopatria no només pel què fa a femelles de tortuga babaua sinó també per mascles. Això fa que hi hagi un aïllament genètic per distància entre zones de nidificació i suggereix que l'aparellament s'estaria donant a zones

properes a les zones de nidificació. Tot i que mascles i femelles són filopàtrics, certes excepcions es poden donar. Aquest és el cas de Grècia, on les femelles mostren una forta filopatria i fidelitat a petita escala (Crete i l'oest de Grècia es poden diferenciar amb ADNm) mentre que els mascles faciliten el flux gènic entre aquestes zones de nidificació, fent que no es detecti un diferenciació significativa amb ADNn.

LA IMPORTÀNCIA DELS PATRONS DE CIRCULACIÓ AQUÀTICA EN L'ESTRUCTURACIÓ DE LES ZONES D'ALIMENTACIÓ

Els patrons de circulació d'aigua s'han identificat tradicionalment com a factors físics rellevants en la dispersió de nounats i juvenils de petita talla (Carr i Meylan 1980;. Bolten et al. 1992) degut a la flotabilitat positiva i les limitades habilitats natatòries d'aquests (Milsom 1975). Els resultats presentats al *Capítol 2.1* demostren que les tortugues no es distribueixen homogèniament al mar Mediterrani i que hi ha diferències no només en la distribució de juvenils provinents de zones de nidificació de l'Atlàntic i el Mediterrani, sinó també entre els de colònies mediterrànies. Aquesta distribució heterogènia és consistent amb els principals patrons de corrents d'aigua tant a gran com a petita escala.

Els resultats del MSA suggereixen que els juvenils d'origen atlàntic que es troben a la conca mediterrània provenen principalment de colònies nord-americanes, fet que seria d'esperar tenint en compte que Florida acull la major població nidificant d'aquesta espècie i que el corrent del Golf uneix la costa americana amb Europa. No obstant això, el MSA no detecta joves de Cap Verd a la Mediterrània i això podria semblar sorprenent ja que Cap Verd alberga la segona major població nidificant (Marco et al. 2012), amb 14.000 nius anuals a les seves platges (Laurent et al. 1999). Aquest fet es podria explicar perquè l'arxipèlag està connectat amb el continent americà pel Corrent Equatorial del Nord enlloc de amb el mar Mediterrani (Mansfield i Putman 2013), fet que remarca la rellevància dels corrents en la distribució de juvenils.

Així, a escala regional, els resultats del MSA suggereixen la prevalença de tortugues de l'oest de Grècia al mar Adriàtic fet que reflexa el pas d'un front aquàtic al llarg de la costa occidental grega abans d'entrar al mar Adriàtic. De la mateixa manera, la prevalença de tortugues líbies al mar Jònic podria estar relacionada amb

els remolins presents al Mar Jònic (Robinson et al. 2001; Hamad et al. 2006; Hays et al. 2010), que podrien estar atrapant les cries i juvenils a la sub-conca tot evitant-ne així la seva dispersió pel Mediterrani oriental.

Aquests resultats són congruents amb el fet que els juvenils es distribueixen en funció dels patrons de circulació, tot flotant-hi passivament. No obstant això, els individus mostrejats al *Capítol 2.1* mesuraven entre 30 i 69cm; talles suficients com per què es poguessin dispersar de forma independent dels corrents mediterranis (excepte a l'Estret de Gibraltar i al mar d'Alborà; Revelles et al. 2007d). Conseqüentment, i juntament amb el fet que l'estructuració genètica en zones d'alimentació és molt consistent amb la distribució de les masses d'aigua i el patró dels corrents superficials, podrien existir altres mecanismes que perpetuarien la distribució de cries i juvenils en estadis posteriors.

Estudis recents han assenyalat que la distribució d'adults podria estar relacionada amb els patrons de corrents com a resultat de l'impronta de l'hàbitat en estadi primerencs. Cada vegada hi ha més evidència que les tortugues joves queden improntades pels hàbitats visitats en les seves migracions durant els primers estadis (determinades pels corrents), que al seu torn determinen els hàbitats en els quals reclutaran i on s'alimentaran en estadis adults (Hatase et al. 2002; Hays et al. 2010; Fossette et al. 2010; Eder et al. 2012). Les tortugues d'origen mediterrani comencen a reclutar a zones d'alimentació aproximadament als 40cm de mida (Casale et al. 2008a), fet que suggereix que l'estructuració genètica descrita al *Capítol 2.1* podria demostrar el procés d'impronta. Això però, no podria aplicar-se a les tortugues d'origen atlàntic, ja que les seves zones de nidificació són a més de 6.000 km de les zones d'alimentació mediterrànies usades durant els estadis juvenils. Això es tradueix en un equilibri entre filopatria i coneixement de l'hàbitat, que finalment els porta a sortir del mar Mediterrani una vegada que són prou grans com per superar els corrents al mar d'Alborà i a l'Estret de Gibraltar (Bowen et al. 2005). En conseqüència, les tortugues adultes d'origen atlàntic són molt escasses al mar Mediterrani.

Patrons de circulació i conseqüències derivades de la distribució i els usos de l'hàbitat

La combinació d'esqueletocronologia i anàlisis genètiques al *Capítol 3.1* han desvelat diferents taxes de creixement entre tortugues de diferent origen. Així, les tortugues d'origen atlàntic que s'alimenten al mar Mediterrani presenten menors taxes de creixement, no només en comparació amb les tortugues d'origen mediterrani sinó també en comparació amb les tortugues d'origen atlàntic que no entren a la conca mediterrània. Això podria explicar-se per diferències en l'ús de l'hàbitat i la productivitat de les zones d'alimentació utilitzades. Les tortugues d'origen atlàntic solen ser oceàniques al mar Mediterrani (Carreras et al 2006, 2011) mentre que les tortugues d'origen mediterrani de la mateixa mida solen estar ja assentades en zones nerítiques (més productives que les zones oceàniques; Bosc et al. 2004). En conseqüència, com que les tortugues d'origen mediterrani es recluten abans als hàbitats nerítics, més productius que els oceànics, també creixen més ràpid. Les diferències en la productivitat també podrien explicar per què les tortugues d'origen atlàntic que habiten el mar Mediterrani creixen més lentament que no pas aquelles que no entren a la conca. Com que el mar Mediterrani és oligotròfic en comparació amb les aigües nerítiques de l'Atlàntic (Longhurst, 1998), això podria estar afectant les taxes de creixement i el temps de residència de les tortugues atlàntiques que entren a la conca mediterrània. Això, a la vegada, pot tenir conseqüències importants en les tortugues que s'alimenten al mar Mediterrani ja que aquestes s'enfronten a altes taxes de captura accidental que podrien tenir un impacte negatiu notable en les poblacions de l'Atlàntic (vegeu més endavant).

Pel què fa a la distribució al mar Mediterrani de les tortugues d'origen mediterrani, l'anàlisi d'isòtops estables ha permès localitzar les zones d'alimentació de les femelles nidificants a través de l'anàlisi de nounats morts al *Capítol 3.2*. L'anàlisi d'isòtops estables ha identificat el sud del mar Jònic com la principal zona d'alimentació per a la majoria de les colònies estudiades (tot i presentar algunes de les zones menys productives de la conca; Bosc et al. 2004). Per contra, el mar Adriàtic i nord del Jònic, altament productius, només són principalment utilitzats per les tortugues nidificant a les costes gregues (vist també per Zbinden et al. 2011) i rarament per tortugues d'altres zones (com a Margaritoulis i Rees 2011; Patel et al. 2012; Hochscheid et al. 2012). L'ús limitat dels mars Adriàtic/Jònic nord per les

femelles de colònies diferents de Zakynthos i Lakonikos és intrigant perquè el mar Adriàtic és de fet la zona més productiva de l'oest del mar Mediterrani i les femelles que allà s'alimenten són més grans i posen més ous que les femelles que s'alimenten en altres zones (Margaritoulis et al. 2003). Si la distribució de la tortuga babaua depengués només d'un equilibri entre la disponibilitat d'aliments i la distància a la zona de nidificació, s'esperaria que les tortugues de les costes orientals s'alimentessin als mars Adriàtic/Jònic nord i al mar Jònic sud en proporcions iguals. Tot i així, això sembla ser cert només a l'oest de Grècia. L'explicació a aquesta distribució en concret també podria restar en la hipòtesi de la impronta de l'hàbitat.

Les diferències trobades entre la distribució de les diferents poblacions són molt congruents amb els patrons de corrents descrits al mar Mediterrani. Així, la majoria de les tortugues podrien estar alimentant-se al mar Jònic sud, encara que menys productiu, com a conseqüència de que el mar Adriàtic es troba en una posició perifèrica dins de les principals corrents superficials de la conca (Hamad et al. 2006) i, per tant, desconeguda per la major part de les tortugues provinents de les costes orientals. Per contra, el mar Adriàtic és de fàcil accés per a les cries de les platges gregues ja que aquests es troben, en arriba a l'aigua, amb un corrent d'aigua bifurcat, amb una branca que flueix cap al nord al Mar Adriàtic i un altre que flueix cap al sud-est (Hays et al. 2010). Com a conseqüència, la meitat de les tortugues adultes que surten de Grècia occidental migren al mar Jònic després de nidificar i l'altra meitat cap al mar Adriàtic (Zbinden et al 2011; Schofield et al. 2013).

És important destacar que, si la hipòtesi de l'impronta de l'hàbitat és certa, les rutes individuals seguides durant les primeres etapes com a nedadors passius podrien explicar la variabilitat observada al *Capítol 3.2* entre la distribució dels individus d'una mateixa població. Els resultats presentats suggereixen que les diferències entre les zones de nidificació no es mostren a nivell de població sinó a nivell individual. Així, les tortugues que s'alimenten en zones d'alimentació d'alta productivitat nidifiquen a les mateixes platges que aquelles tortugues que s'alimenten en zones menys productives. En funció dels corrents trobats i la estocasticitat dels fenòmens naturals, les cries d'una mateixa platja de nidificació seguiran diferents rutes migratòries (Wyneken et al. 2008; Hays et al. 2010; Putman et al. 2012a). Això resulta en una alta variabilitat de zones visitades i diferències en

el coneixement individual de la conca (McClellan i Lee 2007; McClellan et al. 2010); fets que poden influir en el moment de reclutar a una zona d'alimentació concreta seguint l'experiència pròpia i el coneixement de l'heterogeneïtat de l'habitat.

Els patrons de distribució descrits al *Capítol 3.2* (i també al *Capítol 2.1*) posen així de manifest l'existència d'un fort vincle entre les zones d'alimentació utilitzades per les tortugues i la ubicació de les seves colònies. Això té conseqüències sobre el rendiment reproductiu ja que s'ha trobat una forta correlació entre la mida mitjana de la posta i la senyal isotòpica de les femelles nidificants, fet que alhora depèn de la zona on s'alimenten aquestes. Conseqüentment, les femelles que s'alimenten als mars Adriàtic/Jònic nord tenen més nombre d'ous per niu que les femelles de la mateixa població que s'alimenten en zones menys productives com podria ser el mar Jònic sud. A més, les zones d'alimentació utilitzades no només poden tenir un efecte sobre l'eficàcia biològica de les poblacions de tortugues babaues que nidifiquen al mar Mediterrani sinó que també poden afectar la seva probabilitat de supervivència.

LA TORTUGA BABAUA I LES PESQUERIES MEDITERRÀNIES

Les taxes de captura incidental són molt variables dins del mar Mediterrani (Casale 2011) i l'impacte de les interaccions amb la pesca sobre les poblacions de tortuga babaua que s'hi alimenten dependrà de la superposició entre les zones de pesca i la distribució de les tortugues i també de la taxa de natalitat de les poblacions involucrades (Wallace et al. 2008, 2013). L'últim capítol de la present tesi (*Capítol 4.1*) ha permès un profund anàlisi de la composició de la captura accidental de tortugues al mar Mediterrani i, mitjançant l'anàlisi d'isòtops estables i les assignacions individuals a través d'anàlisis genètiques, s'ha descobert l'ús de l'hàbitat i l'origen d'aquestes, respectivament.

Els dos apropaments metodològics demostren que arts de pesca oceànics (palangres de superfície) i nerítics (arrossegament de fons i tremall) utilitzats dins d'una mateixa regió capturen tortugues provinents de les mateixes poblacions. Per tant, s'han detectat diferències en la composició de la captura accidental entre les regions però no entre els arts de pesca dins de cada regió. Aquests resultats

suggereixen que les diferències en la caracterització genètica de les captures en palangre i arrossegament prèviament descrites per Laurent et al. (1998) podrien haver sorgit degut a què els arts de pesca comparats provenien de regions diferents i no pas perquè poblacions diferents usessin habitats diferents. Així, els resultats del *Capítol 4.1* remarquen que la comparació entre arts de pesca de diferents regions s'ha d'evitar en estudis futurs per tal d'eliminar-ne el biaix.

La composició de la captura accidental trobada al Mediterrani mostra que l'impacte de la pesca depèn altament de la superposició entre les zones de pesca i zones d'alimentació. D'aquesta manera, la distribució de les poblacions de tortuga babaua, determinades pels corrents i els coneixements adquirits durant les migracions primerenques (d'acord amb els *Capítols 2.1* i *3.2*) modula la susceptibilitat a la captura accidental en funció de la zona d'alimentació utilitzada. Conseqüentment, les tortugues d'origen atlàntic representen una gran proporció de la captura accidental de tortugues al sud de les Illes Balears i a la conca algeriana degut a que aquesta zona ha estat descrita com un *hot spot* de juvenils atlàntics (*Capítol 2.1*; Laurent et al. 1993; Carreras et al. 2006, 2011; Monzón-Argüello et al. 2009, 2010) a causa dels patrons específics de la circulació de les masses d'aigua (Revelles et al. 2007d). De la mateixa manera, la proporció de tortugues d'origen atlàntic disminueix en la captura incidental al llarg del corrent ciclònic principal que va des de l'Estret de Gibraltar fins al mar Adriàtic (Carreras et al. 2006; Maffucci et al. 2006).

El fet que arts de pesca oceànics i nerítics estiguin capturant juvenils d'origen atlàntic i mediterrani també suggereix que no només les tortugues d'origen mediterrani ocupen els hàbitats nerítics de la Mediterrània, tal i com es pensava anteriorment (Laurent et al. 1998). Degut a que individus atlàntic són capturats en ambdós habitats, l'impacte que la pesca mediterrània pot tenir en les poblacions de l'Atlàntic pot ser més alt del què es pensava. El nombre de femelles que nidifiquen al sud de Florida ha disminuït un 43% des de 1998 tot i que el nombre de tortugues verdes i llaüt que nidifiquen a les mateixes platges ha incrementat (Witherington et al. 2009). Com a conseqüència d'això i, degut a que la causa de la disminució no es troba a les platges de nidificació, la captura accidental de la Mediterrània (entre altres amenaces que afecten les tortugues en altres àrees d'alimentació) podria explicar part d'aquest notable descens. El Mediterrani occidental és l'àrea amb la

major pressió de pesca de palangre (Casale 2011) i és aquí on es troben les contribucions atlàntiques més altes de tortuga babaua (*Capítol 2.1* i *Capítol 4.1*). Si es té en compte que l'esforç pesquer en aquesta zona va assolir el seu punt màxim a principis de 1990 (Farrugio et al. 1993), creiem que la forta caiguda en el nombre de femelles que nien a Florida observada des de 1998 podria ser deguda, en part, a les altes taxes de captura accidental en la Mediterrani occidental .

A més, tal i com s'ha vist al *Capítol 3.1*, les tortugues d'origen atlàntic que s'alimenten al mar Mediterrani es desplacen a hàbitats nerítics a l'Atlàntic nord-occidental a una edat molt més tardana que els que romanen a les aigües de l'Atlàntic. Per tant, les tortugues babaues d'origen atlàntic que entren al mar Mediterrani estan exposades a alts nivells de mortalitat accidental durant un temps molt més llarg (Álvarez de Quevedo et al. 2013), fet que podria augmentar els efectes negatius de la captura accidental en la població. No obstant això, la rellevància d'aquesta mortalitat en les unitats de gestió americanes dependrà de la proporció de tortugues babaues que entrin al mar Mediterrani i això encara ens és desconegut.

Pel què fa a l'impacte de la pesca sobre les poblacions del Mediterrani, els resultats del MSA del *Capítol 2.1* suggereixen que dependrà de la contribució específica de cada població a les zones d'alimentació compartides i la taxa de captura accidental en cada una d'aquestes zones. D'aquesta manera, la Mediterrània occidental no només podria ser una amenaça per a les poblacions que nidifiquen a Amèrica del Nord sinó també per aquelles de Líbia i, particularment, de Misurata. De la mateixa manera, la captura accidental al mar Adriàtic (que deriva principalment de la pesca d'arrossegament; Casale 2011) podria afectar principalment a la població nidificant a l'oest de Grècia, mentre que la captura accidental al mar Llevantí podria afectar les poblacions que nidifiquen a Turquia, Líban i Israel. Tot i que la captura accidental pot tenir un impacte negatiu en les poblacions nidificants, la magnitud d'aquest impacte pot variar segons l'esforç de pesca, el tipus d'art, el temps d'immersió i la profunditat dels art. A més, tot i que tortugues de diferent origen siguin capturades per arts de pesca oceànics i nerítics, tots aquests factors tenen la seva taxa de captura i mortalitat associada (Lewison et al. 2004a) i, per tant, l'impacte que la pesca pugui tenir sobre aquestes poblacions pot variar significativament depenent de la naturalesa de la pesca. D'altra banda , la

mida de la població és també un factor rellevant a l'hora de determinar els impactes de la pesca sobre una població. Els resultats d'isòtops estables que es presenten en el *Capítol 3.2* mostren que la mida de posta està altament correlacionada amb la productivitat de la zona d'alimentació. En colònies on una gran proporció de femelles s'alimenten en terrenys de baixa productivitat, com Israel o Xipre, els nius tenen menys ous i per tant, la importància demogràfica de les captures accidentals pot ser majors per a aquestes colònies. No obstant això, les mateixes taxes de captura podrien no ser perjudicials per a altres poblacions (per exemple Grècia, amb un elevat nombre d'ous per niu). Conseqüentment, estudis futurs haurien d'intentar calcular les taxes de mortalitat de cada unitat de gestió en cada zona en concret i modelar-ne les seves conseqüències demogràfiques a escala local.

Per tal de reduir la captura de tortugues babaues al mar Mediterrani, les flotes pesqueres haurien de prendre precaucions específiques per intentar reduir les captures accidentals però també els governs haurien d'implementar regulacions legals més fortes que les actuals per controlar i minimitzar aquestes captures. Algunes de les mesures de reducció de la captura accidental inclouen la modificació dels ormejos o el tipus d'esquer emprat, establir restriccions geogràfiques i variar el moment i la profunditat de pesca (Gilman et al. 2006). En el cas de les pesqueries de palangre de superfície, el canvi dels hams en J usats tradicionalment per a hams circulars, més grans, disminueix la probabilitat d'ingestió i captura de tortugues sense afectar les taxes de captura de pesca (Watson et al. 2005; Swimmer et al. 2011). El tipus d'esquer també pot ser rellevant en aquest context ja que l'ús d'esquers com el calamar implica probabilitats de captura accidental més altes que no pas l'esquer de peix a causa de l'elasticitat i resistència dels teixits de calamar (Gilman et al. 2006, 2010). Degut a aquestes característiques, l'ús de calamar com a esquer empeny a les tortugues a mossegar l'ham diverses vegades alhora que augmenta el risc acumulat de lesions i ingestió (Gilman et al. 2006). Això no succeeix amb l'esquer de peix, amb teixits més tous i fàcils d'arrencar de l'ham. La profunditat en la qual es fixen els hams també és rellevant en les taxes de captura i mortalitat associades als palangres de superfície. Així, els palangres instal·lats a menys de 50m de profunditat tenen majors taxes de captura que aquells més profunds ja que les tortugues passen la major part del seu temps dins dels primers 40 metres de la columna d'aigua (Polovina et al. 2003). Finalment, pujar a bord

totes les tortugues capturades accidentalment i extreure'n els hams enganxats també ajudaria a reduir dràsticament les taxes de mortalitat post-alliberament (Álvarez de Quevedo et al. 2013).

Pel què fa a la pesca d'arrossegament en zones nerítiques, la mesura de mitigació de captura accidental més exitosa és l'ús de dispositius TED, que permeten a les tortugues escapar de les xarxes d'arrossegament en cas de quedar atrapades. Com que la mortalitat associada a la pesca d'arrossegament és generalment causada per asfíxia, tot i que depèn en gran mesura del temps d'immersió (Robins-Troeger et al. 1995), els TED són eines de gestió eficaces que permeten a les tortugues escapar de la xarxa a través d'una finestra i sortir a la superfície per respirar. Els TED han estat àmpliament utilitzats en aigües dels Estats Units i el ple compliment i la correcta aplicació de les lleis associades han permès una dràstica reducció de captures accidentals en les últimes dues dècades (Finkbeiner et al. 2011). No obstant això, els TED podrien resultar en una significativa reducció dels desembarcaments de peix al mar Mediterrani degut a que les seves flotes de pesca d'arrossegament persegueixen espècies de peix de grans dimensions. Conseqüentment, la limitació en el temps d'immersió de les xarxes en lloc d'utilitzar TEDs podria ser una regulació més exitosa en certes regions de la conca mediterrània (Álvarez de Quevedo et al. 2010).

Tot i que ja s'ha demostrat en altres indrets del món que aquestes mesures de mitigació redueixen notablement les taxes de captura de tortugues babaues, aquestes encara no es tenen en consideració en molts dels països mediterranis. Si les poblacions de tortugues babaues de l'Atlàntic i del mar Mediterrani han de ser preservades, es requereix una forta implementació nivell legal i social amb l'objectiu de garantir una pesca sostenible. La reducció de la flota pesquera, la restricció de les temporades de pesca, la disminució del temps d'immersió o la promoció de la utilització d'hams i esquers específics són molt recomanables a la Mediterrània per disminuir la captura accidental de tortugues. Només amb això i un coneixement profund sobre els patrons de distribució de la tortuga babaua i els seus usos de l'hàbitat en permetran la seva conservació a la conca. No obstant això, també cal tenir present la necessitat de protegir i millorar la pesca artesanal si es vol que aquests canvis s'acceptin i es portin a terme per les flotes mediterrànies.

EFECTES POTENCIALS DEL CANVI CLIMÀTIC AL MAR MEDITERRANI

L'escalfament global i els seus efectes col·laterals han estat motiu de preocupació durant l'última dècada. S'ha pronosticat que la temperatura de l'aire haurà augmentat de 1.1 a 2.9ºC al 2099 (IPCC 2007) i, amb ella, també la temperatura del mar i la temperatura de la sorra de les platges de nidificació de les tortugues babaues.

Les tortugues marines s'han adaptat a fluctuacions climàtiques anteriors (Dutton et al. 1999; Encalada et al. 1996; Reece et al. 2005) i els resultats genètics del *Capítol 1.1* suggereixen que aquest també ha estat el cas al mar Mediterrani, on les tortugues babaues podrien haver sobreviscut les èpoques glacials plistocèniques en refugis càlids de la costa nord d'Àfrica. No obstant això, la velocitat de la fluctuació del clima i els nivells de pressió humana han canviat notablement des de llavors. Degut a que la nidificació és altament depenent de la temperatura, s'esperaria que algunes poblacions de tortugues babaues s'estenguessin cap al nord, (en àrees actualment massa fredes per a la nidificació) a mesura que augmentés la temperatura. Malauradament, la major part de la costa nord del mar Mediterrani està intensament explotada per la indústria del turisme i només queden algunes platges adequades per a la implantació de noves poblacions en l'actualitat (Mazaris et al. 2009). D'altra banda, la superfície total de les platges podria disminuir a mesura que augmenti el nivell del mar i els edificis, carreteres i altres infraestructures impedeixin a les platges retrocedir cap a l'interior. En aquest context, s'espera que la competència entre la indústria turística i les tortugues babaues nidificants augmenti, amb resultats incerts per a les tortugues babaues.

Amb l'augment de la temperatura de la sorra, la proporció de sexes pot ser també altament afectada a causa de la determinació sexual depenent de la temperatura d'aquesta espècie (Hawkes et al. 2009). Temperatures més altes poden conduir a grans canvis en la proporció de sexes, esbiaixant-la cap a la producció majoritària de femelles. Una disminució en la producció masculina podria dur a una pèrdua de diferenciació genètica entre zones nidificants tal i com sembla ocórrer a Xipre al *Capítol 1.2*. A mesura que el nombre de mascles disminueix, l'aparellament oportunista en zones d'alimentació podria homogeneïtzar la diversitat genètica de certes poblacions. La rellevància d'aquests efectes serà

potencialment més fort en les poblacions petites, com les presents en les zones de nidificació del llevant (Israel i el Líban; Margaritoulis et al 2003), poblacions que ja han estat severament reduïdes causa de l'explotació directa durant la dècada de 1920 (Sella 1982).

A més d'aquests impactes, el canvi climàtic també podria afectar fortament a les poblacions de tortuga babaua a través d'una notable variació dels patrons de circulació d'aigua. Com se suggereix en la present tesi, la distribució de tortugues babaues i, conseqüentment, la seva eficàcia biològica i probabilitat de supervivència, està estretament lligada als patrons de corrents aquàtics. L'escalfament climàtic podria conduir a canvis en els patrons globals de vent i en la força, la direcció i el comportament dels principals sistemes de corrents d'aigua (Hoegh-Guldberg, 2011). Com a conseqüència, la biologia, el comportament migratori i el rendiment reproductiu de *Caretta caretta* podrien veure's severament afectats per aquests canvis. No obstant això, la baixa predicibilitat d'aquests canvis ambientals i l'escàs coneixement sobre com les tortugues responen a les variacions permanents en els corrents d'aigua fan que sigui impossible predir els efectes d'aquests impactes.

ESTUDIS FUTURS

Pel què fa a l'estructuració genètica de les poblacions de tortugues babaues, els resultats aquí presentats han remarcat la necessitat d'utilitzar un gran nombre de marcadors en estudis futurs. Encara que l'ús de marcadors microsatèl·lits en la investigació de tortugues marines ha anat augmentant lentament durant aquesta dècada (Carreras et al. 2007, 2011; Monzón-Argüello et al. 2008; Garofalo et al. 2013), encara hi ha un predomini d'estudis que només se centren en l'anàlisi d'ADNm. A més, la majoria d'ells utilitzen fragments curts d'ADNm i per tant en limita la seva resolució tal i com s'ha vist al *Capítol 1.1*. Conseqüentment, l'anàlisi de tortugues de certes àrees amb marcadors que amplifiquin fragments llargs d'ADNm seria altament recomanable. Això, juntament amb l'ús de diversos marcadors microsatèl·lits, ajudaria a augmentar la resolució de la diferenciació genètica no només entre les zones de nidificació sinó també entre les zones d'alimentació. La caracterització genètica de colònies no mostrejades amb llargs fragments d'ADNm podria també, potencialment, permetre el descobriment de

nous haplotips exclusius i per tant millorar les estimes dels MSA en disminuir el nombre d'haplotips orfes (que s'han trobat a àrees d'alimentació però no en platges de nidificació). Només aconseguint un coneixement complet sobre l'estructuració genètica de les àrees de nidificació podran les assignacions individuals i els MSA ser conclusius.

Per desentranyar algunes de les incerteses plantejades al *Capítol 1.2*, nous estudis sobre la distribució de mascles serien recomanables. La telemetria per satèl·lit de mascles adults seria idònia. No obstant això, la necessitat de disposar d'un gran suport econòmic per capturar i marcar els mascles al mar fa que les investigacions anteriors s'hagin centrat principalment en femelles adultes, fàcils de marcar durant la posta d'ous en platges monitoritzades (Godley et al. 2008).

Referent a l'estructura poblacional al mar, el seguiment per satèl·lit de les cries des de zones de nidificació sabudes podria provar directament la relació entre els patrons de circulació d'aigua i de distribució dels juvenils en la conca. La telemetria per satèl·lit també permetria corroborar la hipòtesi presentada als *Capítols 2.1* i *3.2* sobre el reclutament d'individus basat en els coneixements previs sobre heterogeneïtat de l'hàbitat. Així, individus marcats i seguits per satèl·lit durant les primeres etapes del desenvolupament podrien ser re-capturats en estadis tardans o adults i comparar-ne les seves distribucions actuals amb les trajectòries seguides anteriorment. En cas de corroborar doncs la hipòtesi, des del punt de vista de la conservació, això podria ser utilitzat per predir futures distribucions de poblacions específiques i desenvolupar plans de gestió de disseny específic.

Tanmateix, fins i tot en el cas de conèixer tot això, la conservació d'aquesta espècie encara no seria possible sense una avaluació acurada dels impactes humans que afecten les tortugues babaues al mar Mediterrani. Estimes fiables sobre les taxes de captura, la distribució de les flotes pesqueres, el nombre d'hams utilitzats i els temps d'immersió són encara escasses en la majoria dels països mediterranis i en alguns fins i tot són inexistents (Casale i Margaritoulis 2010). Degut a que l'impacte de la pesca sobre poblacions de tortugues de diferent origen depèn de la distribució d'aquestes poblacions i la superposició entre les zones de pesca i les zones d'alimentació de les tortugues (*Capítol 4.1)*, cal un control i una regulació més

estricta des dels partits governants per permetre una millor comprensió d'aquestes interaccions.

D'altra banda, estudis futurs s'haurien de centrar també en la modelització dels efectes de la mortalitat per captura accidental en la dinàmica poblacional de les tortugues. Per a això, variables com ara la mortalitat associada a cada art de pesca, les taxes de mortalitat natural, la durada de cada etapa del cicle de vida i la seva distribució han de ser revelades amb anterioritat. Aquests paràmetres ja són coneguts per algunes poblacions i arts de pesca utilitzats al mediterrani (Casale et al. 2007; Casale et al. 2008a; Álvarez de Quevedo et al. 2013) però per determinar del cert si les pesqueries mediterrànies estan causant l'abrupte declivi enregistrat a Florida, cal conèixer bé el nombre de tortugues que entren a la conca cada any. Això, juntament amb les contribucions de l'Atlàntic estimades en diferents zones d'alimentació mediterrànies al *Capítol 2.1* i les taxes de captura accidental prèviament publicades (Casale 2011) permetran fer prediccions fiables utilitzant models de poblacionals. El mateix s'hauria d'aplicar també a les poblacions mediterrànies.

Finalment, degut a que aquesta és una espècie altament migratòria, les diferents amenaces poden afectar poblacions de tortugues babaues en llocs molt distants, cadascun d'ells amb les seves pròpies amenaces associades. Per tal d'obtenir un panorama fiable de tota la Mediterrània, la cooperació internacional és fonamental i, per tant, el desenvolupament d'aquesta tesi ha estat molt lligat a nombrosos coautors internacionals que van col·laborar en el mostreig i discussió dels resultats. Només d'aquesta manera, els futurs plans de conservació a escala local i internacional podran tenir èxit i la supervivència d'aquesta fascinant espècie podrà ser assegurada.

CONCLUSIONS

- La tortuga babaua colonitzà el mar Mediterrani des de l'Atlàntic fa aproximadament 65.000 anys (20.000-200.000), durant el Plistocè, i va sobreviure les fases més fredes en refugis temperats de la costa nord-Africana.

- L'estructura genètica actual a les zones de nidificació mediterrànies de *Caretta caretta* reflecteix els processos de colonització i extincions locals durant les glaciacions plistocèniques i posteriors re-colonitzacions des de refugis temperats.

- El flux gènic mediat per mascles és molt limitat entre zones de nidificació i l'estructuració genètica basada en ADNn demostra la presència d'una forta filopatria tant en mascles com en femelles al mar Mediterrani.

- L'aparellament sembla ser que estaria succeint en àrees properes a les zones de nidificació tot i que aparellaments esporàdics en zones d'alimentació també es podrien donar. La detecció d'aquest aparellament oportunista podria dependre de la competència d'esperma en les àrees de reproducció, influenciat pel nombre de mascles presents.

- La distribució de juvenils d'origen atlàntic i mediterrani no és homogènia a la conca mediterrània: hi ha una prevalença de juvenils d'origen atlàntic a la conca algeriana, de Líbia a la resta del Mediterrani occidental i al Mediterrani oriental, de Grècia al mar Adriàtic i de les platges orientals (Turquia, Líban i Israel) al sud del mar Llevantí.

- Els resultats presents en aquesta tesi són congruents amb la hipòtesi de que les tortugues joves queden improntades pels hàbitats que visiten durant la seva migració primerenca (determinada per corrents d'aigua) que, al seu torn, determinen els hàbitats als quals reclutaran en l'edat adulta. Conseqüentment, la forta estructuració genètica que es troba en zones d'alimentació reflecteix els principals corrents presents a la conca mediterrània.

- Diferències en la distribució i ús de l'habitat de tortugues de diferent origen pot portar a diferències en la taxa de creixement i el rendiment reproductiu.

- Les tortugues d'origen atlàntic presenten taxes de creixement més baixes en comparació a les tortugues mediterrànies però també en comparació a les tortugues d'origen atlàntic que no entren al mar Mediterrani. Diferències en la productivitat dels diferents habitats podrien explicar aquesta variació intra- i inter-poblacional.

- Entre les tortugues d'origen mediterrani existeix una forta correlació entre la productivitat de les zones d'alimentació i el rendiment reproductiu (mida de la posta). Així, tortugues alimentant-se en zones altament productives com els mars Adriàtic/Jònic nord presenten nius amb un elevat nombre d'ous.

- Tot i que els mars Adriàtic/Jònic septentrional són els més productius de la conca, aquests són usats només per tortugues nidificant a Grècia. Per contra, el sud del mar Jònic (menys productiu) és usat per la majoria de femelles nidificants a tot l'oest de la conca. L'explicació a aquesta particular distribució també podria trobar-se en la impronta causada pels hàbitats visitats durant les fases inicials de la migració de desenvolupament .

- La composició isotòpica i genètica de les tortugues capturades accidentalment a les pesqueries mediterrànies demostren que els arts de pesca nerítics i oceànics emprats a una mateixa regió no difereixen en l'origen de les tortugues capturades. En canvi, sí hi ha diferències en la composició poblacional de les captures accidentals entre zones hidrogràficament distintes.

- La presència habitual de tortugues d'origen atlàntic en la captura accidental dels arts de pesca nerítics demostra que la plataforma continental no és utilitzada únicament per tortugues d'origen mediterrani, com s'havia cregut tradicionalment.

- L'heterogeneïtat regional en l'origen de les tortugues capturades accidentalment posa de manifest que els impactes de la pesca dependran de la distribució de cada població de tortugues, del seu ús de l'habitat, de la superposició entre zones de pesca i zones d'alimentació i la mortalitat associada a cada art de pesca.

INFORME DELS DIRECTORS SOBRE LA CO-AUTORIA I FACTOR D'IMPACTE DE LES PUBLICACIONS DERIVADES DE LA TESI

El doctorand Marcel Clusa Ferrand ha participat activament en el desenvolupament de la feina associada a cadascun dels articles presentats en aquesta tesi doctoral.

En concret, la seva participació ha consistit en:

- Plantejament d'objectius
- Processat de mostres
- Planificació i anàlisi de laboratori
- Anàlisi de resultats obtinguts
- Redacció i revisió dels articles

Val a dir que cap informació d'aquestes publicacions ha estat ni serà utilitzada en altres Tesis Doctorals exceptuant l'atricle titolat *Different growth rates between loggerhead sea turtles (Caretta caretta) of Mediterranean and Atlantic origin in the Mediterranean Sea*. Aquest article també forma part de la tesi doctoral de la seva primera autora, Susanna Piovano, de la Universitat de Turí (Itàlia). La contribució en aquest article del doctorand Marcel Clusa Ferrand ha estat l'anàlisi genètic i estadístic de les mostres usades per Susanna Piovano per a fer esqueletocronologia, juntament amb la redacció i revisió de la secció de genètica del manuscrit.

Barcelona, 5 de desembre de 2013

Els Directors de la Tesi:

Dr. Lluís Cardona Pascual
Departament de Biologia Animal
Universitat de Barcelona

Dra. Marta Pascual Berniola
Departament de Genètica
Universitat de Barcelona

LLISTAT D'ARTICLES INCLOSOS:

Clusa M, Carreras C, Pascual M, Demetropoulos A, Margaritoulis D, Rees AF, Hamza AA, Khalil M, Aureggi M, Levy Y, Türkozan O, Marco A, Aguilar A, Cardona L (2013) Mitochondrial DNA reveals Pleistocenic colonisation of the Mediterranean by loggerhead turtles (*Caretta caretta*). *Journal of Experimental Marine Biology and Ecology* 439, 15-24. **Factor d'impacte: 2.263**

Clusa M, Carreras C, Cardona L, Demetropoulos A, Margaritoulis D, Rees AF, Hamza AA, Khalil M, Levy Y, Türkozan O, Aguilar A, Pascual M (submitted) Philopatry in loggerhead turtles (*Caretta caretta*): beyond the gender paradigm. Article enviat pendent de decisió en primera revisió.

Clusa M, Carreras C, Pascual M, Gaughran J, Piovano S, Giacoma C, Fernández G, Levy Y, Tomás J, Raga JA, Maffucci F, Hochscheid S, Aguilar A, Cardona L. (2013) Fine-scale distribution of juvenile Atlantic and Mediterranean loggerhead turtles (*Caretta caretta*) in the Mediterranean Sea. *Marine Biology* (en premsa, publicat online) DOI: 10.1007/s00227-013-2353-y. **Factor d'impacte: 2.468**

Piovano S, Clusa M, Carreras C, Giacoma C, Pascual M, Cardona L (2011) Different growth rates between loggerhead sea turtles (*Caretta caretta*) of Mediterranean and Atlantic origin in the Mediterranean Sea. *Marine Biology* 158, 2577-2587. **Factor d'impacte: 2.468**

Cardona L, Clusa M, Eder E, Demetropoulos A, Margaritoulis D, Rees AF, Hamza AA, Khalil M, Levy Y, Türkozan O, Marín I, Aguilar A. (2013) Distribution patterns and foraging ground productivity determine clutch size in Mediterranean loggerhead turtles. *Marine Ecology Progress Series* (en premsa) DOI: 10.3354/meps10595. **Factor d'impacte: 2.546**

Clusa M, Carreras C, Pascual M, Gaughran J, Piovano S, Avolio D, Ollano G, Fernández G, Levy Y, Tomás J, Raga JA, Aguilar A, Cardona L. (en revisió) Population make-up of turtle bycatch in the Mediterranean Sea: relevance of fishing ground and fishing gear. Article en revisió final pels altres autors i pendent d'enviar.

Population structure of *Caretta caretta* in the Mediterranean Sea: from nesting beaches to foraging grounds

General Introduction and Objectives

GENERAL INTRODUCTION

Oceans cover a wide proportion of the planet, hosting 50-80% of the planet's biodiversity (Sala and Knowlton 2006) and regulating many biochemical and physical vital processes (Falkowski et al. 2008). With more than 40% of the world's population living in coastal areas, oceans have become an indispensable resource for humankind (IOC/UNESCO 2011) but many coastal and marine regions are suffering from multiple anthropogenic threats. Pollution, coastal tourism, agricultural practices, development of ports, manufacturing and aquaculture threaten marine species worldwide and have caused the decline of many species, some of them currently on the verge of extinction (Gray 1997).

Fisheries are responsible for many of these declines, directly affecting populations through the overfishing of their stocks or indirectly through habitat degradation, particularly for benthic species (Pauly et al. 2005). However, fisheries not only may affect targeted species but also un-targeted species, threatened through overfishing of food resources or lethal direct interactions. Bycatch, the unintentional catching of non-targeted species during fishing operations (Hall et al. 2000), has been described as one of the most important threats causing the decline of many species, specially of large marine vertebrates (Fig. 1): sharks (Dulvy et al. 2008), sea turtles (Wallace et al. 2013), birds (Tasker et al. 2000) and marine mammals (Read et al. 2006). This is because large marine vertebrates are the most vulnerable group to bycatch due to their life-cycle characteristics, presenting a long lifespan, late age at maturity and low reproductive output (Lewison et al. 2004a). These characteristics make of large marine vertebrates a sensitive group as they require high rates of sub-adult and adult survival to overcome their low fecundity and these are the stages typically affected by bycatch (Heppell et al. 1999).

As a consequence of these population declines due to anthropogenic interactions, many species of large marine vertebrates are listed as endangered in the IUCN Red List of Threatened Species (http://www.iucnredlist.org). However, conservation needs for marine megafauna are particularly difficult to assess as these species usually occur in remote oceanic habitats, are distributed across entire oceans and often have complex life cycles involving migrations that cover thousands of

kilometres (Bayliff 1994; Croxall et al. 2005). Thus, because sea turtle life stages may occur in different areas, a detailed knowledge on the life cycle, distribution and habitat use of these species is highly relevant as populations might face different anthropogenic impacts depending on the areas used.

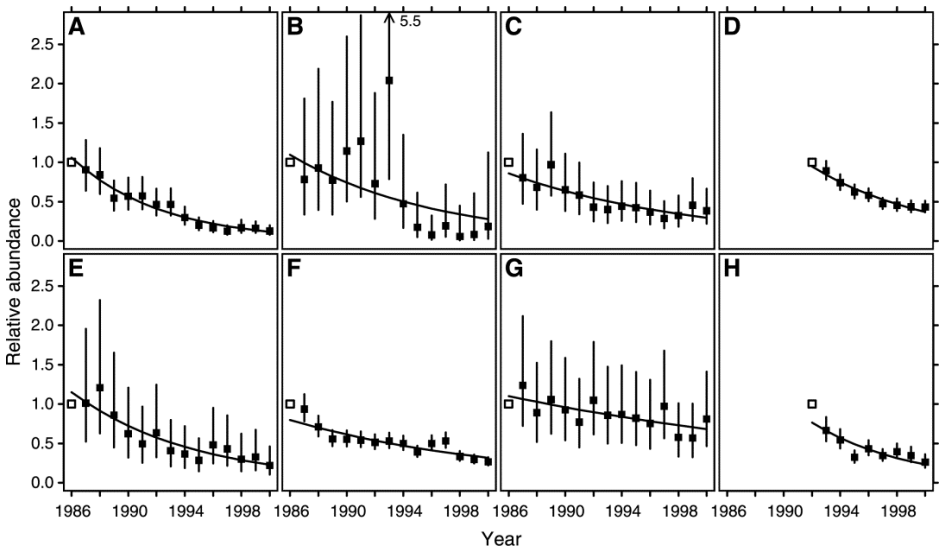

Fig.1. Declines in estimated relative abundance for coastal shark species: (A) hammerhead, (B) white, (C) tiger, and (D) coastal shark species identified from 1992 onward; and oceanic shark species: (E) thresher, (F) blue, (G) mako, and (H) oceanic whitetip. For each species, the overall trend (solid line) and individual year estimates (squares ± 95% CI) are shown. Extracted from Baum et al. (2003).

SEA TURTLES: COMPLEX LIFE CYCLES

Sea turtles are marine reptiles that are partly tied to the terrestrial environment for reproduction, with male turtles never abandoning the aquatic domain but with females emerging to nesting beaches for oviposition (Pritchard 1997). Seven species of sea turtles exist (the loggerhead turtle, *Caretta caretta*; the green turtle, *Chelonia mydas*, the leatherback turtle, *Dermochelys coriacea*; the hawksbill turtle, *Eretmochelys imbricata*; the kemp's ridley, *Lepidochelys kempi*; the olive ridley, *Lepidochelys olivacea*; and the flatback turtle, *Natator depressus*) and most of them share similar life cycles, with the exception of the leatherback and the flatback turtle. Sea turtles life cycle consists of a first stage as early juveniles drifting in oceanic habitats, followed by a later juvenile developmental stage that is neritic. Once turtles reach sexual maturity, they recruit to adult foraging habitats from where they might seasonally migrate to breeding areas to mate, probably close to their natal beaches (Bowen et al. 2005). After mating, females will nest in sandy beaches and later migrate to adult foraging grounds while males will return to the

adult foraging grounds directly after mating (Arendt et al. 2012a; Schofield et al. 2010).

Of all sea turtle species, the loggerhead turtle might present the most complex cycle described (Box 1; Fig. 2), thoroughly studied through the use of satellite telemetry, mark-recapture techniques, stable isotope analyses and genetics (Bolten 2003; Mansfield and Putman 2013).

Box 1.

1.- Stage I: Hatching and emergence – Terrestrial Zone

- Hatchlings emerge of the nest, usually at night or early morning to reduce risk of predation, and venture to the water.

2.- Stage I: Hatchling swim frenzy – Neritic Zone

- Hatchlings enter in the water and actively swim for approximately 48h, a period known as the *swim frenzy*.
- It allows hatchlings to reach the major offshore currents.
- During this period, hatchlings do not feed as they are nutritionally dependent on the remains of their yolk.

3.- Stage I: Post-hatchling transitional stage – Neritic Zone

- Hatchlings start to feed and spend most of the time at the surface.
- During this stage, hatchlings passively drift with surface marine currents.
- It can last from a few days to months and this stage ends when entering the oceanic zone.

4.- Stage II: Oceanic juvenile stage – Oceanic Zone

- Starts when reaching the oceanic zone (>200m deep).
- This stage is epipelagic, i.e. juveniles spend 75% of their time within the top 5m of the water column.
- Small juveniles passively drift with surface water currents but may reorient to remain within preferred currents.
- 15-63cm straight carapace length (SCL).

5.- Stage III: Juvenile transitional stage – Neritic or Oceanic Zone

- Ontogenetic relaxed shift between the oceanic and neritic zone.
- Some individuals might return to the oceanic zone after a neritic period.
- Variable duration.
- 41-82cm SCL.

6.- Stage IV: Large juvenile transitional stage – Neritic or Oceanic Zone

- Turtles feed both in the bottom and the water column when prey is available.
- Depending on the population/region, late juveniles might share foraging areas with adult turtles.
- 63-100cm SCL.

7.- Stage V: Adult – Neritic, Oceanic or Terrestrial Zone

- Reproductively mature turtles usually remain in neritic zones although some might stay in the oceanic zone until breeding.
- Adults migrate to breeding grounds to mate and females go to nesting beaches to lay their eggs.
- >82cm SCL.

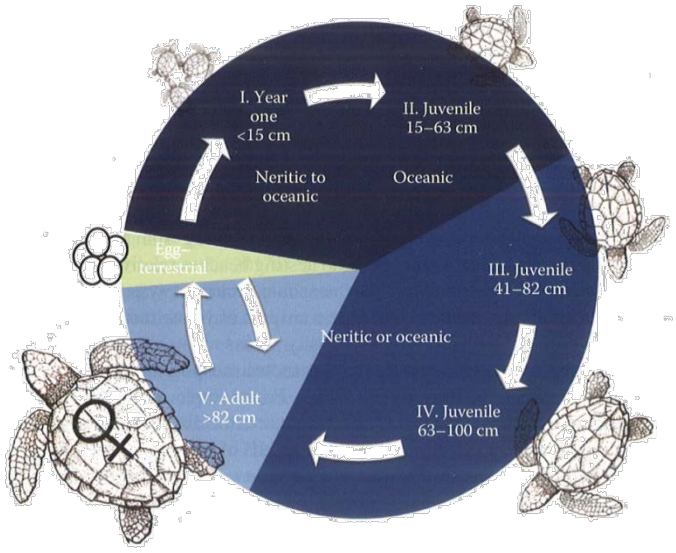

Fig.2. Life cycle diagram for loggerhead turtles nesting in the north-western Atlantic (Mansfield and Putman 2013).

In this generally accepted life history model, adult females return to lay their eggs to, or close to, the nesting beaches in which they were born (Carr 1967; Miller et al. 2003). This is known as philopatry and results in a strong site fidelity to the same nesting beaches within and between seasons (Bowen et al. 1993a; Papi et al. 1997). Philopatry has been described as a successful strategy for female sea turtles to ensure egg viability by nesting in beaches of demonstrated suitability but also as a convenient behaviour for males, as philopatry increases the chances of finding available females to mate with (Schofield et al. 2009). However, whilst previous research has revealed a strong female philopatry in loggerhead turtles, less is known about site fidelity of males as research has been traditionally skewed to females (easier to sample while laying their eggs in monitored nesting beaches). It is now globally accepted, though, that males follow similar migration patterns as females (Hatase et al. 2002; Godley et al. 2008; Schofield et al. 2010) although timings and frequency of these migrations may vary between species and populations (Hays et al. 2010). Whether mating only occurs in breeding grounds close to nesting beaches (Bowen et al. 2005) or also in foraging grounds or en-route during reproductive migrations is still unclear.

The geographic precision of philopatry is also unclear to date. Previous research on sea turtles has suggested that site fidelity of nesting females is frequently recorded in the vicinity of their natal beaches; within several hundred kilometres (Bowen and Avise 1996; Lohmann et al. 2008). However, accurate precision to specific nesting beaches might be less common and hence females may nest relatively close to their natal areas rather than the exact same beach where they were born (Lohmann et al. 2013). Nonetheless, such a reproductive strategy requires high navigational skills as turtles migrate from remarkably distant foraging grounds to specific nesting areas for oviposition. It is accepted that individuals may be strongly imprinted on their natal beaches or regions during the first stage as hatchlings and that this might set the geographical position of natal areas in the turtles (Carr 1967). This imprinting may be determined by two non-exclusive mechanisms: geomagnetic imprinting and chemical imprinting. The first is based on the capacity of turtles to detect differences in magnetic fields, which allows turtles to locate themselves along the north-to-south axis (Lohmann and Lohmann 2003). Accordingly, hatchlings are geo-magnetically imprinted in their natal rookeries and are able to discern whether they are in a northward or southward

position from these rookeries as they grow. This gives adults the possibility to track back the original position of their natal area (Lohmann et al. 2004). However, this mechanism might not be enough to explain precise site fidelity. Whilst magnetic imprinting might lead turtles to areas close to natal regions, chemical imprinting might be the responsible for accurate philopatry. Thus, turtles have been suggested to recognise specific nesting areas at a local scale on the basis of distinctive chemical cues such as odours and chemicals dissolved in water (Grassman et al. 1984; Southwood et al. 2008; Endres et al. 2009). Even if these imprinting mechanisms might explain site fidelity, further research is still needed to unveil all the processes that intervene in orientation and navigation.

DISTRIBUTION OF THE LOGGERHEAD TURTLE

The life cycle and reproductive migrations above described occur in terrestrial, neritic and oceanic zones and hence, different life stages are typically distributed in different areas. Sea turtle distributions can be driven by many factors depending on size, life stage and region and, accordingly, distribution patterns must be studied specifically for each stage and population.

The loggerhead turtle is a circumglobally distributed species, present in all tropical to temperate waters of the planet. The main nesting aggregations can be found in the south-eastern United States and the Gulf of Mexico (32,000-56,000 nesting females; with southern Florida hosting 49,000-83,000 nests per year), Cape Verde (5,000 nesting females) and Brazil (4,000 nesting females; mainly in northern Bahia). However, substantial nesting is also found in eastern and Western Australia, Japan, Oman, South Africa and in the Mediterranean Sea (Miller et al. 2003). In regards to their foraging grounds, loggerhead turtles can be found foraging in all oceans although populations are not randomly distributed, mainly affected by factors such as currents or prey availability.

The distribution patterns of hatchlings are mediated by surface water currents as soon as they enter the aquatic realm (Bolten 2003). Because hatchlings are positively buoyant and present limited swimming and diving abilities (Milsom 1975), individuals passively drift within main currents across entire oceans, at least in the early stages (Carr and Meylan 1980; Bolten et al. 1992). Little is known about

the exact dispersal routes followed by hatchlings and early juveniles as the available tracking devices are heavy and large, hence only useful to track large juveniles or adults. Accordingly, what we know is mainly based on strandings, opportunistic in-water encounters, genetic analyses and migratory predictions based on virtual particle tracking (Godley et al. 2010). Recent research has mainly focused on the latter and prediction models have drawn dispersal patterns worldwide (Fig. 3 but also Hays et al. 2010; Putman et al. 2012b). However, new light is to come as Mansfield et al. (2012) tested small-scale solar-powered satellite tags on 4-6 months old hatchlings. These have already been proven successful in south-eastern United States (Mansfield et al. *in review*) and corroborated the particle modelling previously published for this area (Fig. 3).

In south-eastern United States, hatchlings enter the "frenzy period" after reaching the waterline and are pushed by secondary currents into the Gulf Stream System, where they flow within the northern branch until reaching the coasts of Western Europe (Carr 1986; Bolten et al. 1998). Once there, the negative water balance of the Mediterranean Sea, which generates a permanent eastward flow of Atlantic water at the Strait of Gibraltar (Millot and Taupier-Letage 2004), connects the Gulf Stream with the Mediterranean basin. As a result, some juveniles of Atlantic origin enter the Mediterranean Sea whilst others remain in Atlantic oceanic zones until adult recruitment to the south-eastern coast of United States occurs (Bolten 2003; Bowen and Karl 2007). Even if hatchlings and juveniles from Cape Verde, the second largest nesting aggregation in the North Atlantic (Marco et al. 2012), also inhabit the north Atlantic these are scarce in the Mediterranean Sea (Monzón-Argüello et al. 2009) as the Cape Verde Archipelago is connected with the American continent by the North Equatorial Current rather than with the Mediterranean Sea (Fig.3).

This shows that the contribution of different nesting beaches to any particular juvenile foraging ground will depend on the size of the population nesting at each beach but also on the pattern of surface currents connecting these beaches with the foraging grounds (Bowen and Karl 2007; Hays et al. 2010). In addition, it also highlights the fact that threats impacting on certain populations (such as the turtles of Atlantic origin that forage in the Mediterranean Sea) might

have an indirect effect in nesting areas located in far-away continents, making conservation difficult unless deep knowledge on distribution and habitat use exists.

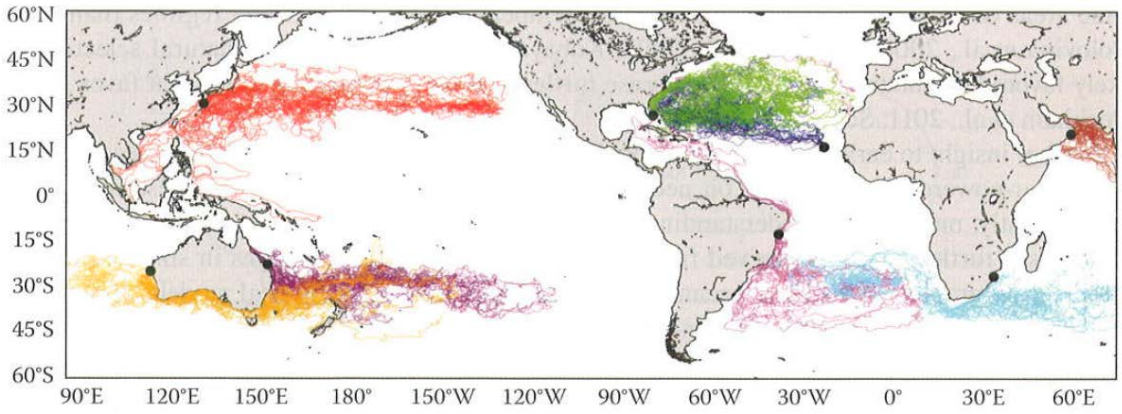

Fig.3. Predicted dispersal patterns from eight major loggerhead rookeries (black dots) under the passive drift assumption. Virtual particles were released during the 3 months of peak hatchling emergence and tracked for 6 years. Image from Mansfield and Putman (2013).

LOGGERHEAD TURTLES IN THE MEDITERRANEAN SEA

Three species of sea turtles inhabit the Mediterranean Sea: the loggerhead turtle, the green turtle and the leatherback turtle; although the latter is only occasionally present and does not nest in the basin. Of these, the loggerhead turtle is the most abundant and mainly nests in central and eastern Mediterranean beaches, with an estimated total of 7,200 nests laid every year (Casale and Margaritoulis 2010).

Loggerhead nesting grounds

The largest nesting aggregations can be found in Greece, Turkey, Cyprus and Libya (Table 1) and almost no nesting activity has been recorded in the western part of the basin, with the exception of a few sporadical nests (Tomás et al. 2008). The nesting season peaks in summer, between June and early August, although some females may lay their eggs in mid-May or September (Margaritoulis et al. 2003).

The analysis of fragments of non-coding mitochondrial DNA (mtDNA) has revealed the existence of genetic structuring among nesting beaches of the Mediterranean Sea. This is because loggerhead turtles are philopatric and mtDNA is a maternally inherited marker (Bowen and Karl 2007). Accordingly, specific

regional management units (RMU; Wallace et al. 2008) with specific haplotypes and in variable frequencies have been defined before (Encalada et al. 1998; Laurent et al. 1998; Carreras et al. 2007; Garofalo et al. 2009; Yilmaz et al. 2011; Saied et al. 2012). The most extensive study of this nature in the region (Carreras et al. 2007) found the existence of four RMUs in the Mediterranean, most of them characterised by the presence of an exclusive haplotype at low frequency. This study, however, had non-conclusive results partly due to small sample sizes and the amplification of short fragments of the mtDNA sequence.

Table 1. Number of nests per year recorded in Mediterranean countries. Period of study and references included.

Nesting area	Nests/year	Period	Reference
Cyprus	694[a]	1993-2008	Demetropoulos and Hadjichristophorou 2010; Fuller et al. 2010
Egypt	67	1998	Clarke et al. 2000
France	1[b]	2002, 2006	Delaugerre and Cesarini 2004; Oliver 2006; Sénégas et al. 2008
Greece	3472[a]	1984-2007	Margaritoulis and Panagopoulou 2010
Israel	57[a]	1993-2008	Levy 2010
Italy	10[a]	2000-2004	Casale 2010
Lebanon	60[c]	1997-2006	Cross and Bell 2006; Aureggi et al. 2005; Newbury et al. 2002; St John et al. 2004; Khalil et al. 2006; Kasparek 2004
Libya	726[a]	2006-2007	Hamza 2010
Spain	4[b]	1991-2006	Tomás et al. 2008
Syria	17[a]	2004-2009	Rees et al. 2010
Tunisia	<15[b]	1993-2008	Bradai and Jribi 2010
Turkey	2145[d]	unknown	Türkozan and Kaska 2010

[a] mean value; [b] maximum value; [c] approximate value; [d] median of range of values

More recently, a new set of primers has been developed (Abreu-Grobois et al. 2006) which amplifies a longer segment of mtDNA (815bp against the previous 380bp). This may potentially increase the resolution of genetic structuring within the Mediterranean Sea as reported for Cape Verde and the north-western Atlantic (Monzón-Argüello et al. 2010; Shamblin et al. 2012). This increase in resolution is due to the fact that the longer fragment of mtDNA contains the commonly used shorter fragment but presents additional polymorphic sites with higher levels of nucleotide diversity outside the shorter segment (Monzón-Argüello et al. 2010). Accordingly, recent research using longer fragments of mtDNA in the Mediterranean Sea has revealed a complex structuring among Turkish rookeries, previously undetected with shorter markers (Yilmaz et al. 2011). Unfortunately, the lack of information on long fragments of mtDNA to date has precluded the unveiling of deeper structuring among the remaining Mediterranean rookeries.

Even if mtDNA is a powerful marker to study population structure and phylogeographic processes, it does not take into consideration the contribution of males to the genetic structure of populations. This can be studied through the analysis of nuclear DNA (nDNA), which informs of both male- and female-mediated gene flow; of relevance when designing conservation and management plans as both sexes might not behave equally (Prugnolle and de Meeus 2002; Lawson Handley and Perrin 2007).

The first signs of population structuring based on nDNA among Mediterranean rookeries were detected in Turkey using randomly amplified polymorphic DNA (RAPD) markers (Schroth et al. 1996). The existence of a significant genetic structure based on nDNA was also corroborated by Carreras et al. (2007) in a study comprising a larger number of Mediterranean nesting areas but using only 7 microsatellite markers. Nonetheless, some studies failed to identify genetic differentiation and restriction in male-mediated gene flow within the Mediterranean Sea (Yilmaz et al. 2011; Garofalo et al. 2013). Discrepancies can be partly due to sampling size effects as well as reduced number of markers (Dutton et al. 1999 and Roberts et al. 2004). Despite new microsatellite markers have been recently isolated for this species (Monzón-Argüello et al. 2008), these have not been

used to analyse Mediterranean rookeries and thus, further research is still needed to reveal even deeper structuring.

Loggerhead foraging grounds

Foraging grounds for loggerhead turtles can be found across the whole Mediterranean Sea, although population structuring exists and different life stages are certainly unevenly distributed. The distribution of juveniles and adults in foraging grounds of the Mediterranean Sea has been widely studied through the use of satellite telemetry (Bentivegna et al. 2002; Cardona et al. 2005; Revelles et al. 2007b; Cardona et al. 2009; Casale et al. 2013), mark-recapture techniques (Margaritoulis et al. 2003; Casale et al. 2007; Revelles et al. 2008) and genetics (Carreras et al. 2006; Maffucci et al. 2006; Casale et al. 2008b; Saied et al. 2012; Garofalo et al. 2013).

Turtles from different populations share the same foraging grounds within the basin, with turtles from as far as the north-western Atlantic foraging there; as reported above. Juveniles of Atlantic origin enter the Mediterranean Sea pushed by a permanent eastward current at the Strait of Gibraltar (Millot and Taupier-Letage 2004) and will remain in Mediterranean waters until they grow up to 40-60cm SCL; a size large enough to overcome the surface current and swim out of the basin (Revelles et al. 2007d). The distribution of these Atlantic individuals has been studied with molecular markers; which have revealed that even if turtles of Atlantic origin may share common foraging areas with turtles of Mediterranean origin, they seldom interbreed (Carreras et al. 2011).

The contribution of different rookeries to mixed foraging grounds has been assessed through mixed stock analyses based on mtDNA (MSA, Grant et al. 1980; Pella and Masuda 2001). MSA assigns turtles sampled in foraging grounds to their natal region, based on the fact that significant, sometimes exclusive, haplotype shifts among rookeries exist. Previous research in the western Mediterranean Sea showed that juvenile turtles of Atlantic origin mainly inhabit foraging grounds off the north-African coast and juvenile turtles of Mediterranean origin forage mainly along the European coasts (Carreras et al. 2006). Nonetheless, little is still known

about the distribution and proportion of Atlantic juveniles in other areas within the Mediterranean Sea (Laurent et al. 1998; Maffucci et al. 2006; Casale et al. 2008b).

In regards to the distribution of turtles from Mediterranean rookeries, MSA has previously revealed a high contribution of Greek individuals to the foraging grounds in the Tyrrhenian, Adriatic and the rest of the central Mediterranean Sea (Maffucci et al. 2006), with also a remarkable presence of turtles from Turkish and Libyan rookeries (Saied et al. 2012). However, most of the studies used the short (380bp) fragment of mtDNA (Laurent et al. 1998; Maffucci et al. 2006; Carreras et al. 2007; Casale et al. 2008b; Saied et al. 2012; but see Garofalo et al. 2013). Thus, the limited assignment power of this marker and a limited number of rookeries sampled has precluded a fine-scale assessment of the contribution of Mediterranean rookeries to the major Mediterranean foraging grounds.

The use of different foraging grounds has also been studied through mark-recapture and satellite telemetry tracking. Previous research has mainly focused on post-nesting migrations of females from nesting to foraging grounds (Fig. 4). These have identified the central Mediterranean Sea as a hot spot for adult turtles from Greece (Hays et al. 2010; Margaritoulis and Rees 2011; Zbinden et al. 2011) and in particular from Zakynthos, one of the largest, best studied rookeries in the region. Unfortunately, little is known from other areas although a few turtles have been tracked from eastern Mediterranean rookeries, showing a potential preference for foraging grounds in the Levantine Sea and the southern Ionian Sea (Broderick et al. 2007). This lack of information is probably due to the large funding budgets required to undertake satellite tracking or the low probability of encountering tagged turtles in any particular foraging ground (Schroeder et al. 2003).

Because of these limitations, the analysis of stable isotope signatures has been recently applied to study sea turtle populations (Hatase et al. 2002; Revelles et al. 2007a; McClellan et al. 2010; Zbinden et al. 2011). The stable isotope ratios in animal tissues provide information on diet but also can be used to track foraging ground locations, as tissue signatures reflect those of the specific food webs present in a certain area (Hobson 1999; Fry 2006).

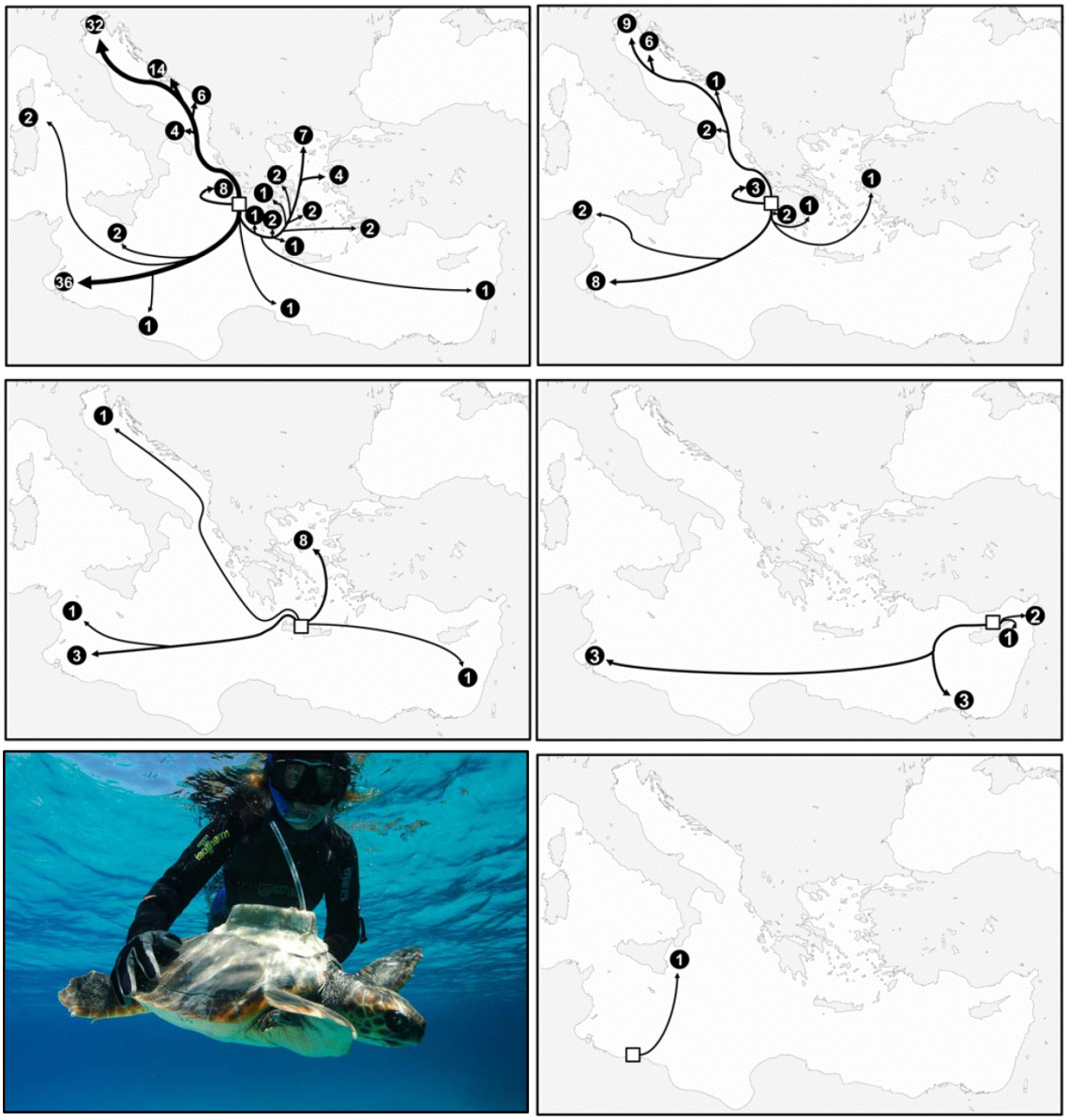

Fig.4. Post-nesting movements of loggerhead turtles from different Mediterranean rookeries (white squares) adapted from previous tagging (left) and telemetry (right) studies. Circles show number and location of individuals recovered (left) or satellite tracking end points (right). Arrows represented to scale but do not indicate migratory routes. References from top-left to bottom-right: Margaritoulis et al. 2007; Hays et al. 2010 and Zbinden et al. 2011; Margaritoulis and Rees 2011; Fuller et al. 2010; Hamza 2010. Image showing satellite tracking device courtesy of Lluís Cardona.

Elements such as carbon, nitrogen, oxygen, hydrogen and sulphur have been used in foraging ecology both in terrestrial and marine environments (Newton 2010). In the case of carbon, the ratio of ^{13}C to ^{12}C (expressed as $\delta^{13}C$) informs about the source of carbon entering the food chain, hence allowing to distinguish between coastal and oceanic food webs. Regarding the ^{15}N to ^{14}N ratio ($\delta^{15}N$), this experiences a stepwise enrichment at each trophic level due to the preferential excretion of the lighter isotope (Fry 2006). Accordingly, $\delta^{15}N$ can be used to define

the trophic position of any individual within the trophic web of a certain area. However, foraging grounds may differ in the isotopic baseline for nitrogen, and hence individual differences in $\delta^{15}N$ values may emerge because of differences in the foraging areas used (Hobson and Wassenaar 2008).

In the case of sea turtles, analyses of stable isotope ratios have unveiled foraging differences among individuals (Reich et al. 2010), between life stages (Arthur et al. 2008) and between populations (Wallace et al. 2006). Accordingly, individuals enriched in both ^{13}C and ^{15}N are usually considered neritic foragers whilst turtles depleted in ^{13}C and ^{15}N are classified as oceanic foragers (McClellan et al. 2010; Eder et al. 2012). This can reveal the areas used by turtles of different sizes (or stages) or by different populations, which may be of remarkable importance when designing management plans. Due to disparities in prey abundance and habitat productivity, differences in the foraging areas used may affect life history traits such as growth rate, stage duration, time to maturity or survival (Snover et al. 2007b; Snover 2008) and thus, may lead to different conservation needs. Previous research in the Mediterranean Sea has revealed different patterns of habitat uses among female loggerhead turtles nesting in Zakynthos, Greece; with those foraging in the Adriatic/northern Ionian Sea growing larger and laying more eggs than those foraging in the southern Ionian Sea (Zbinden et al. 2011). This suggests that the differences previously noted for female size and clutch size between Mediterranean rookeries (Margaritoulis et al. 2003) could be due to the differential use of foraging grounds of contrasting quality by females from different rookeries.

Anthropogenic impacts

Natural threats such as beach erosion, predation or organic debris affect loggerhead turtle populations in the Mediterranean Sea (Casale and Margaritoulis 2010). However, the major impacts that have led this species to a severe decline derive from human activities. Threats in Mediterranean nesting beaches include coastal development, beach restructuring and poaching. Contrarily, boat collisions or pollution typically occur in marine areas. Even if the intentional killing of loggerhead turtles is almost inexistent in the majority of Mediterranean countries, some direct exploitation still exists in Egypt and Greece (Casale and Margaritoulis 2010). Nonetheless, no other threat is currently as relevant as bycatch.

Casale (2011) estimated that over 132,000 sea turtles (the majority loggerheads) are caught every year in the Mediterranean Sea (Fig. 5), of which 44,000 die. Of the wide array of fishing gears used in the Mediterranean Sea, drifting longlines are considered the most threatening as approximately 60,000 turtles are incidentally caught every year (Lewison et al. 2004b; Casale 2011), with a 35% estimated mortality rate associated (Álvarez de Quevedo et al. 2013). Although drifting longlines are set to catch generally tuna and swordfish, sea turtles can also be hooked while trying to ingest bait from baited hooks or become entangled with their flippers. The subsequent mortality will mainly depend on longline soak time, set depth and type of injury caused (Lewison and Crowder 2007).

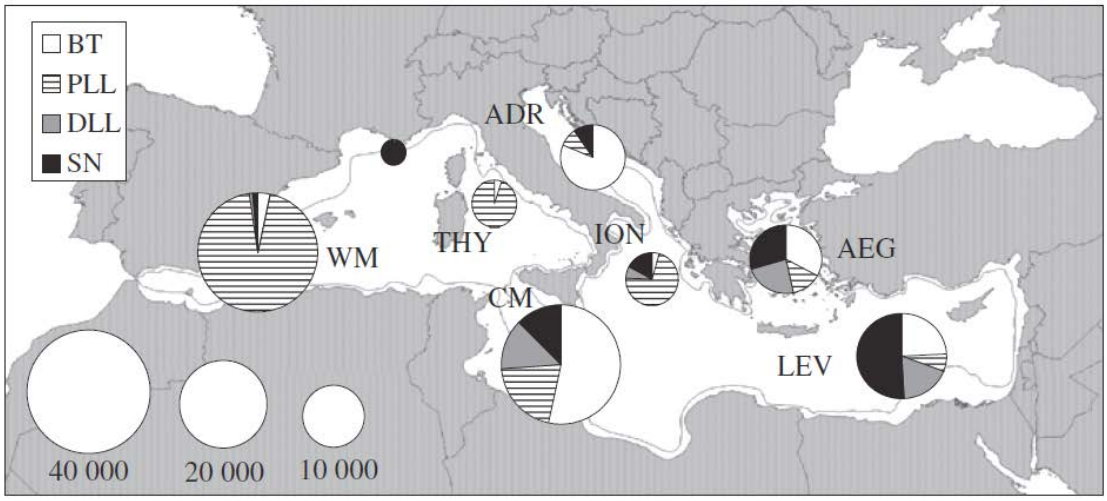

Fig.4. Proportion of turtles captured annually in the Mediterranean Sea by foraging ground and fishing gear estimated from fishery statistics and catch rates. Fishing gears: BT (bottom trawl), PLL (pelagic longline), DLL (demersal longline), SN (set net). Foraging grounds: WM (western Mediterranean), THY (Tyrrhenian Sea), CM (central Mediterranean), ADR (Adriatic Sea), ION (Ionian Sea), AEG (Aegean Sea), LEV (Levantine Sea). The 200m bathymetry line is shown. Figure extracted from Casale 2011.

Even if bycatch derived from trawling and set nets has been considered less relevant than that caused by drifting longlines because of its lower catch rates (39,000 captures per year; Casale 2011), it should not be ignored. Mortality rates associated with trawling nets are significantly higher than in longlines and the main cause of death derives from underwater suffocation after being trapped in the cod-end for a long period of time (Carreras et al. 2004; Wallace et al. 2013). However, whether different fishing gears affect populations of contrasting origin or what is the effect of these fishing gears in each particular population is still unclear.

Laurent et al. (1998) reported a contrasting population make-up for the turtle bycatch of drifting longliners and that of bottom trawlers. Laurent and colleagues suggested that drifting longlines captured a mixture of turtles of Atlantic and Mediterranean origin, whereas bottom trawling captured only turtles of Mediterranean origin. However, no regional differences in the distribution of loggerhead turtles of Atlantic and Mediterranean origin were assumed and the longline bycatch composition from the western and central Mediterranean was compared with that of bottom trawling from the central and eastern Mediterranean.

As seen above, recent research has revealed complex distribution patterns of loggerhead turtles within the Mediterranean Sea, with a prevalence of turtles of Atlantic origin in some areas of the western Mediterranean and the prevalence of turtles of Mediterranean origin in the eastern Mediterranean (Carreras et al. 2006, 2011; Maffucci et al. 2006). Accordingly, the differences observed by Laurent et al. (1998) could be also attributed to differences in the distribution of turtles of Atlantic and Mediterranean origin and not to contrasting patterns of habitat use, as suggested. However, the lack of knowledge on fine-scale distribution of turtles of contrasting origin within the Mediterranean Sea, combined with differences between gears in regards to bycatch rates (Casale 2011) and mortality rates (Carreras et al. 2004, Casale et al. 2004; Álvarez de Quevedo et al. 2013), generates a complex scenario that has make it difficult to allocate the impact of bycatch to the populations involved, to date.

OBJECTIVES

The main objective of the current thesis is to describe the population structure of loggerhead turtles in nesting and foraging areas of the Mediterranean Sea, understand the causes of such structuring and assess its consequences for the conservation of the species.

The thesis is organised around four main topics: the population structure in nesting areas (*Chapter 1*), the population structure in foraging grounds (*Chapter 2*), the biological consequences of different patterns of habitat use (*Chapter 3*) and the evaluation of fishing bycatch on the populations inhabiting the Mediterranean Sea (*Chapter 4*). The specific objectives of each chapter are:

POPULATION STRUCTURE IN NESTING AREAS

- To unveil the colonisation processes that led to the current genetic structure of Mediterranean rookeries through the analysis of mtDNA.
- To define the genetic units present in Mediterranean rookeries by analysing pairwise differentiation among nesting areas with mtDNA and microsatellite markers.
- To evaluate both female- and male-mediated gene flow between nesting areas by combining mtDNA and nDNA analyses. This information will be relevant for conservation purposes as the levels of isolation present in each rookery will be revealed.

POPULATION STRUCTURE IN FORAGING GROUNDS

- To evaluate the contribution of Atlantic and Mediterranean rookeries to seven Mediterranean foraging grounds through mixed stock analysis with mtDNA markers.
- To infer the mechanisms defining the juveniles distribution and relate them to the biology of the species.

BIOLOGICAL CONSEQUENCES OF DIFFERENT PATTERNS OF HABITAT USE

- To assess the differences in growth rates between turtles of Atlantic and Mediterranean origin feeding in Mediterranean foraging grounds through the use of genetics and skeletochronology.

- To estimate the age at sexual maturity of turtles of Atlantic and Mediterranean origin; not only important to understand population dynamics in the Mediterranean Sea but also relevant as different growth rates might reflect differential habitat uses.
- To investigate, through the analysis of stable isotope signature, the existence of differences in clutch size among rookeries in the eastern Mediterranean Sea as a consequence of differential use of foraging grounds with contrasting productivity s.

FISHING BYCATCH

- To characterise the population make-up and the patterns of habitat use of the turtles caught with oceanic (drifting longlines) and neritic (bottom trawling and trammel nets) fishing gears in three different Mediterranean foraging grounds by using genetic markers and stable isotopes analyses.
- To test whether different fishing gears capture turtles from different populations and to assess the effects of bycatch on the conservation of these populations.

CHAPTER 1. Phylogeography and population structure in Mediterranean nesting areas

1.1. Mitochondrial DNA reveals Pleistocenic colonisation of the Mediterranean by loggerhead turtles (*Caretta caretta*)

Títol: L'anàlisi d'ADN mitocondrial revela una colonització plistocènica del mar Mediterrani per part de la tortuga babaua (*Caretta caretta*).

Resum: La tortuga babaua (*Caretta caretta*) és una espècie filopàtrica, fet que comporta una forta estructuració genètica de les seves poblacions nidificants. Així, l'anàlisi d'ADN mitocondrial (ADNm) pot ser usat per a estudiar esdeveniments evolutius i processos colonitzadors. En aquest estudi utilitzem un enfocament genètic per entendre l'estructura poblacional actual de *C. caretta* al mar Mediterrani i per esbrinar si hi podria haver hagut una colonització del Mediterrani durant el Pleistocè, tot sobrevivint les fases més fredes en refugis temperats. Es va amplificar un fragment llarg (815pb) d'ADNm en 168 nounats morts mostrejats entre una selecció de colònies del Mediterrani oriental: Líbia, Israel, Líban, Xipre i Grècia. Dades prèviament publicades de Turquia i Calàbria (sud d'Itàlia) també es van incloure en els anàlisis. La població nidificant a Líbia va ser detectada com la més antiga del Mediterrani, datant del Pleistocè, fa aproximadament 65.000 anys (20.000-200.000). Això revela que la població líbia es podria haver assentat a la conca Mediterrània abans de la fi de l'últim període glacial. La resta de zones de nidificació, excepte Calàbria, haurien estat posteriorment colonitzades a mesura que l'espècie s'anà expandint. Les poblacions que nidifiquen a l'est de Turquia i a la Grècia occidental s'haurien establert fa aproximadament 30.000 anys (10.000-100.000), mentre que les poblacions restants s'haurien originat com a resultat d'expansions recents durant l'Holocè. Degut a que Calàbria presenta un haplotip exclusiu de l'Atlàntic, no present a cap altra zona de nidificació del Mediterrani, considerem que aquesta zona és el resultat d'una colonització independent des de l'Atlàntic i no pas una expansió a partir de poblacions Mediterrànies. Això revela que l'actual estructura genètica de les zones de nidificació de *C. caretta* al Mediterrani seria el resultat, com a mínim, de dos esdeveniments colonitzadors des de l'Atlàntic: el més antic a Líbia i un de més recent a Calàbria, combinats amb extincions locals durant glaciacions plistocèniques i re-colonitzacions des de refugis glacials a Líbia, l'est de Turquia i la Grècia occidental.

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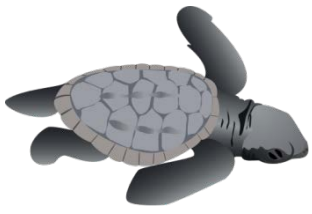

See original publication in Appendix A1

Mitochondrial DNA reveals Pleistocenic colonisation of the Mediterranean by loggerhead turtles (*Caretta caretta*)

Marcel Clusa[a*], Carlos Carreras[a], Marta Pascual[b], Andreas Demetropoulos[c], Dimitris Margaritoulis[d], Alan F. Rees[d], Abdulmaula A. Hamza[e], Mona Khalil[f], Monica Aureggi[g], Yaniv Levy[h], Ogüz Türkozan[i], Adolfo Marco[j], Alex Aguilar[a], Luis Cardona[a]

[a] Department of Animal Biology and IRBio, University of Barcelona, Av.Diagonal 643, E-08028 Barcelona, Spain
[b] Department of Genetics and IRBio, University of Barcelona, Av.Diagonal 643, E-08028 Barcelona, Spain
[c] Cyprus Wildlife Society, Emmanuel Xanthou 11, P.O. Box 24281, 1703 Nicosia, Cyprus
[d] ARCHELON, Solomou 57, GR-10432 Athens, Greece
[e] Libyan Seaturtle Program, Alfateh University, P.O. Box 13793, Tripoli, Libya
[f] MEDASSET, P.O. Box 19, Tyre, Lebanon
[g] Naucrates, Via Corbetta 11, 22063 Cantù , CO, Italy
[h] The Israel Sea Turtle Rescue Centre, Nature Parks Authority, Mikhmoret 40297, Israel
[i] Adnan Menderes University, Faculty of Science and Arts, Department of Biology, Aydin, Turkey
[j] Biological Station of Doñana-CSIC, Apdo. 1056, E-41013 Seville, Spain

ABSTRACT

As the loggerhead turtle (*Caretta caretta*) is a philopatric species with a strong genetic structure, the analysis of mtDNA can be used to track evolutionary and colonisation events. In this study we use a genetic approach to understand the population structure of *C. caretta* in the Mediterranean Sea and to test whether loggerheads could have colonised the Mediterranean during the Pleistocene and survived the cold phases in warm refugia. We amplified a long mtDNA D-loop fragment (815 bp) from 168 dead hatchlings sampled from a selection of rookeries in the Eastern Mediterranean: Libya, Israel, Lebanon, Cyprus and Greece. Previously published data from Turkey and Calabria (Southern Italy) were also included in the analyses. The population nesting in Libya emerged as the oldest population in the Mediterranean, dating from the Pleistocene *ca*. 65,000 years ago (20,000-200,000). This reveals that the Libyan population might have settled in the Mediterranean basin before the end of the last glacial period. The remaining nesting sites, except Calabria, were subsequently colonised as the population expanded. The populations nesting in Eastern Turkey and Western Greece settled *ca*. 30,000 years ago (10,000-100,000), whereas the remaining populations originated as a result of a more recent Holocenic expansion. As Calabria presented a unique Atlantic haplotype, found nowhere else in the Mediterranean, we consider this nesting site as the result of an independent colonisation event from the Atlantic and not the recent spread of Mediterranean populations. This reveals that the current genetic structure of *C. caretta* rookeries in the Mediterranean would be the result of at least two colonisation events from the Atlantic, the oldest one in Libya and a most recent in Calabria, combined with local extinctions during Pleistocenic glaciations and re-colonisations from glacial refugia in Libya, Eastern Turkey and Western Greece.

Keywords: *Caretta caretta*; genetic structuring; glacial refugia; molecular clock; mtDNA; phylogeography.

1. Introduction

The Pleistocene extended from 2.5 mya to 12 kya and was characterised by multiple glacial-interglacial cycles that caused dramatic changes in the distribution of organisms (Taberlet et al., 1998; Wilson and Eigenmann Veraguth, 2010). As ice sheets spread during glacial cycles, species often retreated towards the Equator although some populations survived in areas that acted as refugia (Haffer, 1982). Furthermore, a dryer climate and lower sea levels during glacial periods caused dramatic changes in species distribution even in areas that were not covered by ice (Hewitt, 1996; Maggs et al., 2008). When ice retreated due to post-glacial temperature rises, species re-expanded their distribution polewards, occupying previously inhospitable areas (Hewitt, 2000). These patterns are well established for terrestrial organisms, but the response to Pleistocenic glacial-interglacial cycles is still unclear for many marine species.

After the Messinian Salinity Crisis (5.33-5.59 mya), the Mediterranean basin was colonised by subtropical biota of Atlantic origin (Pérès, 1985). During the following climatic fluctuations, species distributions were affected by changes in the sea level, water temperature and salinity (Grant and Bowen, 1998). According to the fossil records, the most thermophilic groups became extinct during the first cold period of the Pleistocene and waves of extinction and invasion changed the composition of the Mediterranean biota in every climatic phase (Pérès, 1985). Nevertheless, recent molecular evidence has suggested that at least some of the subtropical species currently found in the Mediterranean are not recent Holocenic invaders, but have a pre-glacial origin and survived the glacial peaks in warmer refugia within the Mediterranean (Almada et al., 2001; Domingues et al., 2007; Wilson and Eigenmann Veraguth, 2010). Molecular data indicate that the southern parts of the Mediterranean, being warmer than northern areas during the Pleistocene (Thiede, 1978), acted as refugia for sea grasses (e.g. *Posidonia oceanica,* Arnaud-Haond et al., 2007; *Cymodocea nodosa*, Alberto et al., 2008) and that the Ionian and Aegean Sea, acted in the same way for some fish species (Bahri-Sfar et al., 2000; Magoulas et al., 1996).

Marine turtles have tropical affinities and females are highly philopatric, returning to specific geographical locations to nest (Carr and Ogren, 1960;

FitzSimmons et al., 1997; Meylan et al., 1990). This results in strong genetic structuring when mtDNA is considered (Bowen and Karl, 2007; Lee, 2008), allowing evolutionary and colonisation events to be traced (Garofalo et al., 2009). The loggerhead turtle (*Caretta caretta* L.) is the least thermophilic cheloniid and regularly nests in subtropical and warm temperate regions where sand temperature is higher than 24ºC for a sufficiently long period of time (Miller et al., 2003). Paleoclimatic reconstructions of sea surface temperatures indicate that loggerhead turtles could not use the Western Mediterranean even as a foraging ground due to low sea surface temperatures during the last glacial peak (summer surface temperature < 17ºC; Thiede, 1978). Only the Eastern Mediterranean was warm enough to allow turtle nesting, as summer sea surface temperatures were usually higher than 22ºC (Thiede, 1978); the minimum threshold for loggerhead turtle nesting (Miller et al., 2003). Thus, in the case that *C.caretta* had already colonised the Mediterranean prior to glaciation events, these Eastern regions could have acted as refugia for loggerhead turtles through the cold phases of the Pleistocene. Nevertheless, Bowen et al. (1993a) proposed a recent Holocenic origin for loggerhead turtles currently nesting in the Mediterranean. However, their conclusion was based on the analysis of just one nesting ground from the Ionian Sea (Bay of Kyparissia), the only rookery sampled at that time. New genetic data on the Mediterranean populations have come to light since (Carreras et al., 2007; Chaieb et al., 2010; Encalada et al., 1998; Garofalo et al., 2009; Laurent et al., 1998; Saied et al., 2012; Yilmaz et al., 2011).

To track the colonisation history of the Mediterranean by loggerhead turtles and to test the possible existence of warm refugia during the cold phases we have analysed mtDNA sequences from multiple nesting grounds in the Eastern Mediterranean, including previously poorly sampled locations.

2. Material and Methods

2.1 Sample collection

Samples of skin and/or muscle were taken from 168 dead hatchlings and embryos from unhatched eggs during post-hatch nest excavations of nesting grounds in the Mediterranean Sea between 2003 and 2006 (Fig. 1, Table 1). These included Libya (west of Sirte), Israel (scattered sites along the whole coastline),

Lebanon (El Mansouri), Cyprus (Alagadi and Akamas) and Greece, with samples from Western Greece (Zakynthos and Lakonikos Bay) and Crete (Rethymno). Samples were stored in 95% ethanol and samples from Greece, Israel and Lebanon previously analysed by Carreras et al. (2007) were also used for this study. Independency among samples can be assumed because sampling included protocols to avoid pseudoreplication. These included female tagging and samples taken from clutches laid within a 15-day window to avoid hatchlings from the same individual turtle, as females rarely nest at intervals shorter than this period (Dutton, 1995). However, the new samples from Lebanon were collected in different years from those from Carreras et al. (2007) and hence, additional pseudoreplication tests were undertaken to ensure independency between samples. Pseudoreplication was assessed by amplifying the new samples with seven microsatellite loci (Carreras et al., 2007) and comparing them with the Lebanon samples in Carreras et al. (2007). A pairwise relatedness analysis implemented in GENALEX v6.4 (Peakall and Smouse, 2006) was used for the comparison.

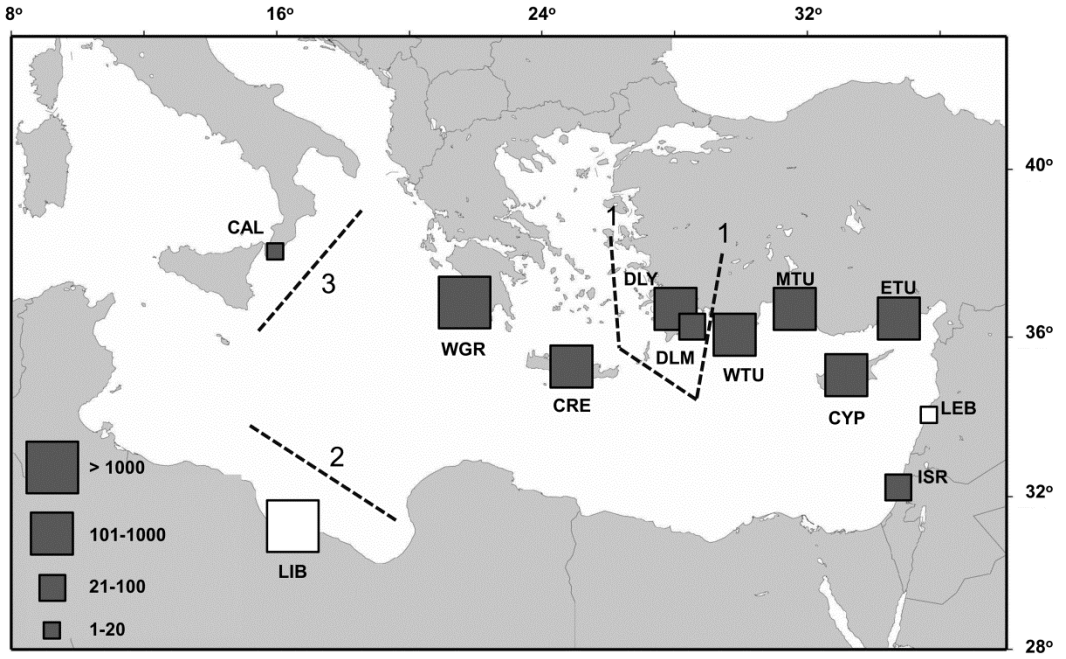

Fig. 1. Sampled nesting areas of loggerhead turtles in the Mediterranean Sea. Nesting areas: Libya (LIB), Israel (ISR), Lebanon (LEB), Cyprus (CYP), Eastern Turkey (ETU), middle Turkey (MTU), Western Turkey (WTU), Dalaman (DLM), Dalyan (DLY), Crete (CRE), Western Greece (WGR: Zakynthos and Lakonikos Bay), Calabria (CAL). Data for Calabria and Turkey from Garofalo et al. (2009) and Yilmaz et al. (2011), respectively. Grey squares feature the average values of nests per season derived from monitoring projects and white squares are estimates (adapted from Casale and Margaritoulis, 2010; Margaritoulis et al., 2003). Dashed lines represent the location of the three strongest genetic breaks revealed by BARRIER. The lowest number indentifies the strongest barrier.

2.2 DNA extraction and amplification

DNA was extracted with the QIAamp extraction kit (QIAGEN®) and 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'-CTCGGATTTAGGGGTTT-3′) (Abreu-Grobois et al., 2006). The analysis of longer sequences has been proven to improve the genetic resolution in *C. caretta* populations (Monzón-Argüello et al., 2010; Saied et al., 2012). The resulting fragment contains the 380 bp fragment traditionally used for population studies on this species (Carreras et al., 2006; Encalada et al., 1998; Norman et al., 1994). PCR cycling parameters were 94ºC for 5 min followed by 35 cycles at 94ºC for 1 min, 52ºC for 1 min, and 72ºC for 90 sec, and a final extension period of 72ºC for 10 min. Resulting products were purified by enzymatic reaction (ExoSAP) and sequencing reactions undertaken with fluorescent dye terminators (BigDye v3.1®). All samples were sequenced in both forward and reverse directions on an ABI 3730 automated DNA Analyser (Applied Biosystems®) to confirm variable sites on both strands of DNA.

2.3 Data analysis

Alignment was conducted using BIOEDIT v5.0.9 (Hall, 1999) and sequences were compared to short and long 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). New haplotypes identified were named following ACCSTR standardised nomenclature (LaCasella et al., 2007) and submitted to GenBank (Accession nos. JF837821-JF83782124).

To understand the genetic relationships between the sampled rookeries, pairwise genetic distances (γ_{st}) were calculated by the DNASP v5 software package (Librado and Rozas, 2009). The significance of genetic differentiation among these regions was assessed using Hudson's nearest neighbour statistics (S_{NN}) with 1,000 permutations in DNASP. Published long sequence data from Southern Italy (Calabria; Garofalo et al., 2009) and Turkey (Yilmaz et al., 2011, which includes Turkish samples from Carreras et al., 2007) were also used in the analyses. Five nesting groups were considered in Turkey as suggested by the authors' conclusions (Yilmaz et al., 2011): Dalyan, Dalaman, Western Turkey (Fethiye, Patara, Kale,

Kumluca and Çirali), middle Turkey (Gazipaşa, Kizilot, Tekirova and Belek) and Eastern Turkey (Anamur, Göksu Deltasi, Alata, Kazanli, Akyatan, Ağyatan and Samandağ). Recently published data from Libya (Saied et al., 2012) were not added to our dataset to avoid pseudoreplication as samples from both datasets were collected from the same location (Sirte) within a three year window. However, genetic differentiation analyses were undertaken with both datasets separately to look for possible differences. Following Narum (2006), modified false discovery rate (FDR) was used to evaluate statistical significance instead of the sequential Bonferroni correction when analysing multiple comparisons. Haplotype diversity (*h*; Nei, 1987) and nucleotide diversity (π; Nei, 1987) were estimated using ARLEQUIN v3.1 (Excoffier et al., 2005) and Fu's *Fs* values for each nesting region were calculated with DNASP. *Fs* detects deviation from neutrality and tends to be negative under an excess of recent mutations (Fu, 1997), which can result from population expansion. A partial correlation test between nucleotide diversity, mean width of the continental shelf (calculated with the ArcGIS software; ESRI, 2011) and the sea surface palaeotemperature (Thiede, 1978) in each nesting area was also carried out with SPSS v15 (SPSS Inc., 2006). The test was used to relate genetic diversities with environmental factors that could have affected nesting patterns. When necessary, variables were log-transformed or arcsine-transformed to satisfy the normality criterion (Zar, 1984).

Genetic structuring on a geographical scale was analysed with a Mantel test using GENEPOP v4.1 (Rousset, 2008). This analysis was conducted with minimum linear (Lat/Long positions) and coastal distances (following the coastline) between locations, calculated using the ArcGIS software (ESRI, 2011). Subsequently, based on a γ_{st} distance matrix, BARRIER v2.2 (Manni et al., 2004) was used to assess the relative order of importance of genetic breaks that could limit gene flow between populations. Previous studies based on mtDNA and microsatellite markers suggested that four is the most likely number of populations present in the Eastern Mediterranean (Carreras et al., 2007), which would imply the existence of three putative barriers. In consequence, we chose a priori to show four barriers since we used additional populations. In order to assess the proportion of genetic variation that explained the differences among nesting grounds, an analysis of molecular

variance (AMOVA) was undertaken with ARLEQUIN considering the four groups identified by the three strongest barriers.

To graphically relate pairwise genetic distances (γ_{st}) between areas, a Principal Coordinate Analysis (PCA) was performed with GENALEX v6.4 (Peakall and Smouse, 2006). Relationships between haplotypes were obtained by the calculation of a haplotype network with the NETWORK v4.5.1.6 software (Bandelt et al., 1999) using a Median Joining method. Less likely events were weighted differently from likely events, changing deletion (double weight) and transversion' weights (3x) according to user guidelines.

Finally, a molecular clock was applied to date the different colonisation events, using two different approaches. In the first one, the substitution rate for the 815 bp mtDNA fragment was calibrated assuming that the divergence between the two major branches of the Atlantic/Mediterranean haplotype tree occurred as a consequence of the rise of the Isthmus of Panama (Bowen, 2003). The Isthmus started rising 15 mya and did not become a complete marine barrier until *ca.* 3 mya (Lessios, 2008). Consequently, we rooted our molecular clock at 3 mya for conservative purposes. The substitution rate was obtained following the methodology previously used for testudines by Avise et al. (1992) considering the 39 fixed mutations existing between the closest related haplotypes (CC-A1.6 and CC-A31.1) of the two major branches of the Atlantic/Mediterranean haplotype tree resulting in a substitution rate of ~0.8% My^{-1}. However, it has been recently pointed out that the molecular evolutionary rate of mitochondrial DNA may be time-dependent (Crandall et al., 2012; Ho et al., 2011; Karl et al., 2012). Consequently, the substitution rate likely overestimates divergence times (Crandall et al., 2012), as calibration was done with an old event (3 mya). No recent calibration points or well-known pedigrees exist to estimate accurate divergence rates for this species. Thus, a second, more conservative approach using the mutation rate to date haplotype coalescence times was used following Emerson (2007). The mutation rate has been described to be 3-10 times faster than the substitution rate in other species (Howell et al., 2003; Lambert et al., 2002). In addition, the mean rate of change for mtDNA genes in three marine invertebrate species calibrated with radiometric dates for sea-level rise yielded values 3 times faster than those estimated from fossils and vicariant events (Crandall et al., 2012). Thus, we estimated the

divergence time between haplotypes of *C. caretta* using a mutation rate three times faster than the substitution rate and obtained lower and upper estimates by also dating coalescence times using the substitution rate and a mutation rate ten times faster than the substitution rate. A Bayesian relaxed-clock model was subsequently applied as implemented in BEAST v1.6.2 (Drummond and Rumbaut, 2007). Four unique Atlantic haplotypes (CC-A1.1, CC-A1.3, CC-A1.4 and CC-A1.6), were chosen as outgroups to root our Mediterranean haplotype tree. Markov-Chain Monte Carlo (MCMC) simulations were run for 10,000,000 generations, with the first 10% discarded as burn-in.

3. Results

A total of 17 haplotypes were found among the analysed Mediterranean rookeries (Table 1). In Lebanon, the comparison of the microsatellite genotypes (data not shown) of the new and old samples from Carreras et al. (2007) indicated that pseudoreplication did not occur in this population (mean LRM = -0.062 ± 0.110). Thus, all samples from Lebanon rookeries were pooled for further analyses. Most long haplotypes found in the current study were concurrent with the short ones previously described for the Mediterranean Sea (Carreras et al., 2007; Encalada et al., 1998; Laurent et al., 1998), as the new fragments include the old 380 bp fragments (Abreu-Grobois et al., 2006). However, some haplotypes identified with the 380 bp sequence were split into additional haplotypes, due to further polymorphism in the additional fragment of the longer sequences (eg. Table 1; CC-A2 split into CC-A2.1, CC-A2.8, CC-A2.9). Three new haplotypes were described because of an increase in sequence length that could be directly related to the 380 bp haplotypes: CC-A29.1 in Israel, CC-A32.1 in Zakynthos and CC-A50.1 in Cyprus (Table 1; GenBank accession nos. JF837821-JF837823). Furthermore, a new haplotype, not previously described for either long or short sequences, was found in Libya (CC-A65.1; GenBank accession no. JF837824); an unsampled or low sampled region in previous studies with short sequences (Carreras et al., 2007; Encalada et al., 1998; Laurent et al., 1998, 1993; but see Saied et al., 2012).

CC-A2.1 was the most frequent haplotype in the dataset (77.33%), followed by CC-A3.1 (12.50%). Of the remaining haplotypes, 13 were unique to a specific nesting beach and two were shared between Mediterranean nesting sites, although

they did not occur at high frequencies. The haplotype network showed a divergent sub-group with two unique haplotypes in Libya (CC-A26.1 and CC-A65.1) and one haplotype also shared with Israel (CC-A2.9) (Fig. 2). Eastern Turkey also presented a sub-group with unique related haplotypes (CC-A3.2 and CC-A52.1). However, Eastern Turkey's unique haplotypes had fewer mutation changes from the ancestral haplotype (CC-A2.1) than haplotypes from Libya. An ambiguity in the haplotype tree was found (am, Fig. 2) between CC-A3.1 and the unshared haplotypes from Western Greece (CC-A6.1 and CC-A32.1). It was resolved as indicated by Carreras et al. (2007) for short fragments based on geographical location similarities, as CC-A32.1 is only present in Western Greece and CC-A3.1 has not been found on these rookeries.

On the other hand, CC-A32.1 and CC-A6.1 share a gap but differ by a transition whilst CC-A32.1 and CC-A3.1 differ by that gap but share the transition. Thus, the most parsimonious explanation to this ambiguity is that the transition independently arose twice, as previously suggested (Carreras et al., 2007).

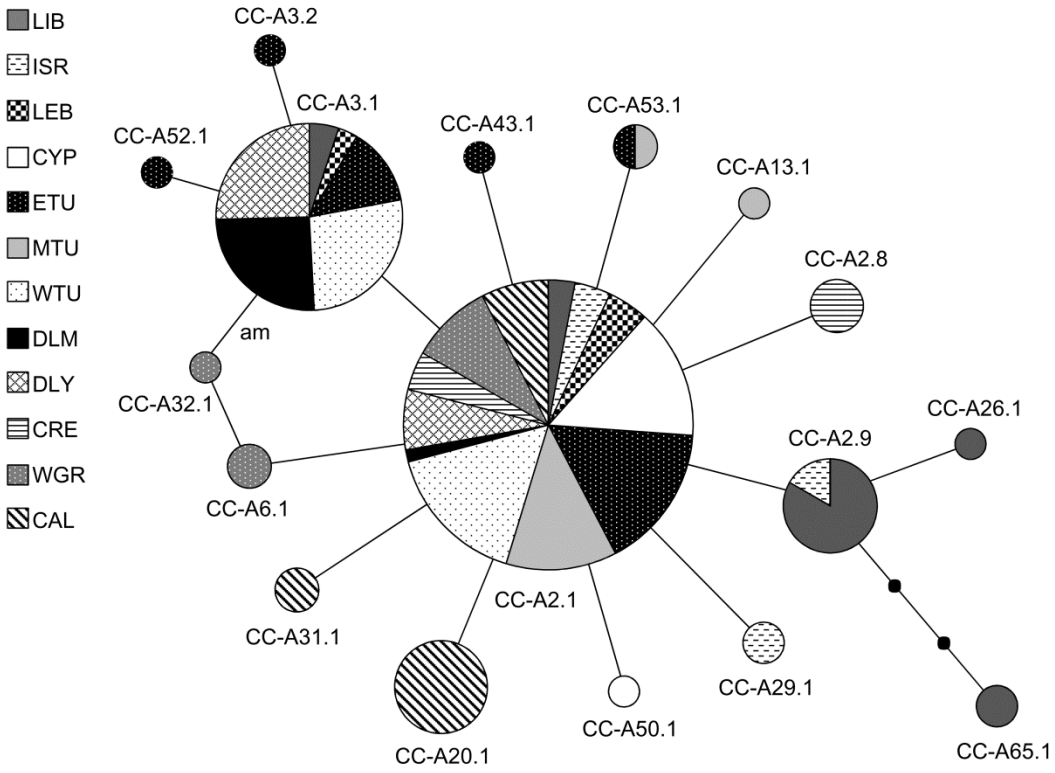

Fig. 2. Unrooted parsimony haplotype network of mtDNA for *Caretta caretta* in the Mediterranean Sea. Connecting lines represent single mutational changes between haplotypes with a probability higher than 95%. Unsampled intermediate haplotypes are indicated by dots and pie graphs represented to scale reflecting haplotype frequencies. [am] *Ambiguity resolved in text*.

Table 1 Absolute frequencies of haplotypes per sampling location for long mtDNA sequences. Short sequence equivalents and total number of individuals (*n*) per sampling location included. Locations: Libya (LIB), Israel (ISR), Lebanon (LEB), Cyprus (CYP), eastern Turkey (ETU), middle Turkey (MTU), western Turkey (WTU), Dalaman (DLM), Dalyan (DLY), Crete (CRE), Lakonikos (LAK), Zakynthos (ZAK), Calabria (CAL).

Short	CC-A2			CC-A3		CC-A6	CC-A13	CC-A20	CC-A26	CC-A29	CC-A31	CC-A32	CC-A43	CC-A50	CC-A52	CC-A53	CC-A65	n
Long	CC-A2.1	CC-A2.8	CC-A2.9	CC-A3.1	CC-A3.2	CC-A6.1	CC-A13.1	CC-A20.1	CC-A26.1	CC-A29.1	CC-A31.1	CC-A32.1	CC-A43.1	CC-A50.1	CC-A52.1	CC-A53.1	CC-A65.1	
LIB	11		10	3					1								2	27 (0)
ISR	15		2							2								19 (19)
LEB	17			2														19 (9)
CYP	44													1				45 (0)
ETU[a]	60			8	1								1		1	1		72
MTU[a]	46						1											48
WTU[a]	60			16														76
DLM[a]	5			15														20
DLY[a]	25			15														40
CRE	16	4																20 (19)
LAK	18					1												19 (19)
ZAK	16					2						1						19 (19)
CAL[b]	22							14			2							38

Re-sequenced samples (previously analysed in Carreras et al., 2007 with the short fragment) are given in brackets

[a]Data from Yilmaz et al. (2011).

[b]Data from Garofalo et al. (2009).

Haplotype (h = 0.04-0.70) and nucleotide (π = 0.000-0.002) diversities were highly variable (Table 2) due to the high number of haplotypes present in Eastern Turkey, Western Greece, Libya and Calabria in comparison to Cyprus, where very low variability was detected. Significant pairwise genetic differences were found in the majority of comparisons including Libya, Calabria and Dalaman (Table 3), thus revealing genetic structure within the basin (Global γ_{st} = 0.262, P < 0.001). Global γ_{st} values of all the Mediterranean rookeries did not differ when changing our dataset from Libya with the data from Saied et al. (2012) (Global γ_{st} = 0.264, P < 0.001). Furthermore, the two sets from Libya did not differ statistically (γ_{st} = 0.001, P = 0.215) despite some unshared haplotypes. Thus, both datasets agree in identifying Libya as the most diverse nesting area in the Mediterranean (Table 2).

Table 2 Haplotype and nucleotide diversities including standard deviations (±), results of Fu's *Fs* test and sample sizes per sampling location. The latitude (Lat.) and longitude (Long.) positions refer to a central point per nesting area, not to the specific position of the beach sampled, as samples came from wide areas pooled under one single location. Population abbreviations as in Table 1. Western Greece (WGR) groups individuals from LAK and ZAK.

	Haplotype diversity	Nucleotide diversity	Fu's *Fs*	n	Lat.	Long.
LIB	0.704 ± 0.054	0.0017 ± 0.0012	-0.909	27	30°59'19"N	17°34'50"E
ISR	0.374 ± 0.130	0.0005 ± 0.0005	-0.671	19	32°02'37"N	34°44'45"E
LEB	0.199 ± 0.112	0.0002 ± 0.0004	-0.055	19	33°16'32"N	35°11'33"E
CYP	0.044 ± 0.042	0.0001 ± 0.0002	-1.548	45	35°04'09"N	33°19'33"E
ETU	0.297 ± 0.067	0.0004 ± 0.0005	**-4.119**	72	36°45'50"N	34°52'37"E
MTU	0.082 ± 0.054	0.0001 ± 0.0002	-2.976	48	36°42'24"N	31°34'16"E
WTU	0.337 ± 0.054	0.0004 ± 0.0005	1.338	76	36°12'31"N	29°34'17"E
DLM	0.395 ± 0.101	0.0005 ± 0.005	0.976	20	36°41'51"N	28°45'33"E
DLY	0.481 ± 0.042	0.0006 ± 0.0006	1.728	40	36°47'28"N	28°37'16"E
CRE	0.337 ± 0.110	0.0004 ± 0.0005	0.721	20	35°21'51"N	24°27'29"E
WGR	0.198 ± 0.083	0.0003 ± 0.0004	-1.407	38	35°59'00"N	21°39'15"E
CAL	0.541 ± 0.049	0.0007 ± 0.0007	0.522	38	37°55'06"N	15°58'45"E

In bold significant values (Fu's *Fs*, P < 0.01)

Due to the lack of statistically significant divergence between Lakonikos and Zakynthos (γ_{st} = 0.027, P = 0.99) they were considered as subsamples of the same population, pooled for further analyses and referred to as Western Greece (WGR). This grouping was supported by the presence of the unique CC-A6.1 haplotype in both nesting areas and the evidence of female exchanges between Aegean Greece and the Ionian islands found in previous tagging studies (Margaritoulis, 1998) and microsatellite analyses (Carreras et al., 2007).

The global Fu's *Fs* test (*Fs* = -15.459, P < 0.01) was significant, indicating deviation from neutrality and a possible recent population expansion in this area, although for each location separately only Eastern Turkey presented a significantly negative Fu's *Fs* value (*Fs* = -4.119, P < 0.01; Table 2). The arcsine-transformed nucleotide diversity estimates were strongly correlated (partial correlation r = 0.847, P = 0.002) with the log-transformed mean width of the continental shelf and the sea surface temperature values during the last glacial period.

Table 3 Pairwise genetic distances between Mediterranean nesting populations (γ_{st}) (below diagonal) and S_{NN} significance (P) values (above diagonal).

	LIB	ISR	LEB	CYP	ETU	MTU	WTU	DLM	DLY	CRE	WGR	CAL
LIB	-	**0.001**	**~0.000**	**~0.000**	**~0.000**	**~0.000**	**~0.000**	**~0.000**	**~0.000**	**~0.000**	**~0.000**	**~0.000**
ISR	0.108	-	0.083	**~0.000**	**0.001**	**0.006**	**~0.000**	**~0.000**	**~0.000**	0.024	**0.011**	**~0.000**
LEB	0.160	0.056	-	0.087	0.913	0.061	0.329	**~0.000**	0.033	0.046	0.538	**~0.000**
CYP	0.243	0.062	0.053	-	**0.006**	0.873	**~0.000**	**~0.000**	**~0.000**	**0.007**	0.718	**~0.000**
ETU	0.160	0.038	0.002	0.040	-	0.039	0.189	**~0.000**	**0.003**	**0.001**	0.136	**0.001**
MTU	0.235	0.054	0.042	0.011	0.039	-	**~0.000**	**~0.000**	**~0.000**	**0.003**	0.690	**~0.000**
WTU	0.166	0.059	0.012	0.085	0.009	0.084	-	**~0.000**	0.075	**~0.000**	**0.007**	**~0.000**
DLM	0.318	0.437	0.422	0.622	0.264	0.582	0.220	-	**~0.000**	**~0.000**	**~0.000**	**~0.000**
DLY	0.187	0.139	0.077	0.224	0.062	0.214	0.031	0.125	-	**~0.000**	**~0.000**	**~0.000**
CRE	0.178	0.082	0.091	0.118	0.056	0.101	0.078	0.464	0.161	-	**0.010**	**~0.000**
WGR	0.227	0.060	0.028	0.012	0.024	0.011	0.059	0.592	0.186	0.113	-	**~0.000**
CAL	0.211	0.124	0.131	0.196	0.145	0.189	0.165	0.388	0.213	0.144	0.184	-

Bold values were significant after FDR correction for a threshold of α = 0.05 (S_{NN}, P < 0.0105)

Geographic and genetic distances were uncorrelated both when using Lat/Long positions (Mantel test, P = 0.160) and minimum coastal distances (Mantel test, P = 0.165). BARRIER indicated that the strongest genetic barrier detected by the Monmonier's maximum difference algorithm (Fig. 1) was found between Dalaman and Dalyan and the remaining populations (Barrier 1, γ_{st} = 0.582). The second barrier separated Libya (Barrier 2, γ_{st} = 0.227) and the third, Calabria from the rest (Barrier 3, γ_{st} = 0.184). The fourth was found between Dalaman and Dalyan (Barrier 4, γ_{st} = 0.125). The four groups (Libya, Dalaman and Dalyan, Calabria and the rest of the populations) identified by the three strongest barriers (Fig. 1) were subsequently used for the AMOVA analysis (Table 4).

Table 4 Analysis of molecular variance (AMOVA) for four Mediterranean genetic groups (Libya, Dalaman and Dalyan, Calabria and the rest of the sampled Mediterranean rookeries) based on the main three breaks inferred by BARRIER

Source of variation	d.f.	Percentage of variation	F-statistic	*P*
Among groups	3	28.68	FCT: 0.28681	< 0.005
Among populations within groups	8	4.74	FSC:0.06653	~0.000
Within populations	450	66.57	FST:0.33426	~0.000

Under this analysis, the highest percentage of variation was found within populations (66.57%) although the percentage of variation between groups was also significant and high (28.68%). PCA based on genetic distances (γ_{st}) between locations (Table 3) identified Dalaman, Dalyan, Libya and Calabria as highly distinct rookeries, with too small an amount of differentiation among the remaining rookeries to be classified as separate units (Fig. 3).

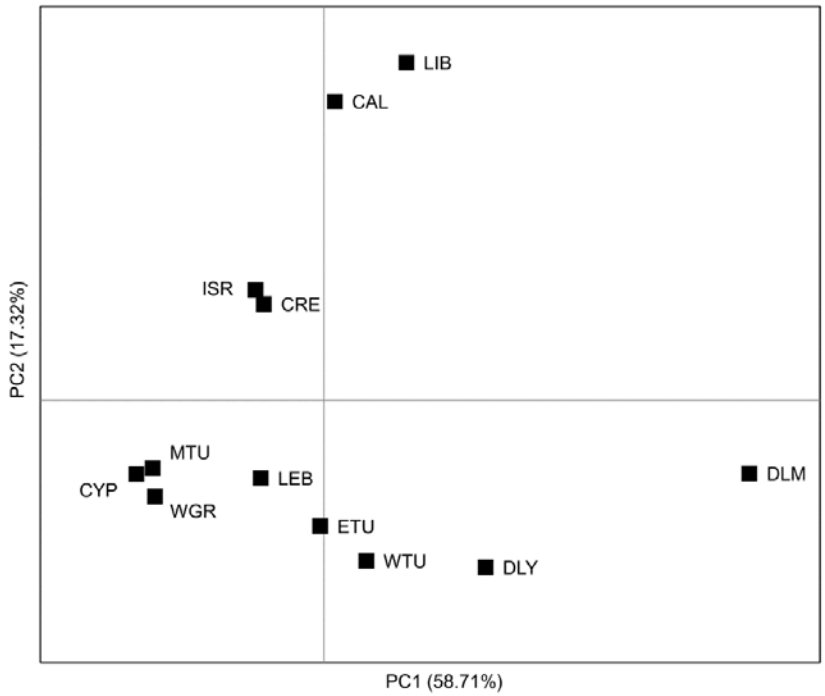

Fig. 3. Principal Coordinate Analysis for pairwise genetic distances (γ_{st}) between nesting colonies in the Mediterranean. First two Principal Coordinates (PC1 and PC2) and the percentage of variation explained by the 2 axes included.

Finally, based on the haplotype network and the number of mutations between haplotypes, a molecular clock was applied to date haplotype divergences. Dates were estimated with a mutation rate three times faster than the substitution rate. We used the inferred substitution rate calculated for this species (~0.8% My^{-1}) as a lower bound and a mutation rate 10 times faster than the substitution rate as an upper bound. Haplotype CC-A65.1 (exclusive to Libya), with four changes from the ancestral CC-A2.1, revealed Libya as the oldest population while haplotypes CC-A32.1 (exclusive to Western Greece) and CC-A3.2 and CC-A52.1 (exclusive to Eastern Turkey), with two changes from the Atlantic ancestor, suggested that these areas would have been more recent. Thus, Libya could have been colonised *ca*. 65,000 years ago (20,000-200,000) and Western Greece and Eastern Turkey *ca*. 30,000 years ago (10,000-100,000). The remaining populations originated as a result of a more recent, Holocenic expansion. All results were supported by the relaxed-clock model tree implemented in BEAST (Fig. 4), with haplotypes unique to Libya, Eastern Turkey and Western Greece diverging before the rest, thus revealing these as the oldest populations of the Mediterranean.

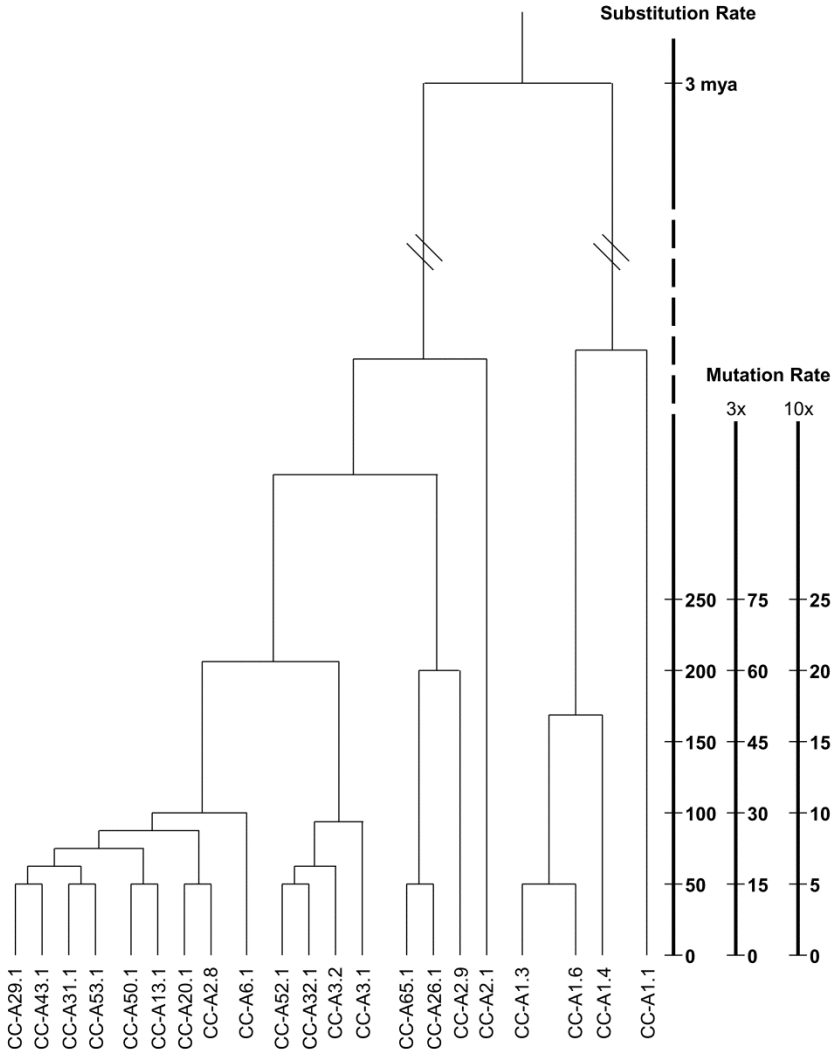

Fig. 4. Haplotype tree adapted from the Bayesian relaxed-clock model results inferred by BEAST. Time bars show different estimated dates (kya) for haplotype coalescence under a substitution rate of ~0.8% My^{-1} (left) and mutation rates 3 and 10 times faster (centre and right, respectively) following Emerson (2007).

4. Discussion

The study of molecular genetic differentiation between populations of endangered species has been described as a powerful tool for conservation planning (Crandall et al., 2000; Moritz, 1994). However, the markers selected and the length of the DNA sequences analysed can significantly alter the results (Monzón-Argüello et al., 2010; this study). For loggerhead turtles, the existence of genetic structure within the Mediterranean was previously detected with short sequences (380 bp) of the mtDNA control region (Carreras et al., 2007; Chaieb et al., 2010; Encalada et al., 1998; Laurent et al., 1998). Nonetheless, the higher nucleotide diversity present in the longer mtDNA fragment (815 bp), along with the analysis of

individuals from previously poorly sampled populations, allowed us to unveil a deeper structuring within the Mediterranean Sea.

4.1 Genetic structuring

The use of longer sequences allowed the splitting of the short CC-A2 and CC-A3 haplotypes into long haplotypes (CC-A2.1, CC-A2.8, CC-A2.9 and CC-A3.1 and CC-A3.2), which in turn revealed further structuring within Crete and Israel (CC-A2.8, CC-A2.9) and Eastern Turkey (CC-A3.2). Furthermore, the inclusion of Calabria (Garofalo et al., 2009) and Libya in the analyses revealed high levels of structuring previously undescribed (Bowen et al., 1993a; Carreras et al., 2007), as these two regions emerged as the most genetically diverse. This is because of the presence of two unique haplotypes in each of the two regions (CC-A26.1 and CC-A65.1 in Libya, and CC-A20.1 and CC-A31.1 in Calabria) and also because of a higher degree of divergence between these haplotypes and the other Mediterranean haplotypes. However, even though Libya and Calabria were found to be the rookeries with the highest diversity indexes, PCA and BARRIER analyses identified Dalaman and Dalyan as a higher differentiated unit. This is because of the high occurrence of CC-A3.1 in these two regions and in particular in Dalaman, where the proportion of CC-A3.1 was even higher than that of CC-A2.1 (Yilmaz et al., 2011), something not observed in any other rookery of the basin. The nesting area of Eastern Turkey hosted three unique haplotypes (CC-A3.2, CC-A43.1 and CC-A52.1) and one only shared with middle Turkey (CC-A53.1). Nonetheless, their frequencies were remarkably low and thus Eastern Turkey did not emerge as a major genetic unit. Cyprus was confirmed as a region with low genetic variability despite the large increase in sample size in relation to previous studies (Carreras et al., 2007; Encalada et al., 1998). However, could be slightly differentiated from most of the other nesting areas by the overwhelming dominance of the CC-A2.1 haplotype. In conclusion, we identify four major clusters of nesting grounds: Libya, Dalaman and Dalyan, Calabria and the rest of the Eastern Mediterranean, although some genetic differentiation exists within the latter cluster (Table 3).

4.2 Evolutionary History

The short (380 bp) haplotypes CC-A2, CC-A3 and CC-A20 are shared by Mediterranean and Atlantic rookeries (Bowen et al., 2004; Carreras et al., 2007;

Garofalo et al., 2009; Monzón-Argüello et al., 2010; Shamblin et al., 2011), indicating that this could have probably been the minimal ancestral haplotypic composition of the stock of loggerhead turtles that colonised the Mediterranean from the Northern Atlantic. Nevertheless, Carreras et al. (2007) also suggested an alternative hypothesis in which the origin of CC-A3 in the Mediterranean could have been independent from the Atlantic, in a clear case of homoplasy. Only a future long sequence screening of the variants of the CC-A3 and CC-A20 short haplotypes present in the Atlantic nesting beaches will clarify which hypothesis is correct. Nevertheless, at least two different CC-A3 variants have already been detected in the Mediterranean (Table 1 from Yilmaz et al., 2011).

Regarding the species history within the Mediterranean Sea, the analysis of individuals from previously poorly sampled nesting grounds (Libya, Turkey) revealed an earlier colonisation of the basin than previously suggested (Bowen et al., 1993a). This dating relies not only on the new haplotypes found in these nesting grounds, but also on the divergence rates applied. The substitution rate estimated (~0.8% My^{-1}) is higher than previously published estimates for other testudines (0.2 to 0.4%, Avise et al., 1992; Bowen et al., 1993b) probably due to the use of different markers and the length of the sequences analysed. Bowen et al. (1993b) analysed the cytochrome b region, which presents a lower substitution rate than the control region of the mtDNA (Dutton et al., 1996). Furthermore, differences in nucleotide diversity along the control region can alter the estimates depending on the length and region sequenced (Monzón-Argüello et al., 2010). As a consequence, the long sequences of the control region presented here had higher nucleotide diversity than the shorter fragments and thus, the substitution rate estimated in the present study is higher. Nevertheless, this makes our estimates of the substitution rate among Mediterranean haplotypes more conservative and thus, the older coalescence times inferred are solely due to the presence of previously unsampled haplotypes from Libya and Turkey. The time estimates changed when using mutation rates 3 and 10 times higher than the phylogeographically calibrated substitution rate (Crandall et al., 2012; Emerson, 2007). The presence of four mutations in a Libyan haplotype (CC-A65.1) from its Atlantic ancestor haplotype (likely to be CC-A2.1) places the oldest colonisation of the Mediterranean as a pre-Holocenic event occurring *ca*. 65,000 years ago (20,000-200,000). Thus, regardless of the molecular rate used, *C.*

caretta seems to have been present in the Mediterranean before the end of the last glacial period (~18,000 years ago; Thunell, 1979). According to this 3x molecular rate, turtles could have survived several cold periods in the Mediterranean (Cacho et al. 2000). The nesting grounds in Western Greece also present a haplotype (CC-A32.1) that is separated from its Atlantic ancestor by two changes, indicating that the population in that area has been stable for a long period of time. The presence of this haplotype dates the colonisation of Western Greece at *ca*. 30,000 years ago (10,000-100,000). This might also be true for Eastern Turkey, as haplotypes CC-A3.2 and CC-A52.1 are also separated by two mutations from the Atlantic ancestor, if it is CC-A2.1, or by one mutation if CC-A3.1 was already present in the colonisers. In the latter, the colonisation of Eastern Turkey would be more recent, 15,000 years ago (5,000-50,000), but discriminating between these two scenarios is dependant of future long sequences analyses of individuals from the Western Atlantic rookeries. The possible pre-Holocenic colonisation was not suggested by Bowen et al. (1993a) because they only considered palaeoclimatic evidences for a more restricted genetic sampling area. Thus, the presence of cold temperatures off Greece 18-12 kya, which could not have allowed nesting success on its beaches, brought Bowen et al. (1993a) to hypothesise a much more recent colonisation. However, the analysis of genetic markers locates this origin earlier than previously thought, suggesting that loggerhead turtles colonised the Mediterranean *ca*. 65,000 years ago (20,000-200,000) and that might have survived glacial periods by nesting at least in Libya and perhaps in Western Greece and Eastern Turkey as well. Thus, the first colonisation event would have happened during the upper Pleistocene and hence before the last glacial maximum.

The star-like shape of the haplotype network is a strong indication of recent expansions such as those related to post-glacial colonisation events (Kaiser et al., 2010; Maggs et al., 2008). This is corroborated by the global Fu's *Fs* although signal of expansion was only found significant for Eastern Turkey. Furthermore, as geographic and genetic distances were uncorrelated both when using Lat/Long positions and minimum coastal distances, we can discard isolation by distance as an explanation for the overall differentiation pattern. The higher diversity and haplotype divergences found in Libya (Saied et al., 2012; this study), and to a lesser extent in Western Greece and Eastern Turkey, suggest that these three areas could

have acted as refugia during cold events maintaining stable population sizes with mild or null bottlenecks. The glacial phase that affected the area from *ca.* 120 to 20 kya (Woodward and Hughes, 2011) probably caused the extinction of most of the populations in the basin leading to the disappearance of some ancestral haplotypes. However, some populations present in the warmer parts of Northern Africa would have survived during these glacial events. During the ensuing interglacial periods, loggerhead turtles might have recolonised the Eastern Mediterranean, only to become extinct in most of the new nesting grounds with the last glacial maximum. Nevertheless, the presence of haplotype CC-A6.1 in Western Greece and haplotypes CC-A3.2 and CC-A52.1 in Eastern Turkey indicate that these populations might have survived at least the most recent glacial peak. Consequently, the northern part of the Eastern Mediterranean and Western Peloponnese seems to have acted as warm refugia for marine species at that time, as has already been suggested for fishes (Domingues et al., 2008). This hypothesis could explain the genetic structure currently seen in Turkey, with a strong westward decline in haplotype diversity and a high variability in the frequency of CC-A3.1 between adjoining sites.

The existence of the highest frequencies of unique haplotypes in Libya and Eastern Turkey suggests that Western Greece probably was less suitable than the Libyan and Turkish coasts as a refugium. This may be explained by Libya and Eastern Turkey presenting a wider continental shelf which allowed a gentle progression of nesting beaches when the sea level decreased during glacial periods (Patarnello et al., 2007). Conversely, off the coast of Greece (Peloponnese), the continental shelf is much narrower which resulted in major redistribution of beaches and loss of many suitable nesting sites due to sea level fluctuations. This can be corroborated by the results found in our study, showing a strong correlation between the nucleotide diversity, width of the continental shelf and sea surface temperature in each of these refugia. Thus, the presence of warmer temperatures and wider continental shelves off Libya and Eastern Turkey could explain the high genetic variability found in these two areas. According to this correlation, Egypt could also be a potential refugium, but its population was depleted during the first half of the 20th century due to direct exploitation (Nada and Casale, 2010; Sella, 1982). It is worth noting that currently, the largest rookeries in the Mediterranean

are found at these potential refugia (Libya, Turkey and Western Greece; Fig. 1). However, this could be an artefact since population sizes in the easternmost Mediterranean rookeries (Israel and Lebanon) have notably changed in the past centuries due to human impacts such as fishing, direct exploitation and beach excavations (Sella, 1982).

The evolutionary hypothesis presented above is in accordance with previous studies suggesting that populations of several species of marine turtles survived glacial periods in warm refugia worldwide. Reece et al. (2005) found that Mexico, South Florida and the Caribbean may have acted as Pleistocenic refugia for Western Atlantic populations of loggerhead turtles during the climate depression at the Pliocene-Pleistocene border. Green (*Chelonia mydas*) and hawksbill (*Eretmochelys imbricata*) turtles also suffered some population contractions (Reece et al., 2005) and equatorial regions such as Brazil or Guinea Bissau have been proposed as Pleistocenic refugia for Atlantic green turtles (Encalada et al., 1996). Of all sea turtle species, the leatherback turtle (*Dermochelys coriacea*) may have been the most deeply affected by climate fluctuations, since it is the only species that extensively feeds at high latitudes (James and Mrosovsky, 2004). Nonetheless, it has been suggested that leatherbacks might have survived in the Indian-Pacific during the early Pleistocene to later recolonise the Atlantic, with a subsequent genetic bottleneck (Dutton et al., 1999).

Currently, loggerhead turtles from the Atlantic rookeries abound in the Western Mediterranean (Carreras et al., 2006), where sea surface temperatures are high enough to allow them to forage year round (Revelles et al., 2007a). Some Atlantic individuals even venture into the Eastern Mediterranean, but they are scarce there (Carreras et al., 2006; Casale et al., 2008b; Maffucci et al., 2006). Young loggerheads from the Atlantic rookeries reach Western Europe after drifting passively in the Gulf Stream and some may spend several years in the Mediterranean before returning to the Atlantic (Revelles et al., 2007c). This process certainly operated during the Pleistocene and allowed loggerheads to colonise the Mediterranean. However, during the cold phases of the Pleistocene, the sea surface temperature in the Western Mediterranean might have been too low (Thiede, 1978) to allow loggerheads to use it even as a foraging ground. This means that any gene flow between the Atlantic and the Mediterranean populations, mediated by

dispersal of turtles from the Atlantic populations, was interrupted during the cold phases of the Pleistocene thus leading to an increased genetic differentiation between the Mediterranean and Atlantic populations. The gene flow and the colonisation events were probably restored in the following warm phase when the Western Mediterranean again became a suitable feeding ground for Atlantic loggerheads. However, contemporary gene flow rates appear to be insufficient to genetically homogenise the two areas (Carreras et al., 2011).

The presence of haplotype CC-A20.1 in Calabria could be homoplasic, as previously discussed, but may also reveal a new colonisation event from the Atlantic that occurred during the Holocene. This could explain why this Atlantic haplotype is found exclusively in the most regularly visited westernmost nesting site in the Mediterranean. If this hypothesis is true, the current genetic structure of loggerhead turtles in the Mediterranean would be the result of at least two independent colonisation events. One taking place *ca.* 65,000 years ago (20,000-200,000) and a recent one 15,000 years ago (5,000-50,000) combined with local extinction and re-colonisation through the expansion of individuals from a few refugia following climatic fluctuations.

4.3 Conservation Implications

Loggerhead turtles nesting in the Mediterranean are considered an independent regional management unit (Wallace et al., 2010) with highly reduced gene flow with other populations in the North Atlantic (Carreras et al., 2011). The rookeries within this regional management unit generally exhibit stable abundance with high genetic diversity. However, under a relatively high degree of threat due to human activities, these populations could decline in the future if threats are not abated (Wallace et al., 2011). The main human activities impacting loggerhead turtles in the region are incidental bycatch and beach loss due to tourism development. Furthermore, direct take of immatures and adults is still a problem in some countries (Casale and Margaritoulis, 2010). Although the impact of these activities should be reduced everywhere, careful planning is necessary to guarantee that the conservation actions have positive impacts on the target populations. For instance, reducing the high levels of bycatch by bottom trawlers operating in the Adriatic sea (Casale et al., 2004) or off Tunisia (Casale et al., 2008b) will certainly

benefit the Mediterranean management unit. However, the actual relevance of such a hypothetical reduction for each of the four major groups of rookeries in the region (Libya, Dalaman and Dalyan, Calabria and the rest of the rookeries) could only partially be anticipated with the data previously available (Casale et al., 2008b; Maffucci et al., 2006). The data presented here will dramatically improve the resolution of mixed stock analysis (Carreras et al., 2006; Saied et al., 2012) for feeding grounds and hence will allow conservationists to indentify which rookeries will most likely benefit from reducing bycatch at particular feeding grounds or with a particular type of fishing gear.

The consequences of global warming are also a matter of concern, as direct impacts on marine turtles come from the flooding of nesting beaches due to the rise in sea level (Baker et al., 2006) and altered sex ratios because of the temperature-dependant sexual determination of these species (Hawkes et al., 2009). Marine turtles have adapted to previous climate fluctuations (Dutton et al., 1999; Encalada et al., 1996; Reece et al., 2005; this study), but they will have much lower chances in the context of the highly human-modified Mediterranean Sea. As temperature increases, some loggerhead populations are expected to expand northwards, colonising areas currently too cold for reproduction. However, most of the coastline in the northern shore of the Mediterranean has been intensely developed by the tourism industry and few places remain suitable for the nesting of loggerhead turtles. Furthermore, total beach surface will decrease as the sea level rises and buildings, roads and other infrastructures impede beaches moving inland. In this context, competition between the tourism industry and nesting loggerhead turtles will increase, with uncertain results for loggerhead turtles.

Acknowledgements

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1.2. Philopatry in loggerhead turtles (*Caretta caretta*): beyond the gender paradigm

Títol: Filopatria en tortugues babaues (*Caretta caretta*): més enllà del paradigma de gènere.

Resum: Les femelles de tortuga babaua presenten filopatria, fet que comporta que les femelles adultes retornin a zones específiques per nidificar. No obstant això, existeix menys informació referent a la filopatria i les migracions reproductores dels mascles. Estudis genètics sobre el flux gènic mediat per femelles fets a través d'anàlisis d'ADN mitocondrial han revelat una forta estructuració en diverses zones de nidificació del mar Mediterrani. Tot i així, l'avaluació de l'estructuració genètica és incompleta sense considerar els fluxos gènics d'ambdós gèneres: femelles i mascles. Això pot ser estudiat mitjançant l'anàlisi d'ADN nuclear (ADNn). Es van analitzar 152 nounats provinents de les zones de nidificació més rellevants del Mediterrani amb 15 marcadors microsatèl·lits. El grau de diferenciació genètica trobat va revelar l'existència de cinc unitats no descrites prèviament, diferents com a resultat d'aïllament per distància: Líbia i Xipre, Israel, Líban, l'oest de Turquia i Grècia. Els nostres resultats suggereixen que almenys a Israel, Líban, Turquia i Grècia l'aparellament succeeix prop de les zones de nidificació ja que aquestes van poder ser identificades com a unitats diferents a través de múltiples marcadors d'ADNn. Això revela una forta filopatria en ambdós sexes com a conseqüència d'un flux genètic limitat entre zones de nidificació. No obstant això, aquest no és el cas de Líbia i Xipre, ja que aquestes dues zones es van identificar com a pertanyents a la mateixa unitat. Ja que les tortugues de Xipre s'alimenten davant de la plataforma líbia, l'aparellament podria estar passant allà, tot emmagatzemant els espermatozous fins als moment de la fertilització i posta d'ous en platges xipriotes. Es conclou que l'anteriorment acceptada presència de flux gènic entre zones de nidificació mediat per mascles pot no ser certa per algunes poblacions mediterrànies, fet que suggereix que el flux gènic mediat per mascles pot haver estat tradicionalment sobrevalorat en la tortuga babaua.

Title: Philopatry in loggerhead turtles (*Caretta caretta*): beyond the gender paradigm.

Authors: Clusa M, Carreras C, Cardona L, Demetropoulos A, Margaritoulis D, Rees AF, Hamza AA, Khalil M, Levy Y, Türkozan O, Aguilar A, Pascual M.

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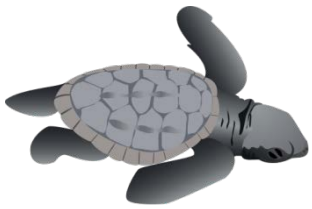

Philopatry in loggerhead turtles (*Caretta caretta*): beyond the gender paradigm

M. CLUSA,[a] C. CARRERAS,[a,b] L. CARDONA,[a] A. DEMETROPOULOS,[c] D. MARGARITOULIS,[d] A.F. REES,[d] A.A. HAMZA,[e] M. KHALIL,[f] Y. LEVY,[g] O. TÜRKOZAN,[h] A. AGUILAR,[a] and MARTA PASCUAL[i]

[a] Department of Animal Biology and IRBio, University of Barcelona, Av.Diagonal 643, E-08028 Barcelona, Spain, [b] Centre for Ecology and Conservation, Penryn Campus, University of Exeter, Penryn, TR10 9EZ, United Kingdom, [c] Cyprus Wildlife Society, Emmanuel Xanthou 11, P.O. Box 24281, 1703 Nicosia, Cyprus, [d] ARCHELON, the Sea Turtle Protection Society of Greece, Solomou 57, GR-10432 Athens, Greece, [e] Libyan Seaturtle Program, Environment General Authority, Alfateh University, P.O. Box 13793, Tripoli, Libya, [f] MEDASSET, P.O. Box 19, Tyre, Lebanon, [g] The Israel Sea Turtle Rescue Centre, Nature Parks Authority, Mevoot Yam, Mikhmoret 40297, Israel, [h] Adnan Menderes University, Faculty of Science and Arts, Department of Biology, Aydin, Turkey, [i] Department of Genetics and IRBio, University of Barcelona, Av.Diagonal 643, E-08028 Barcelona, Spain

Abstract

Female philopatry exists in the loggerhead turtle, with adult females returning to specific locations to nest. However, less is known about philopatry and breeding migrations of males. Genetic studies of female-mediated gene flow using mitochondrial DNA have revealed strong structuring in several nesting areas in the Mediterranean Sea. Nonetheless, genetic structuring assessment is incomplete without considering both male- and female-mediated gene flow. This can be studied through the analysis of nuclear DNA (nDNA). We analysed 152 hatchlings sampled from the major Mediterranean rookeries with 15 microsatellite markers. Fine-scale genetic differentiation revealed the existence of five previously undescribed units: Libya and Cyprus, Israel, Lebanon, western Turkey and Greece. Our results reveal isolation by distance and suggest that at least in Israel, Lebanon, western Turkey and Greece mating might be mainly occurring near the nesting areas as these four areas were identified as genetically differentiated units with multiple nDNA markers. This reveals strong philopatry in both sexes due to limited gene flow. However, this seems not to be the case for Libya and Cyprus as these two nesting areas were identified as belonging to the same group indicating a complex opportunistic pattern of breeding behaviour. Overall, we conclude that the previously suggested widespread male-mediated gene flow between nesting areas might not be true for some Mediterranean populations, which suggests that male-mediated gene flow has been traditionally overrated in the loggerhead turtle.

Keywords: *Caretta*, gene flow, male-mediated, microsatellites, philopatry, rookery

Introduction

Wildlife conservation has become a worldwide priority because of strong population declines during the past decades due to anthropogenic impacts. Thus, knowing population structure and defining management units is of crucial importance in order to infer successful management plans. Many studies have focused on population structuring and gene flow between these units through the use of DNA markers however, differentiation between populations can vary depending on the sample sizes, the number of markers used and the populations analysed (Nybom 2004; Beebee & Rowe 2008).

Levels of genetic differentiation among populations appear to increase with a higher number of samples analysed and markers used (Bernatchez & Duchesne 2000; Falush *et al*. 2007). This was empirically demonstrated on Atlantic cod (*Gadus morhua*) by Ruzzante (1998) showing that the number of individuals and loci remarkably influence genetic differentiation and structuring measures. Failing to detect differentiation between populations could bring to management errors and consequently the genetic structuring of wild populations has to be assessed in depth to ensure the survival of endangered species.

Genetic studies of mitochondrial DNA (mtDNA), a maternally-inherited marker, have revealed strong structuring among wild populations, from honey bees (*Apis mellifera*; Garnery *et al*. 1993) to bottlenose dolphins (*Tursiops sp.*; Krützen *et al*. 2004). However, even if mtDNA is a powerful marker to study population structure and phylogeographic processes, it does not take into consideration the contribution of males to the genetic structure of populations. This can be studied through the analysis of nuclear DNA (nDNA), which informs of both male- and female-mediated gene flow; of important relevance when designing conservation and management plans as both sexes might not behave equally (Prugnolle & de Meeus 2002; Lawson Handley & Perrin 2007).

Sea turtles have been traditionally mentioned as a good model to compare population structure of females and males due to the presence of differential behaviour and sex-biased dispersal in all sea turtle species (Bowen & Karl 2007). Whilst previous research revealed a strong female philopatry in sea turtles, with adult females returning to specific locations to nest (Miller *et al*. 2003), less is known

about philopatry, distribution patterns and breeding migrations of males. Adult turtles typically migrate from foraging grounds to breeding areas (Frick *et al*. 2000; Limpus 1993) and, after mating, males return to their foraging grounds while females remain to nest on sandy beaches (Arendt *et al*. 2012a; Schofield *et al*. 2010). These general patterns have been widely studied through long-term tag-recovery, telemetry and stable isotope analyses (Godley *et al*. 2010). However, research has been traditionally skewed to females as they are easier to sample while laying eggs in monitored nesting beaches. Nonetheless, it is now globally accepted that males follow similar migration patterns as females (Hatase *et al*. 2002; Godley *et al*. 2008) although timings and frequency of these migrations may vary between species and populations (Hays *et al*. 2010). Whether mating occurs in foraging grounds, in breeding grounds close to nesting beaches or en-route to nesting areas is still unclear.

Different sea turtle nesting populations with overlapping habitats might interbreed, increasing gene flow and significantly reducing genetic differentiation between populations. This has been described for green turtles (*Chelonia mydas*; FitzSimmons *et al*. 1997; Karl *et al*. 1992; Roberts *et al*. 2004) and also for loggerhead turtles nesting in the north-western Atlantic (Bowen *et al*. 2005). Consequently, nDNA structuring in nesting areas has been generally accepted to be lower than genetic structuring based on mtDNA in sea turtles as a result of widespread male-mediated gene flow (Birky *et al*. 1989; Jensen *et al*. 2013). However, variable levels of male-mediated gene flow among loggerhead turtle populations have been suggested in the Mediterranean Sea (Schroth *et al*. 1996; Carreras *et al*. 2007; Yilmaz *et al*. 2011).

The endangered loggerhead turtle hosts an independent regional management unit in the Mediterranean Sea, genetically separated from those in the Atlantic Ocean (Wallace *et al*. 2010; Carreras *et al*. 2011). Regular nesting only occurs in the eastern Mediterranean (Margaritoulis *et al*. 2003; Casale & Margaritoulis 2010) although some sporadic nesting has been reported in the western Mediterranean (Delaugerre & Cesarini 2004; Bentivegna *et al*. 2008; Tomás *et al*. 2008; Casale *et al*. 2012b). Genetic studies revealed that nesting areas in the eastern Mediterranean exhibit deep mtDNA genetic structuring (Laurent *et al*.

1998; Carreras *et al*. 2007; Yilmaz *et al*. 2011; Saied *et al*. 2012; Clusa *et al*. 2013), derived from a combination of isolation by distance, sequential colonisation and the use of glacial refugia during the Pleistocene (Carreras *et al*. 2007; Clusa *et al*. 2013).

Despite several isolated management units have been described based on mtDNA markers (Carreras *et al*. 2007; Yilmaz *et al*. 2011; Saied *et al*. 2012; Clusa *et al*. 2013), genetic structuring assessment is incomplete without considering both male- and female-mediated gene flow. The first signs of relevant population structure based on nDNA among Mediterranean rookeries were detected along the Turkish coast using randomly amplified polymorphic DNA (RAPD) markers (Schroth *et al*. 1996). This significant structure based on nDNA was also corroborated by Carreras *et al*. (2007) in a study comprising a larger number of eastern Mediterranean nesting areas and using 7 microsatellite markers. Nonetheless, other studies failed to identify genetic differentiation and restriction in male-mediated gene flow within the Mediterranean Sea (Yilmaz *et al*. 2011; Garofalo *et al*. 2013). Differences among studies could be partly due to sampling size effects as well as to reduced number of markers (Dutton *et al*. 1999 and Roberts *et al*. 2004).

To overcome these discrepancies, in the present work we have analysed 152 hatchlings sampled from the major Mediterranean loggerhead rookeries with 15 microsatellite markers. Specifically, we aim to (1) assess the genetic structuring in Mediterranean loggerhead rookeries by increasing resolution with a higher number of nDNA markers and sampled areas, (2) re-define management units in the Mediterranean Sea, (3) evaluate the genetic connectivity between these management units considering both male- and female-mediated gene flow and (4) assess the implications of such gene flow for loggerhead turtle conservation.

Materials and Methods

Sampling locations

Samples of skin and/or muscle were taken from 152 dead hatchlings from a selection of nesting grounds in the Mediterranean Sea (Fig. 1, Table 1). Nest sampling (2003-2006) included central Libya (west of Sirte), Israel (scattered sites along the whole coastline), Lebanon (El Mansouri), western Turkey (Fethiye),

Cyprus (Alagadi and Akamas) and Greece (Rethymno on the Island of Crete, Lakonikos Bay, and Zakynthos). Cyprus was considered as a single unit because no significant differences existed between samples of Alagadi and Akamas (D_{ST} = 0.011, P = 0.455; see protocols and statistical analysis below). Nests were excavated after hatchling emergence and samples were collected from one dead hatchling per nest and stored in 95% ethanol. The same samples were previously analysed for the mtDNA control region in Clusa *et al*. (2013). Independency among samples can be assumed as sampling included protocols to avoid pseudoreplication, e.g. female flipper tagging and samples taken from clutches laid within a 15-day window to avoid hatchlings from the same individual turtle as females rarely nest at intervals shorter than this period (Dutton 1995).

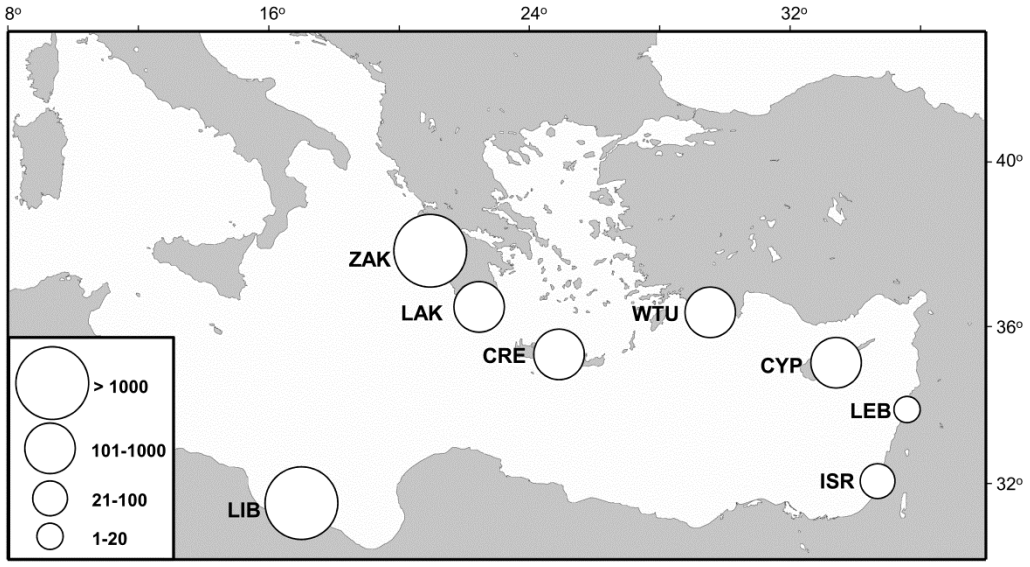

Fig. 1 Sampled nesting areas for the loggerhead turtle (*Caretta caretta*) in the Mediterranean Sea: LIB (Libya), ISR (Israel), LEB (Lebanon), CYP (Cyprus), WTU (western Turkey), CRE (Crete), LAK (Lakonikos), ZAK (Zakynthos). Circles represented to scale reflect average number of nests per season in the sampled nesting areas (adapted from Casale & Margaritoulis 2010).

nDNA analysis

DNA was extracted with the QIAamp extraction kit (QIAGEN®) and 15 microsatellite loci previously used for loggerhead turtle studies were amplified: Cc117, Cm72 and Cm84 (FitzSimmons *et al*. 1995); Ccar176 (modified by Carreras *et al*. 2007 from Moore & Ball 2002); Cc7 and Cc141 (Bowen *et al*. 2005); and Cc2, Cc10, Cc13, Cc16, Cc17, Cc22, Cc25, Cc28 and Cc30 (Monzón-Argüello *et al*. 2008). One primer for each marker was fluorescently labelled with 6-FAM, NED,

PET or VIC. Four PCR multiplex reactions were used to amplify the nine new microsatellite loci designed by Monzón-Argüello *et al*. (2008) following the author's protocol. Single PCRs were performed for failed amplifications and the remaining microsatellite markers following the protocol in Carreras *et al*. (2007). Fragment lengths were measured with an ABI 3730 automated sequencer at the Scientific-Technical Services from the University of Barcelona with GeneScan 500 LIZ (Applied Biosystems) as an internal size standard. Allele sizes were assigned with GENEMAPPER v3.5 (Applied Biosystems).

Data analysis

The mean number of alleles (*k*), observed heterozygote proportions (*Ho*) and Nei's genetic diversity estimates (*He*) were calculated for each nesting area using GENALEX v6.5 (Peakall & Smouse 2012). Differences in diversity among sampling sites were evaluated with Friedman ANOVA test and Wilcoxon pairwise tests with STATISTICA v10 (StatSoft 2011). Deviations from Hardy-Weinberg equilibrium and linkage disequilibrium between loci were assessed with GENEPOP v4.1 (Rousset 2008) and the presence of null alleles was inferred with FreeNA (Chapuis & Estoup 2007). The shortest distances along the coastline from each rookery to Libya were calculated using the ARCGIS v9 software (ESRI 2011). Linear regressions of *Ho* and *He* with the shortest distances along the coastline from each rookery to Libya were calculated with STATISTICA v10.

Pairwise genetic distances (F_{ST}) between nesting areas were calculated with GENEPOP v4.1 and their differentiation significance (G test) assessed by Markov Chain Monte Carlo (MCMC) randomisation. Pairwise genetic distances were also calculated using D_{ST} (Jost 2008) as traditional measures can fail in measuring differentiation when genetic diversity is high. D_{ST} was calculated with DEMETICS (Gerlach *et al*. 2010) and 10,000 iterations were pre-set to calculate significance of pairwise differences. Congruence between F_{ST} and D_{ST} distance measurements was analysed through a Mantel test with GENALEX v6.5. The congruence between pairwise genetic differentiations found with nDNA (this study) and pairwise genetic differentiations previously published for mtDNA for the same samples (γ_{ST}; Clusa *et al*. 2013) was also assayed using the same approach. Principal Coordinate Analyses (PCoA) as implemented in GENALEX v6.5 were used to plot D_{ST} and γ_{ST} pairwise

distances between areas for comparison among markers. Modified false discovery rate (FDR) was always used to evaluate statistical significance when analysing multiple comparisons as suggested by Narum (2006).

Isolation by distance comparing genetic and geographical pairwise distances was assessed through a Mantel test with GENALEX v6.5. The test was independently performed twice with the shortest distance between locations across the ocean and the shortest distance between locations along the coastline, calculated using ARCGIS v9. The most likely number of populations within the area (K = 1 to 8) was inferred with STRUCTURE v2.3.4 (Pritchard *et al*. 2000), a Bayesian algorithm for clustering. 100,000 MCMC iterations with a pre-set 10,000 burn-in were run 20 times for each K. The best K was obtained with the ad hoc statistic ΔK (Evanno *et al*. 2005) in STRUCTURE HARVESTER (Earl & vonHoldt 2012). The 20 runs for the best selected K were compiled with CLUMPP v1.1.2 (Jakobsson & Rosenberg 2007) and the result was represented with DISTRUCT v1.1 (Rosenberg 2004).

The algorithm implemented by STRUCTURE v2.3.4 does not consider geographic data. Consequently, a second analysis at a fine-scale level was undertaken with GENELAND v4.0.3 (Guillot *et al*. 2005), a spatially explicit Bayesian approach to determine the number of clusters in the study area based on genetic and geographic information. The parameter K was allowed to vary between 1 and 8 (maximum number of nesting areas analysed) in 20 runs and 100,000 MCMC iterations were set to calculate K in a spatial domain of 100 pixels along the X-axis and 100 pixels along the Y-axis.

The existence of recent genetic bottlenecks was tested by the Wilcoxon signed-ranked test with 100,000 iterations implemented in BOTTLENECK v1.2.02 (Piry *et al*. 1999) under the assumption of a two-phase microsatellite mutation model (TPM; 26% stepwise, 74% variable), as used for green turtles (FitzSimmons 1998, Roberts *et al*. 2004). Family relatedness within nesting areas was assessed with GENALEX v6.5 by the algorithm of Lynch and Ritland (LRM; Ritland 2000) and differences in relatedness between areas were evaluated by a Kruskal-Wallis test in STATISTICA v10.

Results

The 152 samples were successfully amplified and allele sizes ranged between 161bp (Cc30) and 432bp (Cc10). The mean number of alleles per microsatellite locus ranged from 2.75 (Cm72) to 9.5 (Cc7) (see Table S1). Heterozygote proportions (*Ho*) were not statistically different between nesting areas (Friedman ANOVA: $\chi^2 = 11.027$, $P = 0.137$; Table 1) but differences in Nei's genetic diversity (*He*) were highly significant (Friedman ANOVA: $\chi^2 = 25.993$, $P < 0.001$; Table 1). *Ho* and *He* in each nesting area significantly decreased in relation to the minimum distance following the coastline from Libya (Fig. 2; both $P < 0.01$), in accordance with the sequential colonisation of the Mediterranean Sea (Clusa *et al*. 2013). Accordingly, the nesting areas further away from Libya, i.e. Lakonikos and Zakynthos, showed the lowest mean *Ho* and *He* values. This was not led by only a few loci as *Ho* and *He* values decreased from Libya to Greece in > 65% of the analysed loci (data not shown).

Table 1 Number of analysed individuals (n), mean allele number (k), heterozygote proportion (Ho) and Nei's genic diversity (He) estimated per sampling location. Standard deviations included. Location acronyms as in Figure 1

	n	k	Ho	He
LIB	27	6.600 ± 2.028	0.649 ± 0.210	0.660 ± 0.174
ISR	19	6.267 ± 2.251	0.649 ± 0.230	0.681 ± 0.193
LEB	19	6.200 ± 2.038	0.622 ± 0.152	0.669 ± 0.148
CYP	21	6.200 ± 2.007	0.629 ± 0.229	0.668 ± 0.177
WTU	17	4.733 ± 1.387	0.619 ± 0.208	0.619 ± 0.163
CRE	18	5.267 ± 2.086	0.591 ± 0.226	0.623 ± 0.192
LAK	18	5.133 ± 2.134	0.594 ± 0.222	0.596 ± 0.183
ZAK	13	4.600 ± 1.404	0.557 ± 0.241	0.597 ± 0.200

Independence of loci was assumed as no linkage disequilibrium was found between loci pairs (χ^2: $P > 0.05$ in all cases). However, marker Cc25 was excluded from further analyses since it departed from Hardy-Weinberg equilibrium (χ^2: FDR $P < 0.014$) and presented a high frequency of null alleles in the majority of locations (mean 0.153 ± 0.08) as inferred with FreeNA.

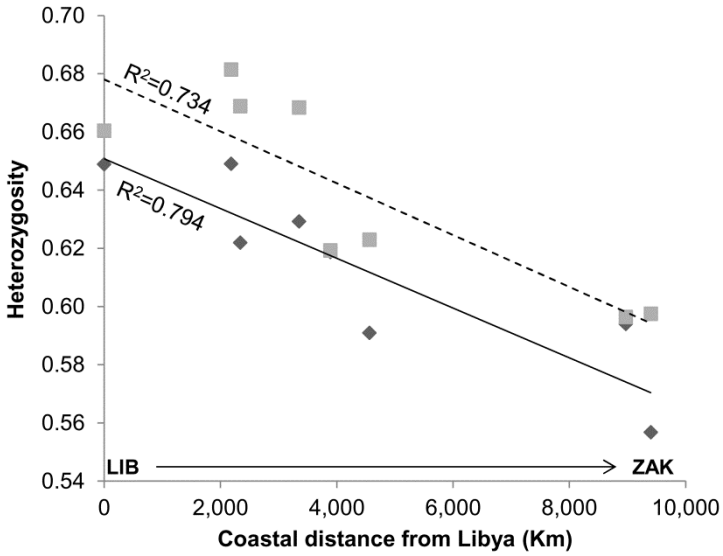

Fig. 2 Linear regressions between the mean observed heterozygosity (*Ho*; diamonds) or mean Nei's genetic diversity (*He*; squares) of each nesting area and their shortest distance along the coastline from Libya (Km).

Pairwise genetic distances between nesting areas based on F_{ST} and D_{ST} (Table 2) were strongly correlated ($R^2 = 0.943$, $P < 0.001$). Significant F_{ST} and D_{ST} genetic differences were found in ca. 80% of the pairwise comparisons between nesting areas except between Greek rookeries (Crete, Lakonikos and Zakynthos) and between Libya, Lebanon and Cyprus. No correlation was found between pairwise genetic distances analysed with nDNA (this study) and previously published genetic distances based on mtDNA using the same samples ($R^2 = 0.006$, $P = 0.340$; Clusa *et al*. 2013).

Table 2 Pairwise genetic distances between Mediterranean nesting populations: F_{ST} values below diagonal and D_{ST} values above diagonal. Location acronyms as in Figure 1

	LIB	ISR	LEB	CYP	WTU	CRE	LAK	ZAK
LIB		**0.061***	0.008	0.019	**0.061***	**0.088***	**0.095***	**0.101***
ISR	**0.015***		**0.053***	**0.043***	**0.080***	**0.086***	**0.107***	**0.138***
LEB	0.002	**0.016***		0.016	0.028	0.031	**0.044***	**0.065***
CYP	0.007	**0.017***	0.005*		0.036*	**0.062***	**0.108***	**0.091***
WTU	**0.017***	**0.028***	**0.006***	**0.010***		0.033	0.021	0.044
CRE	**0.024***	**0.026***	**0.009***	**0.022***	**0.011***		-0.001	-0.017
LAK	**0.033***	**0.039***	**0.016***	**0.044***	**0.014***	-0.002		-0.007
ZAK	**0.035***	**0.045***	**0.021***	**0.035***	**0.022***	-0.011	-0.004	

* Significant $P < 0.05$; in bold significant after FDR correction for a threshold of $\alpha = 0.05$ ($P < 0.013$).

According to the PCoA plot based on pairwise genetic distances with nDNA markers (D_{ST}) between locations (Fig. 3), the three populations from Greece clustered together and were highly differentiated from the other nesting areas. Among the remaining populations, Libya was particularly related to Cyprus and Lebanon whilst Israel was clearly separated from all these Levantine areas. The PCoA plot explained 81% of the variation with the first two axes. The first axis, explaining 63% of the total variation, distributed the populations following their geographical locations according to coastal distances. The same plot was obtained when using F_{ST} (data not shown), explaining 83% of the variation with the first two axes. The PCoA plot based on mtDNA (γ_{ST}, Fig. 3) only explained 72% of the observed variation and different clusters were shown in comparison to the D_{ST} plots, with Libya and Crete being the most differentiated nesting sites.

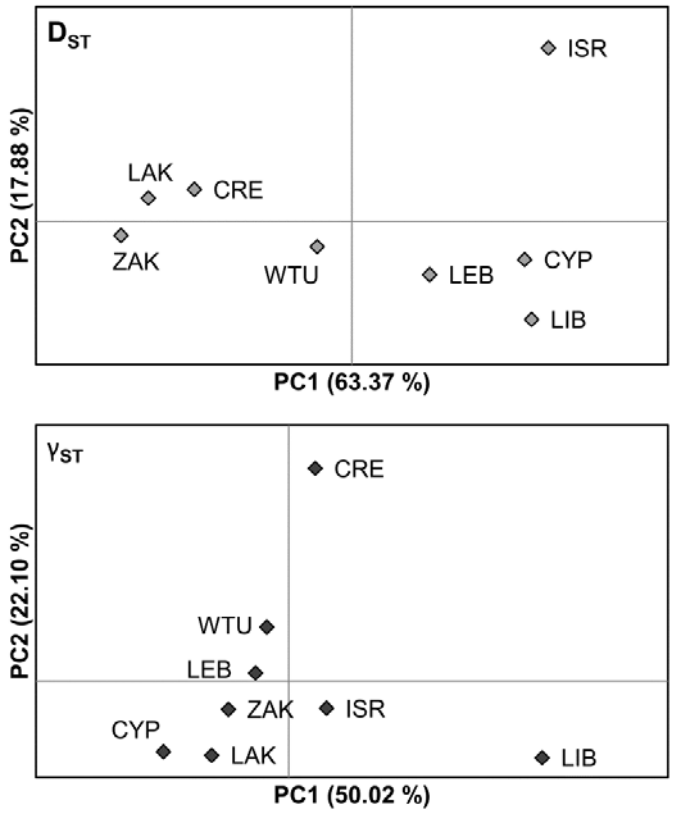

Fig. 3 Principal Coordinate Analyses for pairwise genetic distances between loggerhead turtle Mediterranean nesting areas for nDNA (D_{ST}) and mtDNA (γ_{ST}). First two Principal Coordinates (PC1 and PC2) and the percentage of variation explained by the 2 axes included. Location acronyms as in Figure 1.

The Mantel tests relating geographic and genetic distances (D_{ST}) revealed significant isolation by distance. However, a higher correlation was found when using the shortest distance between locations along the coastline (R^2 = 0.455, P =

0.002) than the shortest distance between locations across the sea (R^2 = 0.165, P = 0.044). Two genetically differentiated clusters were identified by STRUCTURE (K = 2; Fig. 4), separating the southern-eastern rookeries (Libya, Israel, Lebanon and Cyprus) from the northern rookeries (western Turkey, Crete, Lakonikos and Zakynthos).

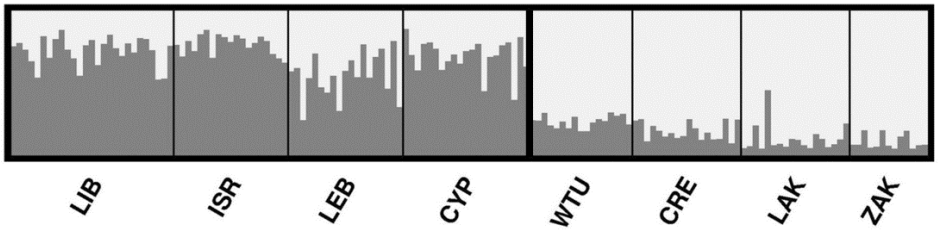

Fig. 4 Bar plot of the estimated membership fraction of each individual to the genetically differentiated clusters (K = 2) identified by STRUCTURE. Each bar shows the probability of each individual to belong to the southern-eastern cluster (dark grey; LIB, ISR, LEB and CYP) or to the northern cluster (light grey; WTU, CRE, LAK, ZAK). Location acronyms as in Figure 1.

GENELAND identified a fine-scale sub-structuring based on genetic and geographic information. With this approximation five clusters were identified (K = 5; Fig. 5): (1) Libya and Cyprus, (2) Israel, (3) Lebanon, (4) western Turkey and (5) Greece (Crete, Lakonikos and Zakynthos).

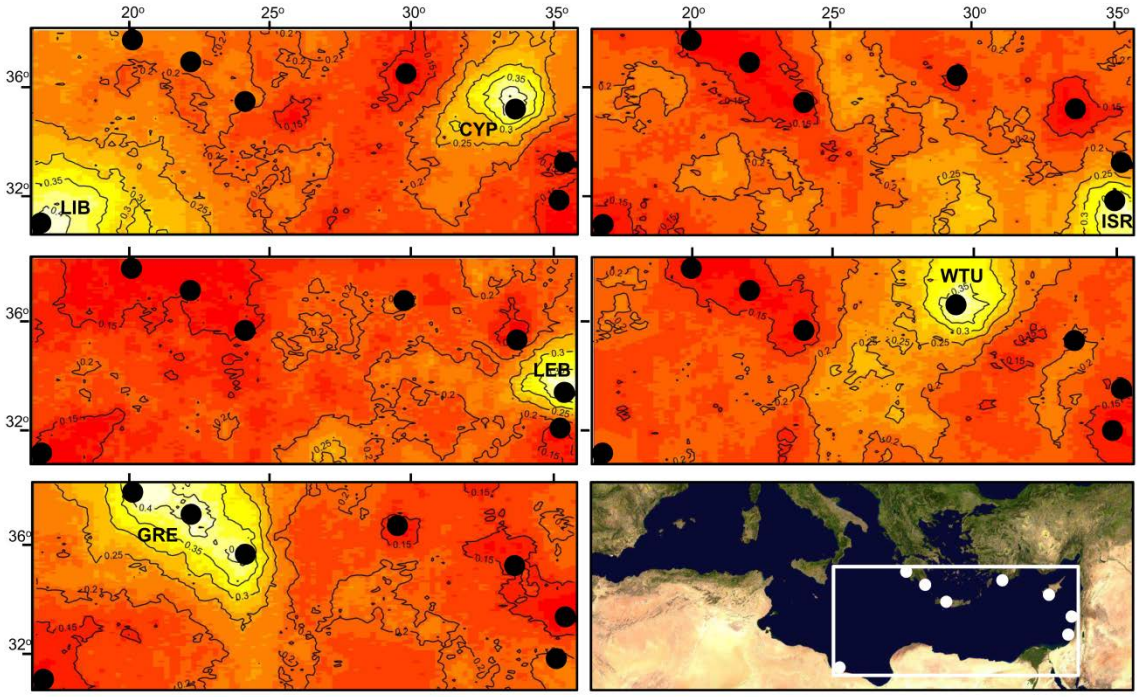

Fig. 5 Maps representing the five clusters identified in the spatially explicit analysis inferred from GENELAND: LIB and CYP (Libya and Cyprus), ISR (Israel), LEB (Lebanon), WTU (western Turkey) and GRE (Crete, Lakonikos and Zakynthos). The contours and colour patterns show the probability of assignment to each cluster. Map of the Mediterranean Sea with the area and rookeries represented by GENELAND in white (bottom left).

No evidences of recent bottlenecks were detected in any nesting area as the proportion of loci with heterozygosity deficiency was not significantly greater than expected as tested by the Wilcoxon rank test (all $P > 0.05$). Differences in relatedness were detected between nesting areas (Kruskal-Wallis: $\chi^2 = 14.162$, $P = 0.048$) with mean pairwise relatedness values within rookeries gradually increasing from Libya to Zakynthos (Fig. 6).

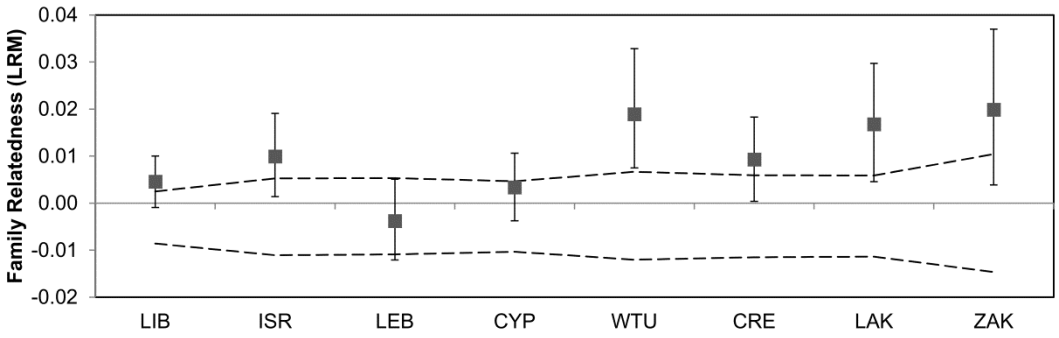

Fig. 6 Pairwise relatedness within each location (mean LRM values and standard error bars determined by permutations). Area between dashed lines represents the 95% CI of the null hypothesis of no differentiation across populations. Location acronyms as in Figure 1.

Discussion

Species with sex-biased dispersal and complex migratory patterns may involve deep genetic structuring of wild populations. Many species present philopatry only in females, with strong mtDNA structuring but not with nDNA due to male-mediated gene flow (Lyrholm *et al*. 1999; Pardini *et al*. 2001). However, male philopatry has been reported in some species such as sea turtles (Miller 1997) although widespread male-mediated gene flow between nesting areas has been traditionally accepted (Karl *et al*. 1992; FitzSimmons *et al*. 1997; Bowen *et al*. 2005).

Philopatry has been described as a successful strategy for female sea turtles to ensure viability of nesting beaches but also as a convenient behaviour for males, as philopatry increases the chances of finding available females to mate with (Schofield *et al*. 2009). Adult loggerhead males present similar migratory behaviour to adult females, increasing temporospatial overlap (Hatase *et al*. 2002; Schofield *et al*. 2009, 2013; Arendt *et al*. 2012a,b; Casale *et al*. 2013; Varo-Cruz *et al*. 2013). However, it is still unclear whether this overlap affects the genetic make-up of a population since it is unknown whether loggerhead mating occurs in foraging grounds, in breeding grounds close to nesting beaches or en-route to nesting areas.

Our results reveal a complex opportunistic pattern of breeding behaviour in loggerhead turtles. We identified strong philopatry of both sexes since significant genetic differentiation was detected between some rookeries using both nuclear and mitochondrial markers. Accordingly, mating occurs in breeding grounds close to nesting beaches or en-route to specific nesting areas. This was not previously recorded in loggerhead turtles since the detection of these levels of differentiation is tightly linked to the number of samples and to the type and number of markers used. Dutton *et al*. (2013) and Roden *et al*. (2013) recently found that population differentiation in leatherback (*Dermochelys coriacea*) and green turtles, respectively, highly increased when increasing the number of markers and samples used in comparison to their previous studies (Dutton *et al*. 1999; Roberts *et al*. 2004). Accordingly, previous studies with five or less markers failed to detect any structuring in loggerhead populations and hence wide-spread male-mediated gene flow was suggested in the Atlantic (Bowen *et al*. 2005). Similarly, Carreras *et al*. (2007) used seven markers and although some structuring was detected in the Mediterranean Sea, high degrees of male-mediated gene flow were accepted. Thus, only sea turtle studies with more than ten nDNA markers (Lee 2008, Dutton *et al*. 2013; Roden *et al*. 2013; this study) have been able to detect strong structuring with highly-restricted male-mediated gene flow among some of their populations. Accordingly, the results here presented corroborate the need for larger sets of nDNA markers in future studies on population structure to ensure better understanding of genetic structuring worldwide.

We have revealed significant genetic differentiation among loggerhead nesting areas in the Mediterranean Sea. Northern and southern-eastern Mediterranean rookeries are strongly differentiated, as revealed by STRUCTURE but finer scale genetic differentiation has been further unveiled with the existence of five units: Libya and Cyprus, Israel, Lebanon, western Turkey and Greece. These units detected with nDNA were not congruent with the three clusters (Libya, Crete and the rest) observed in the PCoA analysis based on mtDNA pairwise distances (same individuals as in Clusa *et al*. 2013). These differences could lie in the power of fine-scale differentiation detection that multiple microsatellites have in comparison to the single mtDNA marker (Goldstein & Pollock 1997; Godley *et al*. 2010). Nonetheless, differences may also arise since mtDNA only considers the genetic

structure based on females whereas nDNA reflects the structure of both sexes. Consequently, despite mtDNA is a powerful marker to infer historical colonisation processes (Bowen & Karl 2007), nDNA is needed to assess not only female but also male reproductive behaviour between nesting areas. This is of important relevance when designing conservation and management plans.

The results here presented show highly restricted male-mediated gene flow among most locations. However, this is not the case among the Greek rookeries as a high degree of male-mediated gene flow may be causing the lack of differentiation detected between Crete, Lakonikos and Zakynthos. Whilst Crete was statistically differentiated from western Greece with mtDNA (Clusa *et al*. 2013), nDNA analyses (this study; Carreras *et al*. 2007) clustered all Greek rookeries together showing that male-mediated gene flow exists between these neighbouring rookeries.

Surprisingly, Israel and Lebanon, also two close neighbouring nesting areas (<100km), were significantly differentiated with nDNA and mtDNA despite no evidence for the existence of an oceanographic barrier between them. Genetic differences for both types of molecular markers between these two localities can be attributed to strong philopatry of both sexes but also genetic drift could account for this result. Extensive turtle exploitation was reported in the eastern Mediterranean Sea during the 1920s (Sella 1982) which may have reduced population census sizes in Israel and Lebanon (Fig. 1). Allele frequency changes driven by genetic drift due to small population sizes could hence explain the genetic differentiation between these rookeries; masking male-mediated gene flow. Unfortunately, no genetic data exists from turtles previous to that period and thus, this hypothesis cannot be tested.

Western Turkey was significantly differentiated from the rest of Mediterranean rookeries as reported in previous studies (Carreras *et al*. 2007) although the differentiation values found here were on average higher, most likely due to the larger number of nuclear markers. The strong differentiation of this population based on mtDNA (Clusa *et al*. 2013) suggests that not only females but also males show philopatric mating behaviour, similar to the other Levantine nesting beaches. Further structuring might even exist within Turkey as previous studies suggested the existence of sub-structuring with a smaller number of microsatellite loci (Yilmaz *et al*. 2011) as well as isolation by distance with RAPD

markers (Schroth *et al*. 1996). Unfortunately, only samples from western Turkey were available for the present study and hence internal sub-structuring of Turkish nesting populations could not be re-assessed.

The lack of genetic differentiation found between Cyprus and Libya is in favour of a complex pattern of breeding behaviour, with females from Cyprus probably mating in foraging grounds off Libya. Satellite tracking has been widely used in the Mediterranean although research has been mainly located in Greek and Cypriot beaches (Broderick *et al*. 2007; Hays *et al*. 2010; Zbinden *et al*. 2011; Margaritoulis & Rees 2011; Patel *et al*. 2012; Schofield *et al*. 2013). Broderick *et al*. (2007) tracked post-nesting females from Cyprus and at least one individual migrated to foraging grounds off Tunisia in two different nesting seasons. This is in accordance with recent stable isotope studies (Cardona *et al. in press*) which have revealed that the majority of females nesting in Cyprus forage in the southern Ionian Sea. Accordingly, even if some females might forage in the Adriatic or northern Ionian Sea (Cardona *et al. in press*; Demetropoulos *unpubl. data*) evidence suggests that the North African coast is a relevant foraging area for adult loggerheads nesting in Cyprus.

Under this scenario, females from Cyprus could be mating in foraging grounds off Libya and sperm stored until egg laying in Cypriot beaches; a reproductive behaviour recorded for this species outside the Mediterranean Sea (Moore & Ball 2002; Lee 2008). Previous studies showed that mating can occur one or two months before the first ovipositional cycle (Miller 1997) by storing sperm in the upper oviduct (Gist & Jones 1989; Pearse & Avise 2001) until fertilisation and egg laying. However, the quality and viability of sperm may decrease in time (FitzSimmons 1998). Accordingly, females mate with the first male they encounter at the beginning of the season to ensure fertilisation (Lee & Hays 2004) but might upgrade their offspring by mating again with secondary, fitter males closer to oviposition (Moore & Ball 2002). Thus, depending on the availability of males closer to nesting beaches, gene flow mediated by mating in distant feeding grounds may vary.

Nests of loggerhead turtles in Alagadi (and the rest of northern Cyprus), where half the samples for the current study were collected, have the highest

female-biased hatchling production in the Mediterranean, with only 11% of males produced per year on average (estimated from incubation durations, Godley *et al*. 2001; Fuller *et al*. 2013). Primary sex ratio data from nesting beaches in southern Cyprus has not been published to date, but these beaches may differ in sand temperature due to differences in sand colour and grain size (Demetropoulos & Hadjichristophorou 1995). Thus, nests laid in southern Cyprus might produce a larger proportion of males. However, no genetic differences were found between individuals from Alagadi and Akamas, thus suggesting a similar pattern of gene flow. Although mating behaviour is observed regularly off Cyprus, particularly at Chrysochou Bay (Demetropoulos *pers. obs*.), the contribution of sperm from Libyan males to clutches laid in northern Cyprus might prevail. However, additional data on primary sex ratios from southern Cyprus is needed to test this hypothesis.

We did not detect any significant gene flow between Libyan and Greek rookeries even though some turtles from western Greece (Casale *et al.* 2013; Margaritoulis *et al*. 2003; Hays *et al*. 2010; Zbinden *et al*. 2011) and Crete (Margaritoulis & Rees 2011; Patel *et al*. 2012) also feed in foraging grounds off the North African coast. Nests of loggerhead turtles in the Greek rookeries produce 25-32% of male hatchlings every year (estimated from incubation durations, Zbinden *et al*. 2007a). Thus, even if Greek females mate in foraging grounds off the North African coast, it is highly likely that they re-mate afterwards with philopatric males of the same rookery. Sperm competition when mating again in Greek waters might occur, diluting the effect of the Libyan contribution. If this is the case, then mating again in front of Greek rookeries with the large abundance of males off Greece (Rees *et al*. 2013) would be enough to compete with the deteriorated and older sperm that females may be carrying from earlier mating in Libya.

The differential relevance of sperm competition depending on sex ratios close to rookeries is supported by the increased relatedness found in Greece. Turtles from Greece might show a strong isolation from turtles of the other nesting areas because of increased mating between Greek individuals. This might have an effect on the heterozygosity detected in the area (the lowest recorded in this study), which highlights the fact that Greece might be the largest nesting area but the one with the lowest effective population size. However, the higher individual relatedness in

Greek rookeries may be circumvented with high polyandry to ensure genetic variability of the offspring and reduce inbreeding depression as multiple paternity has been recorded in 93% of the nests laid in Zakynthos (Zbinden *et al*. 2007b).

In summary, we have defined five management units to be considered: Libya and Cyprus, Israel, Lebanon, western Turkey and Greece (Crete, Lakonikos and Zakynthos). Accordingly, future regional conservation plans should address all units to ensure the conservation of the genetic diversity found within the Mediterranean Sea. However, anthropogenic threats affecting turtles at foraging grounds, mainly interactions with fisheries, should be also taken into consideration as foraging grounds are reservoirs of mating for some Mediterranean populations. This highlights the need for international co-operation and underlines the importance of natural reproductive and migratory corridors for population conservation.

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Table S1 Relative allele frequencies, number of chromosomes (N), allele number (*k*), heterozygote proportion (*Ho*) and Nei's genetic diversity (*He*) per locus in each location. LIB (Libya), ISR (Israel), LEB (Lebanon), CYP (Cyprus), WTU (western Turkey), CRE (Crete-Rethymno), LAK (Lakonikos), ZAK (Zakynthos)

	Allele	LIB	ISR	LEB	CYP	WTU	CRE	LAK	ZAK
Cc2									
N		54	34	36	42	34	36	34	26
k		5	4	4	4	3	4	3	5
	224	0.000	0.000	0.056	0.095	0.088	0.028	0.000	0.038
	226	0.130	0.118	0.000	0.000	0.000	0.167	0.147	0.077
	228	0.074	0.059	0.083	0.143	0.147	0.083	0.000	0.038
	230	0.093	0.059	0.167	0.071	0.000	0.000	0.176	0.077
	232	0.685	0.765	0.694	0.690	0.765	0.722	0.676	0.769
	323	0.019	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Ho		0.407	0.353	0.500	0.381	0.412	0.444	0.529	0.462
He		0.474	0.394	0.480	0.489	0.386	0.443	0.490	0.393
Cc7									
N		48	32	36	36	32	36	36	26
k		11	11	11	11	7	8	11	6
	167	0.021	0.063	0.056	0.139	0.000	0.278	0.083	0.077
	171	0.146	0.281	0.139	0.194	0.188	0.111	0.056	0.192
	173	0.292	0.125	0.250	0.194	0.250	0.139	0.250	0.269
	175	0.000	0.000	0.000	0.028	0.031	0.000	0.000	0.000
	177	0.063	0.031	0.028	0.028	0.063	0.000	0.028	0.000
	183	0.083	0.063	0.056	0.083	0.250	0.056	0.194	0.269
	185	0.083	0.000	0.056	0.056	0.000	0.000	0.028	0.038
	187	0.042	0.188	0.083	0.083	0.000	0.083	0.083	0.000
	189	0.021	0.031	0.056	0.000	0.000	0.028	0.000	0.000
	191	0.042	0.031	0.028	0.000	0.000	0.000	0.028	0.000
	193	0.125	0.125	0.028	0.083	0.031	0.028	0.028	0.000
	197	0.083	0.031	0.222	0.083	0.188	0.278	0.194	0.154
	199	0.000	0.031	0.000	0.000	0.000	0.000	0.000	0.000
	201	0.000	0.000	0.000	0.028	0.000	0.000	0.028	0.000
Ho		0.875	0.938	0.833	1.000	0.750	0.722	0.833	0.769
He		0.849	0.842	0.847	0.872	0.799	0.802	0.841	0.787
Cc10									
N		48	26	36	40	34	30	34	18
k		5	5	6	5	4	4	4	5
	412	0.000	0.000	0.028	0.025	0.000	0.000	0.000	0.000
	416	0.229	0.154	0.083	0.200	0.088	0.333	0.235	0.333
	422	0.104	0.115	0.167	0.225	0.147	0.133	0.059	0.167
	428	0.521	0.462	0.472	0.450	0.588	0.500	0.618	0.389
	430	0.021	0.115	0.028	0.000	0.000	0.000	0.000	0.056
	432	0.125	0.154	0.222	0.100	0.176	0.033	0.088	0.056

Ho		0.708	0.769	0.722	0.700	0.235	0.600	0.412	0.667
He		0.649	0.713	0.691	0.696	0.593	0.620	0.552	0.704
Cc13									
N		50	32	32	42	34	28	32	22
k		8	7	7	7	5	9	6	6
	401	0.080	0.094	0.031	0.024	0.000	0.036	0.000	0.000
	403	0.000	0.000	0.000	0.000	0.000	0.036	0.000	0.000
	405	0.060	0.031	0.063	0.024	0.088	0.036	0.031	0.091
	407	0.220	0.188	0.219	0.286	0.294	0.179	0.250	0.273
	409	0.340	0.188	0.281	0.095	0	0.143	0.063	0.045
	411	0.140	0.063	0.156	0.190	0.265	0.321	0.469	0.364
	413	0.080	0.406	0.125	0.214	0.147	0.143	0.125	0.136
	415	0.000	0.000	0.000	0.000	0.000	0.036	0.000	0.000
	417	0.060	0.031	0.125	0.143	0.206	0.071	0.063	0.091
	419	0.020	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Ho		0.760	0.813	0.563	0.810	0.647	0.929	0.750	0.818
He		0.769	0.750	0.813	0.791	0.772	0.814	0.693	0.756
Cc16									
N		50	36	36	40	34	28	30	14
k		4	6	4	4	5	5	4	3
	340	0.000	0.000	0.056	0.025	0.029	0.071	0.000	0.000
	342	0.100	0.194	0.194	0	0.147	0.286	0.200	0.143
	344	0.480	0.278	0.528	0.700	0.500	0.357	0.367	0.429
	346	0.000	0.056	0.000	0.000	0.000	0.000	0.000	0.000
	348	0.200	0.278	0.139	0.200	0.147	0.071	0.100	0.000
	350	0.080	0.167	0.000	0.075	0.176	0.214	0.200	0.214
	352	0.000	0.028	0.000	0.000	0.000	0.000	0.000	0.000
Ho		0.760	0.722	0.611	0.350	0.765	0.643	0.933	0.714
He		0.694	0.776	0.645	0.464	0.675	0.735	0.758	0.704
Cc17									
N		44	34	34	36	28	30	20	14
k		7	9	5	4	3	3	3	3
	319	0.136	0.206	0.088	0.167	0.036	0.067	0.050	0.071
	321	0.227	0.118	0.235	0.222	0.393	0.267	0.250	0.286
	323	0.455	0.382	0.588	0.583	0.571	0.667	0.700	0.643
	325	0.023	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	331	0.000	0.029	0.000	0.000	0.000	0.000	0.000	0.000
	333	0.068	0.059	0.000	0.028	0.000	0.000	0.000	0.000
	337	0.000	0.029	0.000	0.000	0.000	0.000	0.000	0.000
	341	0.23	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	345	0.068	0.029	0.059	0.000	0.000	0.000	0.000	0.000
	347	0.000	0.088	0.029	0.000	0.000	0.000	0.000	0.000
	349	0.000	0.059	0.000	0.000	0.000	0.000	0.000	0.000

Ho		0.545	0.647	0.471	0.444	0.429	0.400	0.400	0.429
He		0.713	0.780	0.587	0.582	0.518	0.480	0.445	0.500

Cc22

N		54	36	38	40	34	34	36	22
k		7	7	6	7	5	4	5	5
	221	0.019	0.028	0.000	0.050	0.000	0.000	0.028	0.000
	223	0.111	0.417	0.158	0.125	0.147	0.147	0.250	0.136
	227	0.111	0.139	0.184	0.175	0.059	0.294	0.167	0.364
	229	0.556	0.306	0.526	0.325	0.618	0.500	0.528	0.409
	231	0.019	0.028	0.053	0.025	0.059	0.000	0.000	0.045
	233	0.093	0.028	0.026	0.275	0.118	0.059	0.000	0.045
	235	0.093	0.056	0.053	0.025	0.000	0.000	0.028	0.000
Ho		0.667	0.667	0.737	0.750	0.706	0.647	0.667	1.000
He		0.649	0.708	0.658	0.769	0.576	0.638	0.630	0.678

Cc25

N		50	18	34	38	34	32	32	18
k		6	5	6	5	5	4	6	5
	323	0.000	0.056	0.000	0.000	0.000	0.000	0.000	0.000
	325	0.080	0.111	0.029	0.105	0.029	0.125	0.031	0.111
	327	0.320	0.222	0.353	0.421	0.382	0.188	0.250	0.222
	329	0.020	0.333	0.059	0.079	0.029	0.063	0.063	0.111
	331	0.520	0.278	0.324	0.368	0.441	0.625	0.563	0.444
	333	0.000	0.000	0.059	0.000	0.000	0.000	0.000	0.000
	335	0.020	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	337	0.000	0.000	0.000	0.000	0.000	0.000	0.031	0.000
	341	0.040	0.000	0.176	0.026	0.118	0.000	0.063	0.111
Ho		0.480	0.333	0.412	0.368	0.647	0.250	0.438	0.444
He		0.618	0.747	0.732	0.669	0.644	0.555	0.611	0.716

Cc28

N		54	36	38	42	34	36	36	24
k		4	3	5	5	5	5	4	5
	190	0.333	0.361	0.342	0.381	0.294	0.111	0.139	0.083
	192	0.222	0.250	0.289	0.143	0.265	0.361	0.333	0.250
	194	0.000	0.000	0.000	0.000	0.000	0.028	0.000	0.083
	196	0.000	0.000	0.000	0.024	0.000	0.000	0.000	0.000
	208	0.093	0.000	0.079	0.119	0.088	0.167	0.139	0.250
	210	0.352	0.389	0.263	0.333	0.324	0.333	0.389	0.333
	212	0.000	0.000	0.000	0.000	0.029	0.000	0.000	0.000
	214	0.000	0.000	0.026	0.000	0.000	0.000	0.000	0.000
Ho		0.556	0.722	0.737	0.762	0.706	0.722	0.889	0.667
He		0.707	0.656	0.723	0.709	0.730	0.718	0.699	0.750

Cc30

N		54	34	34	38	32	34	32	22
k		7	5	5	7	5	4	5	3
	161	0.130	0.176	0.029	0.079	0.000	0.000	0.031	0.000
	165	0.074	0.029	0.000	0.000	0.063	0.000	0.031	0.000
	169	0.000	0.000	0.000	0.053	0.000	0.000	0.000	0.000
	171	0.204	0.294	0.353	0.263	0.313	0.382	0.188	0.318
	173	0.037	0.000	0.000	0.026	0.125	0.059	0.000	0.045
	175	0.259	0.265	0.118	0.263	0.250	0.118	0.094	0.000
	179	0.000	0.000	0.000	0.026	0.000	0.000	0.000	0.000
	181	0.278	0.235	0.471	0.289	0.250	0.441	0.656	0.636
	183	0.000	0.000	0.029	0.000	0.000	0.000	0.000	0.000
	185	0.019	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Ho		0.741	0.529	0.588	0.632	0.813	0.706	0.500	0.273
He		0.790	0.756	0.638	0.767	0.758	0.642	0.523	0.492

Cc117

N		54	38	38	36	26	36	34	26
k		8	7	7	9	7	8	6	6
	228	0.093	0.000	0.000	0.028	0.077	0.111	0.000	0.115
	230	0.000	0.026	0.000	0.000	0.000	0.000	0.000	0.000
	232	0.463	0.395	0.395	0.361	0.500	0.444	0.441	0.500
	234	0.130	0.105	0.053	0.111	0.077	0.083	0.029	0.077
	236	0.056	0.026	0.053	0.056	0.038	0.028	0.059	0.115
	238	0.130	0.211	0.211	0.167	0.077	0.111	0.235	0.038
	240	0.019	0.000	0.000	0.000	0.038	0.028	0.000	0.000
	244	0.074	0.211	0.237	0.167	0.192	0.139	0.206	0.154
	246	0.037	0.000	0.026	0.028	0.000	0.000	0.000	0.000
	248	0.000	0.000	0.000	0.056	0.000	0.000	0.000	0.000
	250	0.000	0.026	0.026	0.028	0.000	0.056	0.029	0.000
Ho		0.778	0.789	0.789	0.833	0.615	0.778	0.647	0.538
He		0.747	0.762	0.757	0.816	0.720	0.768	0.724	0.720

Cc141

N		52	24	36	42	28	30	32	18
k		9	9	10	8	6	6	7	4
	181	0.000	0.000	0.028	0.000	0.000	0.000	0.000	0.000
	189	0.173	0.042	0.083	0.190	0.143	0.100	0.063	0.000
	195	0.019	0.000	0.000	0.024	0.000	0.000	0.000	0.000
	197	0.365	0.042	0.222	0.190	0.000	0.000	0.000	0.000
	199	0.077	0.208	0.250	0.071	0.429	0.333	0.438	0.389
	201	0.154	0.292	0.111	0.167	0.179	0.100	0.156	0.000
	203	0.077	0.125	0.139	0.190	0.036	0.267	0.063	0.444
	205	0.077	0.125	0.028	0.143	0.179	0.167	0.156	0.111
	207	0.019	0.083	0.083	0.000	0.000	0.033	0.094	0.056
	209	0.000	0.042	0.000	0.000	0.000	0.000	0.031	0.000
	213	0.038	0.000	0.028	0.024	0.000	0.000	0.000	0.000

	215	0.000	0.042	0.028	0.000	0.036	0.000	0.000	0.000
Ho		0.885	0.917	0.778	0.857	0.857	0.600	0.688	0.556
He		0.793	0.826	0.840	0.837	0.730	0.769	0.742	0.636
Ccar176									
N		50	30	28	38	28	32	32	20
k		8	6	6	6	4	8	6	5
	170	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.000
	172	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.000
	176	0.240	0.267	0.143	0.289	0.179	0.188	0.094	0.200
	178	0.020	0.067	0.107	0.026	0.000	0.063	0.000	0.000
	184	0.000	0.000	0.000	0.132	0.179	0.031	0.063	0.000
	186	0.620	0.533	0.607	0.474	0.607	0.563	0.594	0.650
	190	0.000	0.000	0.000	0.000	0.000	0.031	0.000	0.050
	192	0.020	0.000	0.071	0.026	0.000	0.000	0.000	0.050
	194	0.020	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	196	0.040	0.067	0.036	0.053	0.036	0.031	0.063	0.050
	198	0.020	0.000	0.036	0.000	0.000	0.000	0.156	0.000
	204	0.020	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	206	0.000	0.000	0.000	0.000	0.000	0.031	0.000	0.000
	212	0.000	0.000	0.000	0.000	0.000	0.063	0.031	0.000
Ho		0.720	0.667	0.533	0.684	0.643	0.813	0.563	0.400
He		0.554	0.633	0.564	0.6700	0.566	0.637	0.605	0.530
Cm72									
N		54	38	18	40	30	36	32	26
k		4	3	3	5	2	2	2	1
	223	0.926	0.947	0.833	0.900	0.900	0.972	0.969	1.000
	233	0.019	0.000	0.000	0.025	0.100	0.000	0.000	0.000
	241	0.000	0.026	0.111	0.025	0.000	0.028	0.000	0.000
	243	0.000	0.000	0.000	0.000	0.000	0.000	0.031	0.000
	245	0.000	0.000	0.000	0.025	0.000	0.000	0.000	0.000
	247	0.037	0.026	0.000	0.025	0.000	0.000	0.000	0.000
	249	0.000	0.000	0.056	0.000	0.000	0.000	0.000	0.000
	251	0.019	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Ho		0.074	0.105	0.333	0.200	0.200	0.056	0.063	0.000
He		0.141	0.101	0.290	0.188	0.180	0.054	0.061	0.000
Cm84									
N		54	34	36	42	28	36	30	26
k		6	7	8	6	5	5	4	6
	311	0.037	0.000	0.083	0.048	0.000	0.000	0.000	0.000
	313	0.389	0.294	0.194	0.262	0.357	0.417	0.567	0.577
	315	0.259	0.235	0.361	0.405	0.429	0.306	0.267	0.115
	317	0.074	0.088	0.056	0.071	0.036	0.000	0.000	0.038
	319	0.000	0.000	0.028	0.000	0.000	0.000	0.000	0.038

	321	0.130	0.147	0.111	0.167	0.071	0.194	0.067	0.115
	323	0.111	0.176	0.139	0.048	0.107	0.056	0.100	0.115
	325	0.000	0.029	0.028	0.000	0.000	0.028	0.000	0.000
	327	0.000	0.029	0.000	0.000	0.000	0.000	0.000	0.000
Ho		0.778	0.765	0.722	0.667	0.857	0.556	0.600	0.615
He		0.746	0.796	0.789	0.730	0.671	0.691	0.593	0.624

CHAPTER 2. Distribution of juvenile loggerhead turtles in Mediterranean foraging grounds

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

Títol: Distribució de juvenils atlàntics i mediterranis de tortuga babaua (*Caretta caretta*) al mar Mediterrani.

Resum: Les tortugues babaues nidificant al mar Mediterrani presenten una notable estructuració genètica. Aquest treball parteix de la hipòtesi que els juvenils de tortuga babaua de diferents zones de nidificació no es distribueixen homogèniament entre les principals zones d'alimentació del Mediterrani com a conseqüència d'un complex patró de corrents superficials. Així, es va extreure ADN mitocondrial de 275 tortugues juvenils avarades o capturades accidentalment en sis zones d'alimentació (mar Catalano-Balear, conca algeriana, mar Tirrè, mar Adriàtic, el nord del mar Jònic i el sud del mar Llevantí). Es va usar un *mixed stock analysis* bayesià per estimar les contribucions des de zones de nidificació del Mediterrani, nord-oest de l'Atlàntic i Cap Verd a les zones d'alimentació estudiades. Es van trobar diferències en la contribució relativa de les tortugues juvenils d'origen atlàntic i mediterrani a cada àrea d'alimentació. Una decreixent proporció de juvenils atlàntics va ser detectada al llarg del corrent superficial principal que entra al mar Mediterrani des de l'Atlàntic, amb una elevada presencia de tortugues procedents de l'est de Florida a la conca algeriana i en menor nombre a la resta de zones. Pel què fa a les tortugues d'origen mediterrani, els juvenils de Líbia es concentren en les zones d'alimentació del Mediterrani central i occidental. Per contra, el mar Adriàtic es caracteritza per una notable presència d'individus de Grècia occidental, mentre que al sud del mar Llevantí hi ha una barreja heterogènia de tortugues provinents de zones de nidificació del Mediterrani oriental (Turquia, Líban i Israel). En general, la distribució de juvenils va poder ser directament relacionada amb els patrons de circulació superficials existents al mar Mediterrani i es pot concloure que les pesqueres poden tenir efectes diferencials en cada població depenent del grau de solapament entre les zones d'alimentació i àrees de pesca.

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

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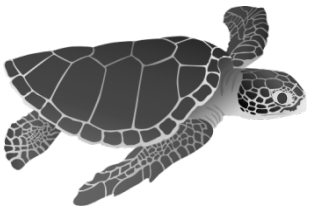

See original publication in Appendix A2

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

Marcel Clusa · Carlos Carreras · Marta Pascual · Stephen J. Gaughran · Susanna Piovano · Cristina Giacoma · Gloria Fernández · Yaniv Levy · Jesús Tomás · Juan Antonio Raga · Fulvio Maffucci · Sandra Hochscheid · Alex Aguilar · Luis Cardona

M. Clusa (✉) · C. Carreras · A. Aguilar · L. Cardona
Department of Animal Biology and IrBio, University of Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain
e-mail: mclusa@ub.edu

M. Pascual
Department of Genetics and IrBio, University of Barcelona, Av. 643, 08028 Barcelona, Spain

S. J. Gaughran
Sackler Institute for Comparative Genomics, American Museum of Natural History, Central Park West at 79th Street, New York, NY 10024 USA

S. Piovano · C. Giacoma
Department of Life Sciences and Systems Biology, University of Torino, Via Accademia Albertina 13, 10123 Turin, Italy

G. Fernández
Fundación Aspro Natura, c/ Garcilaso de la Vega 9, E-07181, Costa d'en Blanes, Calvià, Spain

Y. Levy
The Israel Sea Turtle Rescue Centre, Nature Parks Authority, Mevoot Yam, Mikhmoret 40297, Israel

J. Tomás · J.A. Raga
Cavanilles Institute of Biodiversity and Evolutionary Biology, University of Valencia, Aptdo. 22085, 46071 Valencia, Spain

F. Maffucci · S. Hochscheid
Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Naples, Italy

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 regards 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, whilst 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.

Introduction

Great migrations are often found in the animal kingdom and at very different scales (Hoare 2009). By migrating, species have adapted to increase their fitness and reproductive success for millions of years but nowadays many anthropogenic threats affect populations at their origin, destination and along migratory corridors. Only by understanding the distribution of these migratory species and the overlap with anthropogenic threats will conservation be possible.

Sea turtles are among these highly migratory species, undertaking long distance journeys sometimes spanning entire oceans (Bolten 2003; Plotkin 2003). One of the best known oceanic migrators is the loggerhead turtle (*Caretta caretta*), distributed in all tropical and warm-temperate areas and the most abundant sea turtle in the Mediterranean Sea (Broderick et al. 2002; Casale and Margaritoulis 2010). Loggerhead turtles of different origins co-exist in this area, as juveniles from western Atlantic rookeries share foraging grounds with those clutched within the Mediterranean (Bowen et al. 1993a; Laurent et al. 1993, 1998; Carreras et al. 2006, 2011). Small Atlantic juveniles enter the Mediterranean Sea through the Strait of Gibraltar during their pelagic stage and remain there until they are large enough to swim against the strong and permanent eastward current of the Strait (Revelles et al. 2007d; Eckert et al. 2008). During this period, juvenile turtles of Atlantic origin use the same foraging grounds as juveniles born in Mediterranean rookeries but rarely interbreed (Carreras et al. 2011), maintaining isolation between these two genetically distinct Regional Management Units (RMU; Wallace et al. 2010).

The distribution of juvenile loggerhead turtles of Atlantic and Mediterranean origin in the Mediterranean Sea has been widely studied through the use of satellite telemetry (Cardona et al. 2005; Bentivegna et al. 2007; Revelles et al. 2007b; Cardona et al. 2009; Casale et al. 2013), mark recapture techniques (Margaritoulis et al. 2003; Casale et al. 2007; Revelles et al. 2008) and genetics (Carreras et al. 2006; Maffucci et al. 2006; Casale et al. 2008b; Saied et al. 2012; Garofalo et al. 2013). In the western Mediterranean Sea, juvenile turtles of Atlantic origin mainly inhabit foraging grounds off the north-African coast and juvenile turtles of Mediterranean origin forage mainly along the European coasts (Carreras et al. 2006). However, little is known about the distribution and proportion of Atlantic juveniles in other areas within the Mediterranean Sea (Laurent et al. 1998; Maffucci et al. 2006; Casale et al. 2008b; Piovano et al. 2011). Furthermore, nothing is known about the distribution of young turtles from the different nesting populations existing in the Mediterranean Sea (Carreras et al. 2007; Garofalo et al. 2009; Saied et al. 2012; Clusa et al. 2013).

The relative contribution of each rookery to specific foraging grounds can be studied through Mixed Stock Analysis (MSA; Grant et al. 1980). Previous research in the Mediterranean Sea has mostly used a ~380bp fragment of non-coding mitochondrial DNA (mtDNA) as the genetic marker for MSA (Laurent et al. 1998; Maffucci et al. 2006; Carreras et al. 2007; Casale et al. 2008b; Carreras et al. 2011; Saied et al. 2012; but see Garofalo et al. 2013). However, the limited assignment power of this marker has precluded a fine-scale assessment of the contribution of Mediterranean rookeries to the Mediterranean foraging grounds. A new set of primers has been developed (Abreu-Grobois et al. 2006), which amplifies a longer segment of the mitochondrial control region (815bp) and hence increases the resolution of genetic structuring among the different nesting areas (Monzón-Argüello et al. 2010; Shamblin et al. 2012; Clusa et al. 2013). With this increase in the genetic resolution, origin assignment power of juveniles from Mediterranean foraging grounds is expected to improve at regional and fine-scale levels, potentially unveiling previously unknown distribution patterns.

Bycatch of juvenile turtles at their foraging grounds is one of the most significant anthropogenic threats for sea turtles in the Mediterranean Sea, with over 132,000 annual captures estimated in the area (Casale and Margaritoulis 2010;

Casale 2011). The impact of fisheries bycatch depends on habitat use, type of fishing gear, fishing effort, abundance of the affected populations and origin of these populations (Wallace et al. 2008). Thus, fine-scale information on the composition of bycatch in each fishing ground is essential for a proper impact assessment of turtle bycatch in the Mediterranean Sea.

This paper analyses the origin of juvenile loggerhead turtles from seven distinct foraging grounds within the Mediterranean Sea through a Mixed Stock Analysis with longer fragments of mtDNA with the aim to i) describe the distribution of juveniles of Atlantic origin within the Mediterranean Sea (regional level), ii) unveil the use of Mediterranean foraging grounds by juveniles of Mediterranean origin (fine-scale level), iii) understand the mechanisms of such distributions and iv) evaluate the impact that incidental bycatch in foraging grounds might have on nesting populations.

Material and Methods

Sample collection

Tissue samples were taken from 275 stranded or bycaught juvenile loggerhead turtles from several developmental foraging grounds in the Mediterranean Sea between 2002 and 2012 (Table 1). Only turtles smaller than 69cm curved carapace length (CCL) were sampled, as this is the average minimum size of nesting females in the Mediterranean (Margaritoulis et al. 2003) and turtles of Atlantic origin become adults at a much larger size (Piovano et al. 2011). Sampling was designed to ensure coverage of several juvenile foraging grounds within the major sub-basins in the region (Fig. 1): the Catalano-Balearic Sea (CAB), the Algerian basin (ALG), the Tyrrhenian Sea (TYR), the northern Adriatic Sea (NADR), the southern Adriatic Sea (SADR), the northern Ionian Sea (ION) and the southern Levantine Sea (LEV). No samples could be obtained from the southern Ionian Sea or the Aegean Sea, areas also known to be used by juvenile turtles as foraging grounds (Margaritoulis et al. 2003; Casale et al. 2013).

Muscle samples were collected from dead animals and stored in 95% ethanol. Blood samples were taken from live animals and stored frozen.

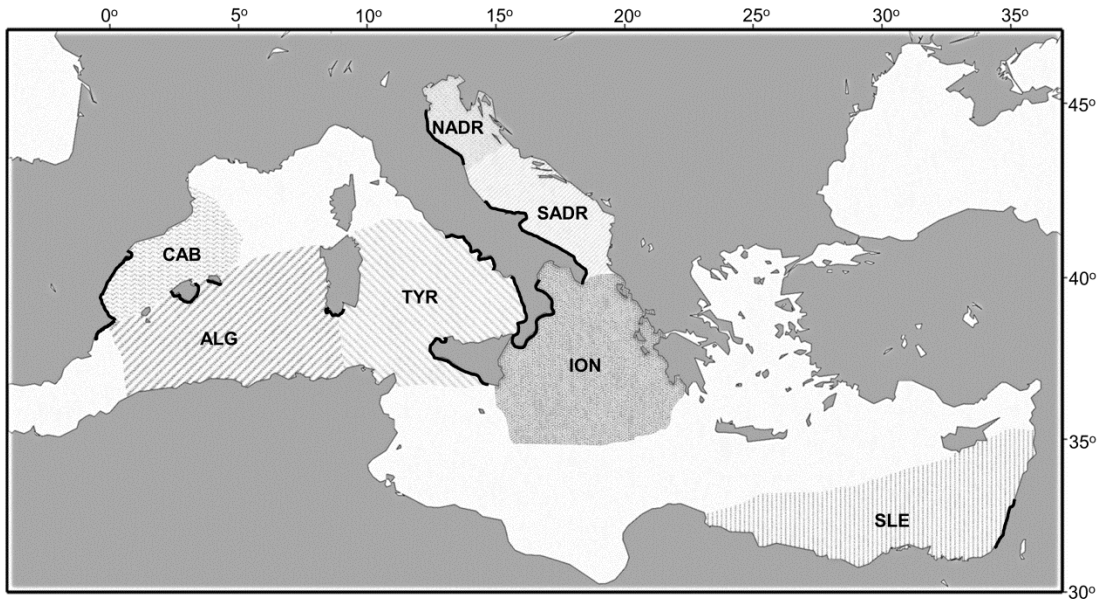

Fig. 1 Foraging grounds for juvenile loggerhead turtles sampled in this study: CAB (the Catalano-Balearic Sea), ALG (the Algerian basin), TYR (the Tyrrhenian basin), NADR (the northern Adriatic Sea), SADR (the southern Adriatic Sea), ION (the northern Ionian Sea) and SLE (the southern Levantine Sea). Black lines represent surveyed coastlines

Laboratory procedures

DNA from samples was extracted with the QIAamp extraction kit (QIAGEN®) following the manufacturer's instructions. An 815bp 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 to confirm variable sites on both strands of DNA on an ABI 3730 automated DNA Analyser at the Scientific-Technical Services at the University of Barcelona or at the Molecular Biology Service of the Stazione Zoologica Anton Dohrn.

Genetic structuring of foraging grounds

Sequences were aligned with BioEdit v7.1.6 (Hall 1999) and compared to the 815bp haplotypes previously described for this species compiled by the Archie Carr Center for Sea Turtle Research of the University of Florida (ACCSTR; http://accstr.ufl.edu). The resulting fragment also contains the 380bp fragment traditionally used in molecular studies on marine turtles (Norman et al. 1994).

Our results of the northern Ionian Sea were compiled with haplotype frequencies previously published from the same area (Garofalo et al. 2013) in order to increase sample size. Pseudoreplication between these two sample sets was not expected as all the individuals in this region were found dead in both studies. Compilation of haplotype frequencies for the other foraging grounds also analysed in Garofalo et al. (2013) was not done as individual carapace sizes fell off the considered range for juvenile loggerheads (Margaritoulis et al. 2003; Piovano et al. 2011).

Haplotype diversity (*h*; Nei 1987) and nucleotide diversity (π; Nei 1987) were estimated for each foraging ground using ARLEQUIN v3.5 (Excoffier et al. 2010) to analyse the genetic diversity of the sampled areas. Pairwise genetic distances (F_{ST}) between foraging grounds were calculated with the DnaSP v5.10 software package (Librado and Rozas 2009). The significance of genetic differentiation among these regions was assessed using Hudson's nearest neighbour statistic (S_{NN}) with 1,000 permutations. Statistical significance when analysing multiple pairwise comparisons was evaluated with a modified false discovery rate (FDR) (Narum 2006). Pairwise genetic distances between foraging grounds (F_{ST}) were plotted with a Principal Coordinate Analysis (PCoA) inferred with GenAlEx v6.5 (Peakall and Smouse 2012).

Stock composition

A Bayesian Mixed Stock Analysis (MSA) was used to assess the composition of each foraging ground as implemented in BAYES (Pella and Masuda 2001). This analysis estimates the proportion of individuals in each foraging ground coming from different rookeries. We used a baseline with a total of 23 rookeries (Supplementary Table 1) analysed in previous studies using the same primer pair (Garofalo et al. 2009; Monzón-Argüello et al. 2010; Yilmaz et al. 2011; Saied et al. 2012; Shamblin et al. 2012; Clusa et al. 2013). This baseline included haplotype frequencies from 10 Atlantic rookeries (Monzón-Argüello et al. 2010; Shamblin et al. 2012) and 13 Mediterranean rookeries (Garofalo et al. 2009; Yilmaz et al. 2011; Saied et al. 2012; Clusa et al. 2013), as loggerheads from both areas may potentially coexist in any of the Mediterranean foraging grounds considered. A 'many-to-many' MSA (Bolker et al. 2007) was not used in the present study because the

genetic characterisation of Atlantic foraging grounds based on 815bp mtDNA fragments is still unknown and this is needed for the 'many-to-many' approach.

Estimates on the size of each rookery (expressed as the mean number of nests per year; Supplementary Table 1) were included in the Bayesian approach as a weighting factor as suggested by previous studies (Bass et al. 2004). Iterated chains were only considered reliable when the Gelman-Rubin criterion was fulfilled (G-R shrink factor <1.2 for all parameters; Gelman et al. 1996). The analyses were undertaken twice: first considering two regional areas (Atlantic and Mediterranean; regional level) and second considering all rookeries as independent units (fine-scale level).

Results

Genetic structuring of foraging grounds

A total of 17 different haplotypes were found in the Mediterranean foraging grounds analysed (Table 1), all of them described in previous studies. Haplotype CC-A2.1 was the most dominant (70.9%), followed by CC-A1.1 (10.2%). Five haplotypes were exclusive to Atlantic rookeries (CC-A1.1, CC-A1.3, CC-A5.1, CC-A10.4 and CC-A14.1), six exclusive to Mediterranean rookeries (CC-A2.8, CC-A2.9, CC-A6.1, CC-A29.1, CC-A31.1 and CC-A32.1) and three shared between Atlantic and Mediterranean rookeries (CC-A2.1, CC-A3.1 and CC-A20.1). The remaining haplotypes (CC-A10.3, CC-A28.1 and CC-A55.1) have only been described in foraging grounds but have not been found in any rookery to date. However, their combined frequency was very low (1.1%). Overall, haplotype and nucleotide diversities in foraging areas were highly variable (h range: 0.095-0.668; π range: 0.0001-0.0248) with the Algerian basin presenting the highest haplotype (0.668±0.041) and nucleotide (0.0248±0.0123) diversities (Table 1). Highly significant genetic structuring was found among 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.

Table 1 Absolute mtDNA haplotype frequencies found in the Mediterranean foraging grounds for juvenile loggerhead turtles: CAB (the Catalano-Balearic Sea), ALG (the Algerian basin), TYR (the Tyrrhenian basin), NADR (the northern Adriatic Sea), SADR (the southern Adriatic Sea), ION (the northern Ionian Sea) and SLE (the southern Levantine Sea). Total number of haplotypes (n), number of turtles found dead (d), haplotype diversity (*h*) and nucleotide diversity (π) found in each foraging ground included at the bottom of the table. Mean standard deviations included (±)

	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						1	
n	40	65	51	29	21	35	34
d	33	48	46	29	21	35	34
h	0.439±0.098	0.668±0.041	0.409±0.084	0.197±0.095	0.095±0.084	0.613±0.083	0.321±0.101
π	0.0095±0.0050	0.0248±0.0123	0.0109±0.0057	0.0002±0.0004	0.0001±0.0002	0.0010±0.0008	0.0033±0.0020

The majority of pairwise statistically significant differences occurred between the Algerian basin and the central-eastern side of the Mediterranean (Table 2).

Table 2 Genetic distances (F_{ST}) among Mediterranean foraging grounds for juvenile loggerhead turtles (below diagonal) and S_{NN} significance p values (above diagonal). CAB (the Catalano-Balearic Sea), ALG (the Algerian basin), TYR (the Tyrrhenian basin), ADR (the Adriatic Sea), ION (the northern Ionian Sea) and SLE (the southern Levantine Sea)

	CAB	ALG	TYR	ADR	ION	SLE
CAB		0.032	0.660	0.037	0.100	0.492
ALG	0.194		0.006*	<0.001*	<0.001*	<0.001*
TYR	-0.019	0.164		0.022	0.005*	0.270
ADR	0.071	0.379	0.099		0.002*	0.422
ION	0.058	0.364	0.088	0.040		0.062
SLE	0.012	0.316	0.036	-0.002	0.003	

* Significant S_{NN} p values after FDR correction for a threshold of α=0.05 ($p < 0.015$)

PCoA ordination also reflected the deepest differentiation between the Algerian basin and the rest of foraging grounds, explaining 93.89% of the observed variation with the first two axes (Fig. 2). This analysis also separated the Catalano-Balearic Sea and the Tyrrhenian Sea from the rest, although only by the second axis, which in turn explained only 11% of the total variation.

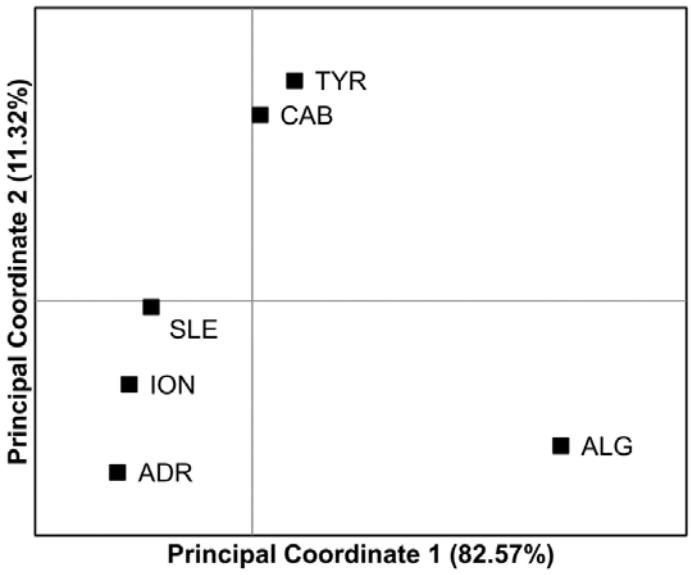

Fig. 2 Principal Coordinate Analysis based on genetic distances (F_{ST}) between juvenile loggerhead turtles in Mediterranean foraging grounds. Percentage of variation explained by each coordinate included in brackets. Foraging ground acronyms as in Table 2

Stock composition

MSA results showed that the deep differentiation between the Algerian basin and the other foraging grounds reported above was due to the overwhelming prevalence of individuals of Atlantic origin in the Algerian basin (Fig. 3). Individuals of Atlantic origin could be detected in all the foraging grounds considered but nowhere was the Atlantic contribution as strong as in the Algerian basin (58.4±11.2%). Overall, the majority of the Atlantic contribution came from central eastern Florida and south eastern Florida (CEF and SEF; Supplementary Table 2). All the other foraging grounds studied hosted mainly Mediterranean individuals, with the strongest Mediterranean contribution (Fig. 3) found in the northern Ionian Sea (96.4±3.6%) and the Adriatic Sea (93.6±16.2%).

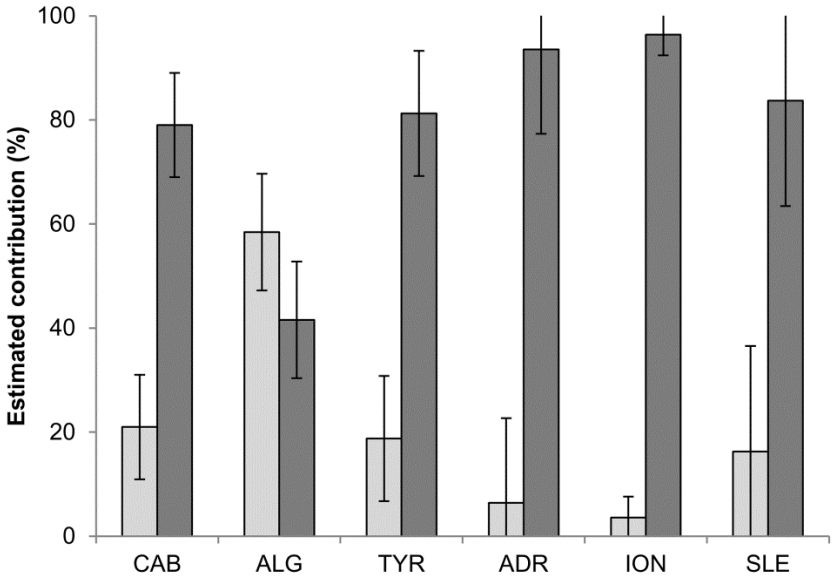

Fig. 3 Atlantic (light grey) and Mediterranean (dark grey) juvenile contributions to each Mediterranean foraging ground estimated by MSA. Standard deviation bars included. Foraging ground acronyms as in Table 2

Results based on un-clustered rookeries (Fig. 4, Supplementary Table 2) showed that juveniles from Mediterranean rookeries were not homogenously mixed in the Mediterranean Sea, with major differences between adjoining foraging grounds. Whilst the Adriatic Sea was inhabited by a high proportion of turtles from western Greece (57.8±33.3%), the northern Ionian Sea hosted individuals mainly from Misurata in Libya (70.4±34.9%).

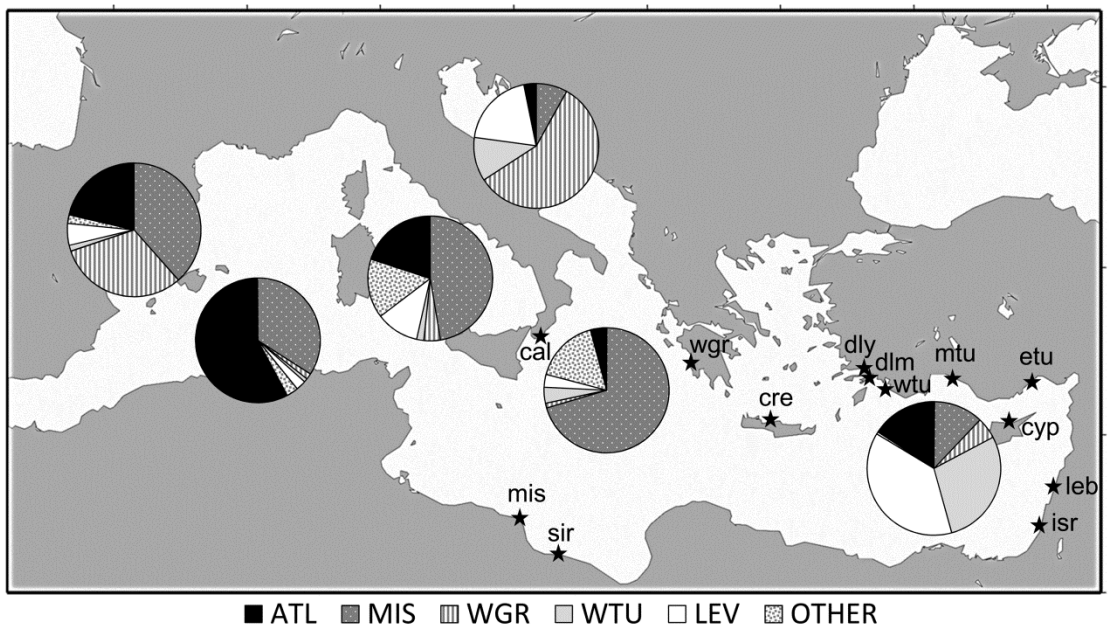

Fig. 4 Fine-scale rookery contributions (%) to Mediterranean foraging grounds estimated by MSA. Rookeries: ATL (Atlantic), MIS (Misurata, Libya), WGR (western Greece), WTU (western Turkey), LEV (Israel, Lebanon, Cyprus and other Turkish rookeries), OTHER (Sirte, Libya; Calabria, Italy; Crete, Greece). Stars show Mediterranean rookery locations

The Tyrrhenian Sea also hosted mainly individuals from Misurata (47.4±31.3%) but there was also relevant contribution from Calabria (14.5±12.5%). Juvenile turtles from Misurata (38.6±29.1%) and from western Greece (31.3±23.7%) had a similar abundance in the Catalano-Balearic Sea. Finally, the southern Levantine Sea showed a particularly different composition as this hosted a high proportion of individuals from the easternmost rookeries in the Mediterranean Sea: Israel, Lebanon and Turkey (Supplementary Table 2). However, their contributions were unequal and western Turkey was the source of 28.4±36.6% of its turtles in comparison to eastern Turkey or Israel and Lebanon (~10% each).

Discussion

The contribution of different nesting beaches to any particular juvenile foraging ground will depend on the size of the population nesting at each beach and the pattern of surface currents connecting these beaches with the foraging ground (Bowen and Karl 2007; Hays et al. 2010). The largest nesting aggregation of loggerhead turtles in the North Atlantic is found along the coasts of North America (Ehrhart et al. 2003) and is connected with the European coasts by the Gulf Stream (Carr 1986; Bolten et al. 1998). Furthermore, the negative water balance of the Mediterranean Sea generates a permanent eastward flow of Atlantic water at the

Strait of Gibraltar (Millot and Taupier-Letage 2004), thus connecting the Mediterranean with the Gulf Stream. The Cape Verde Archipelago hosts the second largest nesting aggregation in the North Atlantic (Marco et al. 2012), but is connected with northern South America by the North Equatorial Current rather than with the Mediterranean Sea (Mansfield and Putman 2013). In this scenario, it is hardly surprising that most of the juvenile loggerhead turtles found in the foraging grounds of the eastern Atlantic and the south-western Mediterranean had a North American origin, with only a few juveniles coming from Cape Verde (Monzón-Argüello et al. 2009 and 2010; Carreras et al. 2011; this study).

Once into the Mediterranean Sea, Atlantic water flows initially eastwards along the slope of northern Africa (Fig. 5) and then splits in two major currents, one flowing northwards into the Tyrrhenian Sea and the other flowing eastwards along the coast of Libya to the southern Levantine Sea (Millot and Taupier-Letage 2004).

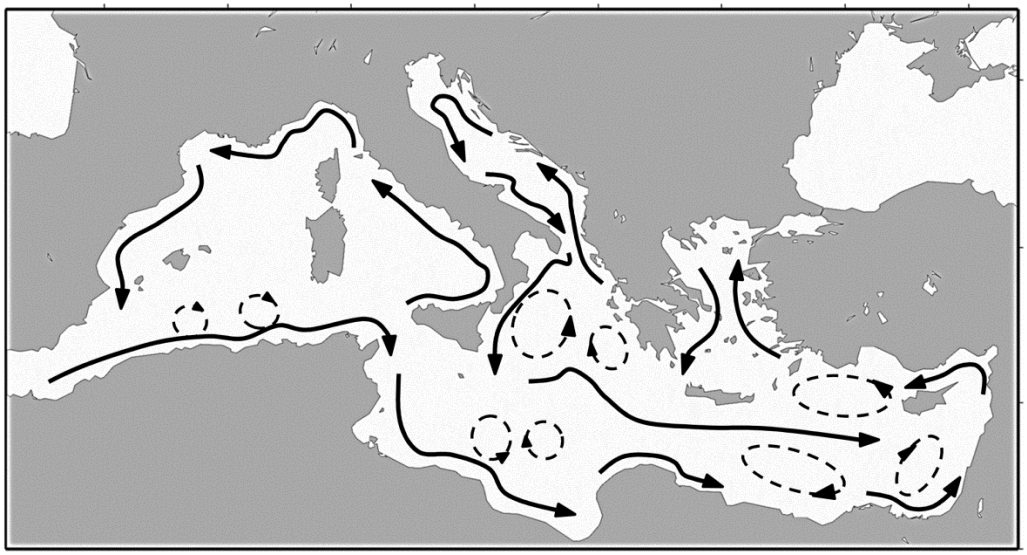

Fig. 5 Main surface circulation patterns of the Mediterranean Sea. Thin dashed lines show transient gyres and eddies. Adapted and modified after Robinson et al. 2001 and Millot and Taupier-Letage 2004

Accordingly, the relative abundance of juvenile loggerhead turtles of Atlantic origin decreases downstream, from the Algerian basin to the Adriatic Sea (Carreras et al. 2006; Maffucci et al. 2006; this study). However, the contribution of Atlantic rookeries to the Algerian basin reported here is lower than that detected in previous studies (Carreras et al. 2006; Carreras et al. 2011). This is because the longer mtDNA fragment allowed the differentiation of the Libyan CC-A2.9 haplotype from the widespread CC-A2.1 haplotype, something impossible with the

short fragment. Thus, some of the turtles occurring in the Algerian basin and previously considered of Atlantic origin come actually from Libya.

Conversely, the occurrence of turtles of Atlantic origin in the eastern Mediterranean is higher than previously reported. This is likely to be a consequence of analysing only turtles shorter than 69cm CCL, as turtles of Atlantic origin migrate back to the Atlantic at an average length of 58.8cm CCL (Revelles et al. 2007c) and hence the proportion of turtles of Atlantic origin in any foraging ground will decline when larger turtles are considered. Casale et al. (2008b), on the basis of data from Laurent et al. (1998), estimated that only 11% of the turtles in the southern Levantine Sea had an Atlantic origin, whereas our MSA results based on long fragments indicate a much higher proportion (20%). It should be noted that the turtles sampled by Laurent et al. (1998) ranged in size from 49.4 to 86.3cm CCL whereas here only turtles shorter than 69cm have been considered. This might also explain why the proportion of turtles of Atlantic origin present in the Adriatic Sea is slightly larger than that previously estimated on the basis of a wider size range (Giovannotti et al. 2010; Yilmaz et al. 2012).

Another methodological difference is the use of population size as a weighting factor for the MSA (Bass et al. 2004), while other studies in the region did not use it (Maffucci et al. 2006). Thus, an underestimation of the contribution of juveniles from Atlantic rookeries could have also occurred in these previous studies as they did not consider the much larger number of nests per year recorded in Atlantic beaches (*ca.* 100,000 nests per year; SWOT 2007) compared to the Mediterranean (*ca.* 7,200 nests per year; Casale and Margaritoulis 2010).

The surface circulation pattern might also explain the distribution patterns of turtles from Mediterranean nesting beaches to the different sub-basins. The prevalence in the Adriatic Sea of turtles from western Greece might be explained by the pattern of water entering the Adriatic Sea having previously flowed past the coast of western Greece (Fig. 5; Millot and Taupier-Letage 2004). Likewise, the prevalence of turtles from Libyan beaches in the Ionian Sea may be linked to the mesoscale eddies present in the Ionian Sea (Robinson et al. 2001; Hamad et al. 2006; Hays et al. 2010), which might trap the hatchlings and juveniles swimming off Libya in the sub-basin and prevent dispersal across the eastern Mediterranean (Fig. 5). A proportion of juveniles from Libya might also be trapped in coastal

systems and pushed by a westward current to the Algerian basin, the Catalano-Balearic Sea and the Tyrrhenian Sea, where its contribution is also relevant. This westwards dispersal perfectly fits the one suggested by Hays et al. (2010) for hatchlings drifting in the Mediterranean Sea.

Nevertheless, if the hypothesis that currents determine the observed distribution patterns of juveniles is true, a higher proportion of juvenile turtles from western Greece would be expected to occur in the northern Ionian Sea, as hatchlings swimming off western Greece encounter a water current bifurcation, with one current flowing northwards into the Adriatic Sea and another one flowing south-eastwards (Fig. 5; Hays et al. 2010). Accordingly, half of the adult turtles departing from western Greece migrate to the Ionian Sea after nesting and the other half to the Adriatic Sea (Zbinden et al. 2011; Schofield et al. 2013). In this scenario, the low estimated contribution of western Greece to the foraging grounds in the northern Ionian Sea might be caused by two non-excluding processes. In one hand, currents flowing off western Greece fluctuate seasonally (Hays et al. 2010) and most hatchlings might emerge when northward flowing prevails, thus drifting to the Adriatic Sea. This hypothesis could be tested combining particle tracking modelling with detailed data about the seasonality of hatchling emergence at rookeries in western Greece. Expanding this kind of studies to the remaining rookeries in the Mediterranean would improve our understanding of hatchling dispersal within the whole basin. On the other hand, a very large nesting population might exist in Libya (Laurent et al. 1999), which might result in the dilution of contributions from western Greece. Although recently published figures don't support that claim (Casale and Margaritoulis 2010), nest numbers in Libya are poorly known due to political unrest and further research in the region is urgently needed.

The turtles considered in this study ranged from 30 to 69cm CCL and hence were capable of dispersing independently of prevailing currents within the Mediterranean, except in the Strait of Gibraltar, the Alboran Sea and the Algerian Stream (Revelles et al. 2007d). However, the results reported here revealed genetic structuring consistent with the distribution of water masses and the pattern of surface currents. There is increasing evidence that young turtles become imprinted by the habitats they visit during their developmental migration, which in turn determines the habitats where they will settle and forage as adults (Hatase et al. 2002; Hays et al. 2010; Fossette et al. 2010; Eder et al. 2012). Turtles of

Mediterranean origin begin settlement at approximately 40cm CCL (Casale et al. 2008a), which suggests that the genetic structuring here reported might emerge from such a process as imprinting. This however, might not apply to turtles of Atlantic origin, as their natal rookeries are more than 6,000km away from the Mediterranean foraging grounds they used as juveniles. This results in a remarkable trade-off between philopatry and habitat knowledge, that finally leads them to leave the Mediterranean once they are large enough to overcome the currents in the Alboran Sea and the Strait of Gibraltar and settle in the western Atlantic (Bowen et al. 2005). Accordingly, adult turtles of Atlantic origin are highly scarce in the Mediterranean Sea.

The contributions from specific rookeries to Mediterranean foraging grounds described here are important not only for a better understanding of the biology of this species but also for its conservation. Fisheries bycatch stands as one of the major anthropogenic factors threatening sea turtle populations worldwide (Lewison et al. 2004a; Lewison and Crowder 2007, Wallace et al. 2008) and available evidence indicates that tens of thousands of turtles are bycaught incidentally every year around the Mediterranean Sea (Carreras et al. 2004, Lewison et al. 2004a; Alessandro and Antonello 2010; Casale 2011; Álvarez de Quevedo et al. 2010 and 2013). However, the impact of these high levels of bycatch is unevenly distributed among nesting areas, according to the heterogeneous admixture revealed by genetic markers in this study. For example, bycatch in the western Mediterranean might be a threat for populations nesting in North-America and in Libya, but less of a threat for those nesting elsewhere. Likewise, the Tyrrhenian Sea is an important foraging area for turtles from Libya but also from Calabria. Thus, bycatch in the Tyrrhenian Sea may directly impact the small nesting population of Calabria. Bycatch in the Adriatic Sea might primarily affect the population nesting in western Greece, whereas bycatch in the Levantine Sea might affect primarily the populations nesting in Turkey, Lebanon and Israel. This shows that knowing the degree of overlap between fishing and foraging grounds is a key factor to protect specific populations nesting in the Mediterranean Sea.

Overall, the present study has revealed previously unknown distributions of Atlantic and Mediterranean juvenile turtles within the Mediterranean Sea at a regional and fine-scale level through the use of population genetics. We highlighted the importance of large studies comprising vast sampling areas (particularly in the

case of migratory species) and the use of long fragments of mtDNA as these highly enhance genetic resolution. We have underlined MSA as a useful tool in conservation biology and with it we suggest that future management plans include updated genetic assessments of wild populations as a conservation method to unveil population structuring and life-stage specific distributions.

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Supplementary Table 1 Published absolute haplotype frequencies and references for each Atlantic and Mediterranean rookery considered for the present MSA. Total number of haplotypes per rookery (n) and rookery sizes (mean nests per year) included. MIS (Misurata, Libya), SIR (Sirte, Libya), ISR (Israel), LEB (Lebanon), CYP (Cyprus), ETU (eastern Turkey), MTU (middle Turkey), WTU (western Turkey), DLM (Dalaman, Turkey), DLY (Dalyan, Turkey), CRE (Crete, Greece), WGR (western Greece), CAL (Calabria, Italy), NOR (South Carolina and Georgia), CEF (central eastern Florida), SEF (south eastern Florida), SAL (Cay Sal Bank, Bahamas), DRT (Dry Tortugas, Florida), QMX (Isla Cozumel and mainland Quintana Roo, Mexico), SWF (south western Florida), CWF (central western Florida), NWF (north western Florida) and CPV (Cape Verde). References included in brackets and specified below the table

	MIS	SIR	ISR	LEB	CYP	ETU	MTU	WTU	DLM	DLY	CRE	WGR	CAL	NOR	CEF	SEF	SAL	DRT	QMX	SWF	CWF	NWF	CPV
CC-A1.1														141	668	27		1		106	248	94	
CC-A1.2															15	1					9		
CC-A1.3															3	16		1	1		2		79
CC-A1.4															20	7	1		13		7	2	6
CC-A1.5																							3
CC-A2.1	12	16	15	17	44	60	46	60	5	25	16	34	22		275	123	18	28	64	80	108	12	2
CC-A2.2																1							
CC-A2.3															1	3			6		2		
CC-A2.4															1	8					3		
CC-A2.5															1	1			10		3		
CC-A2.8											4												
CC-A2.9	1	12	2																				
CC-A2.11																					1		
CC-A3.1	1	2		2		8		16	15	15					37	14	1		3	13	46	1	
CC-A3.2						1																	
CC-A5.1															2				2				
CC-A6.1												3											
CC-A7.1															7	2					6		
CC-A7.2															2								
CC-A8.1																			7		1		
CC-A9.1															2	2		2	8	1	1	1	
CC-A10.1															9	7			41		7		
CC-A10.4															1								
CC-A11.2																							1
CC-A11.3																	1		7		1		
CC-A11.5																			1				
CC-A12.1																			2				
CC-A13.1							1								1	1			2		2		
CC-A14.1															15	1			7	1	5		
CC-A17.1																							30
CC-A17.2																							6
CC-A20.1													14		2	2				5	1		
CC-A21.1																					2		
CC-A26.1		4																					
CC-A27.1																						1	
CC-A29.1			2																				

	MIS	SIR	ISR	LEB	CYP	ETU	MTU	WTU	DLM	DLY	CRE	WGR	CAL	NOR	CEF	SEF	SAL	DRT	QMX	SWF	CWF	NWF	CPV
CC-A31.1													2										
CC-A32.1												1											
CC-A36.1															1								
CC-A36.2																			1		1		
CC-A41.1															1								
CC-A42.1																			1				
CC-A43.1						1										1							
CC-A47.1																							1
CC-A50.1					1																		
CC-A51.1																				2			
CC-A52.1						1																	
CC-A53.1						1	1																
CC-A59.1																						1	
CC-A60.1																			1				
CC-A68.1		1																					
n	14 (A)	35 (A)	19 (B)	19 (B)	45 (B)	72 (C)	48 (C)	76 (C)	20 (C)	40 (C)	20 (B)	38 (B)	38 (D)	141 (E)	1064 (E)	217 (E)	21 (E)	32 (E)	177 (E)	208 (E)	456 (E)	112 (E)	128 (F)
Rookery size	249 (G)	343 (G)	57 (G)	60 (G)	572 (A)	575 (G)	652 (G)	537 (G)	91 (G)	194 (G)	418 (A)	2459 (A)	15 (D)	900 (E)	12177 (E)	3711 (E)	300 (E)	151 (E)	1448 (E)	128 (E)	449 (E)	142 (E)	14000 (H)

References: (A) Saied et al. 2012; (B) Clusa et al. 2013; (C) Yilmaz et al. 2011; (D) Garofalo et al. 2009; (E) Shamblin et al. 2012; (F) Monzón-Argüello et al. 2010; (G) Casale and Margaritoulis 2010; (H) Laurent et al. 1999

Supplementary Table 2 Specific rookery contributions (%) to the Mediterranean foraging grounds analysed with standard deviations included (±) as assessed by MSA. Atlantic and Mediterranean rookeries are separated by a horizontal line. Foraging grounds: CAB (the Catalano-Balearic Sea), ALG (the Algerian basin), TYR (the Tyrrhenian basin), ADR (the Adriatic Sea), ION (the northern Ionian Sea) and SLE (the southern Levantine Sea). Rookeries: NOR (South Carolina and Georgia), CEF (central eastern Florida), SEF (south eastern Florida), SAL (Cay Sal Bank, Bahamas), DRT (Dry Tortugas, Florida), QMX (Isla Cozumel and mainland Quintana Roo, Mexico), SWF (south western Florida), CWF (central western Florida), NWF (north western Florida) and CPV (Cape Verde), MIS (Misurata, Libya), SIR (Sirte, Libya), ISR (Israel), LEB (Lebanon), CYP (Cyprus), ETU (eastern Turkey), MTU (middle Turkey), WTU (western Turkey), DLM (Dalaman, Turkey), DLY (Dalyan, Turkey), CRE (Crete, Greece), WGR (western Greece), CAL (Calabria, Italy)

Rookery	CAB	ALG	TYR	ADR	ION	SLE
NOR	0.12±0.73	0.75±3.72	0.60±2.20	0.05±0.31	0.07±0.42	0.07±0.45
CEF	12.5±7.8	50.26±12.02	10.20±5.57	0.87±1.57	1.35±2.39	5.33±4.76
SEF	3.27±7.58	2.57±6.53	3.79±9.09	0.81±2.81	0.82±2.58	1.84±5.31
SAL	0.75±5.59	0.19±1.74	1.15±7.62	0.21±2.08	0.55±4.82	4.14±16.87
DRT	0.17±2.19	0.17±2.36	1.37±8.54	0.20±2.82	0.07±1.09	0.87±7.17
QMX	1.64±3.89	0.15±0.77	0.12±0.64	0.11±0.60	0.18±0.91	0.19±0.96
SWF	0.05±0.81	0.10±1.38	0.28±2.30	0.02±0.41	0.01±0.24	0.02±0.30
CWF	0.16±1.2	0.78±5.09	0.36±2.14	0.04±0.35	0.06±0.54	0.05±0.50
NWF	0.03±0.36	0.06±0.90	0.09±0.95	0.01±0.10	0.01±0.17	0.01±0.21
CPV	2.62±2.76	2.65±2.35	1.99±2.18	0.71±1.17	1.11±1.84	3.41±3.31
MIS	38.57±29.12	34.14±18.25	47.42±31.34	8.10±22.82	70.38±34.93	11.96±28.52
SIR	1.93±5.84	2.96±7.99	0.42±2.09	0.04±0.46	9.67±22.36	0.08±0.77
ISR	0.88±6.14	0.52±4.28	0.81±6.43	0.01±0.15	0.01±0.32	9.50±20.80
LEB	0.58±5.68	0.05±0.87	2.65±12.94	0.32±4.44	0.19±2.79	9.02±25.80
CYP	1.58±7.92	0.56±3.31	3.28±12.13	3.01±13.85	0.34±2.37	2.84±11.94
ETU	1.04±5.88	0.49±2.95	2.00±9.35	13.75±26.91	1.55±8.10	12.17±27.63
MTU	0.89±5.12	0.73±4.17	2.16±9.67	1.71±8.39	0.57±3.50	3.00±11.90
WTU	1.43±6.71	0.95±4.67	1.85±8.35	11.32±22.16	4.10±13.79	28.39±36.60
DLM	0.06±0.72	0.05±0.71	0.03±0.44	0.15±1.24	0.12±1.41	0.22±1.90
DLY	0.26±2.42	0.21±1.84	0.22±1.98	0.72±4.1	0.57±4.25	1.22±7.60
CRE	0.15±1.36	0.10±1.03	0.46±4.20	0.09±0.91	7.07±11.9	0.43±3.69
WGR	31.32±23.73	1.55±5.57	4.27±12.57	54.75±33.28	1.22±4.36	5.24±14.68
CAL	0.00±0.07	0.00±0.10	14.46±12.45	0.00±0.05	0.00±0.16	0.00±0.12

CHAPTER 3. Influence of foraging ground use on loggerhead populations

3.1. Different growth rates between loggerhead sea turtles (*Caretta caretta*) of Mediterranean and Atlantic origin in the Mediterranean Sea

Títol: Diferents taxes de creixement entre tortugues babaues (*Caretta caretta*) d'origen mediterrani i atlàntic al mar Mediterrani.

Resum: En aquest estudi s'han estimat per primera vegada les taxes de creixement de tortugues babaues d'origen mediterrani i atlàntic habitant al mar Mediterrani tot combinant anàlisis esqueletocronològics i genètics. Els nostres models de creixement suggereixen que la taxa de creixement de tortugues babaues d'origen mediterrani és més ràpida que no pas la de les seves congèneres d'origen atlàntic alimentant-se al mar Mediterrani. L'edat de maduresa sexual estimada per a tortugues d'origen mediterrani és de 24 anys, fet que suggereix que les tortugues babaues que nidifiquen al Mediterrani no només són més petites en comparació a les que nidifiquen al oest de l'Atlàntic nord, sinó que també són més joves i creixen més ràpid.

Title: Different growth rates between loggerhead sea turtles (*Caretta caretta*) of Mediterranean and Atlantic origin in the Mediterranean Sea.

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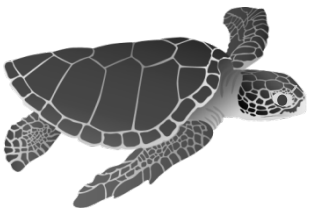

See original publication in Appendix A3

Different growth rates between loggerhead sea turtles (*Caretta caretta*) of Mediterranean and Atlantic origin in the Mediterranean Sea

Susanna Piovano · Marcel Clusa · Carlos Carreras · Cristina Giacoma · Marta Pascual · Luis Cardona

S. Piovano (✉) · C. Giacoma
Dipartimento di Biologia Animale e dell'Uomo, University of Torino, Via Accademia Albertina 13, 10123 Turin, Italy
e-mail: susanna.piovano@gmail.com

M. Clusa · C. Carreras · L. Cardona
Department of Animal Biology and IrBio, Faculty of Biology, University of Barcelona, Avenida Diagonal 645, 08028 Barcelona, Spain

M. Pascual
Department of Genetics and IrBio, Faculty of Biology, University of Barcelona, Avenida Diagonal 645, 08028 Barcelona, Spain

Abstract We estimated for the first time the growth rates of loggerhead sea turtles of Mediterranean and of Atlantic origin found in the Mediterranean Sea, combining both skeletochronological and genetic analyses. Our growth models suggested that the growth rate of loggerhead sea turtles of Mediterranean origin was faster than that of their conspecifics with an Atlantic origin exploiting the feeding grounds in the Mediterranean Sea. The age-at-maturity for Mediterranean origin loggerhead sea turtles, estimated using our best fitting model, was 24 years, which suggests that loggerhead sea turtles nesting in the Mediterranean are not only smaller than those nesting in the western North Atlantic, but they are also younger.

Introduction

Large marine vertebrates have some traits in common, such as late age at maturity and low reproductive rates, that make them highly vulnerable to negative effects of human activities (Lewison et al. 2004a). Many threats, such as incidental catch in fishing gear, hunting and habitat degradation, operate on local populations, resulting in a global population decline. As a consequence, many of those large vertebrates, including all seven sea turtle species, are now listed in the IUCN Red List of Threatened Species (IUCN 2010).

In the present study we focused on the loggerhead sea turtle *Caretta caretta*, a species circumglobally distributed from tropical to temperate waters and currently categorized as "Endangered" (IUCN 2010). The loggerhead sea turtle is the most common sea turtle species in the Mediterranean Sea (Margaritoulis et al. 2003) and it is also a highly migratory species, with individuals capable of migrations spanning thousands of kilometres (Carr 1987; Bolten et al. 1998). Atlantic loggerhead sea turtles enter the Mediterranean Sea through the Strait of Gibraltar (Revelles et al. 2007c; Eckert et al. 2008), so individuals with Atlantic and Mediterranean origin are both present in Mediterranean waters (Laurent et al. 1998; Carreras et al. 2006). However, there is increasing evidence that the proportional contribution of turtles carrying an Atlantic genotype is higher in the western basin and lower in the eastern basin of the Mediterranean Sea (Carreras et al. 2006).

Analyses using mitochondrial DNA (mtDNA) markers have demonstrated that Atlantic females do not nest regularly in the eastern Mediterranean, as some haplotypes that are frequent in Atlantic nesting beaches (Encalada et al. 1998; Monzón-Argüello et al. 2010) are not detected in the Mediterranean ones (Carreras et al. 2007; Garofalo et al. 2009). Furthermore, Mediterranean loggerhead sea turtles are significantly smaller at maturity than loggerhead sea turtles from other populations (Tiwari and Bjorndal 2000; Margaritoulis et al. 2003) and are thought to settle earlier on neritic habitats than their Atlantic conspecifics (Revelles et al. 2007b; Casale et al. 2008a; Cardona et al. 2009). Whether these traits are adaptive or just the result of phenotypic plasticity remains unknown, although biparentally inherited genetic markers indicate a limited gene flow between Atlantic and Mediterranean populations reflected in the high genetic differentiation between them (Carreras et al. in press).

Growth rates in sea turtles have been traditionally estimated from capture-tagging-recapture data (Frazer and Ehrhart 1985; Shaver 1994). This approach suffers from two main problems: variability in recapture interval and researchers' tendency to exclude negative growth rates from data analyses (Snover et al. 2007a). The first leads to an overestimation of annual growth rate if based on summer recaptures and to an underestimation if based on winter-early spring recaptures. The second problem affects the distribution of error in measurements, biasing it

towards errors that overestimate growth. Additionally, capture-tagging-recapture methods require long-term labour-intensive efforts (Bjorndal et al. 2001).

Skeletochronology, which relies on the count of growth marks deposited in bone tissue to estimate age, was applied for the first time by Zug et al. (1986) on loggerhead sea turtles. Since then, the technique has been used for aging green turtles *Chelonia mydas* (Zug and Glor 1998; Zug et al. 2002; Goshe et al. 2010), Kemp's ridley turtles *Lepidochelys kempii* (Zug et al. 1997; Avens and Goshe 2007; Snover et al. 2007a), olive ridley turtles *Lepidochelys olivacea* (Zug et al. 2006), leatherback turtles *Dermochelys coriacea* (Zug and Parham 1996; Avens et al. 2009) and loggerhead sea turtles from the Pacific (Zug et al. 1995) and Atlantic Oceans (Klinger and Musick 1992, 1995; Parham and Zug 1997; Bjorndal et al. 2003; Snover et al. 2007b, 2010; Snover and Hohn 2004). Previous attempts at using skeletochronology on loggerhead sea turtles found in the Mediterranean Sea (Guarino et al. 2004; Casale et al. 2011a) did not include genotype analyses, and hence these findings could not be used to separately characterize age and growth for loggerhead sea turtles from the Mediterranean and Atlantic populations.

The primary aim of this study was to estimate growth rates of loggerhead sea turtles with Mediterranean and Atlantic origin, combining skeletochronological and genetic methods.

Material and methods

Study area

Italy extends into the middle of the Mediterranean Sea, and with its peninsula and main island, Sicily, it geographically divides the eastern from the western part of the Sea. The two parts remain connected through the Strait of Sicily and the Strait of Messina. Data from loggerhead sea turtles stranded along Italian coasts, incidentally captured by Italian fishing vessels or recovered by Italian rescue centres showed that the size of individuals ranged from small juvenile to adult (bancadati.tartanet.it). Moreover, nesting beaches are known to occur along the south Italian coasts (Mingozzi et al. 2007). These characteristics make Italy an ideal candidate area for the investigation of the growth rates of loggerhead sea turtles

carrying a Mediterranean or an Atlantic genotype across sizes spanning small juvenile to adult life stages.

Sample collection

Front flippers from a total of 95 individuals were sampled from 2007 to 2009 for genetic analysis and skeletochronology. Samples were collected from dead loggerhead sea turtles coming from the Adriatic Sea, the Ionian, the Tyrrhenian and the Sardinian Seas, as well as from the Strait of Sicily and the Strait of Messina. The individuals had either been stranded dead (75%) or died at the local Tartanet network of rescue centres during rehabilitation (25%). In addition, two dead-in-nest hatchlings of Atlantic origin found during nest excavation were provided by Brancaleone CTS rescue centre (Calabria, nesting season 2007) and two additionally dead-in-nest hatchlings, previously identified as having a Mediterranean origin, were provided by Riserva Naturale Orientata "Isola di Lampedusa" (Pelagie Islands, nesting season 2006). For all individuals, only curved carapace length (CCL) measured notch-to-tip (Bolten 1999) was available.

The right front flipper was removed during post-mortem examination, muscle or skin samples were collected and stored in 95% ethanol, the humerus bone was dissected, flensed of tissue, boiled, and then allowed to dry in the air for 4 weeks.

Molecular methods

DNA was extracted from the muscle or skin samples using the QIAamp extraction kit (QIAGEN) following the manufacturer's instructions (www.qiagen.com).

We amplified a fragment of 815 bp of the control region of the mitochondrial DNA of all the samples using primers LCM15382 (5'-GCTTAACCCTAAAGCATTGG-3′) and H950(5'-TCTCGGATTTAGGGGTTT-3′) (Abreu-Grobois et al. 2006) which included the 380 bp region historically surveyed for this species in previous studies within the same area (Carreras et al. 2006, 2007; Casale et al. 2008b; Encalada et al. 1998; Laurent et al. 1998). Sequences were aligned by eye using the program BioEdit version 5.0.9 (Hall 1999) and compared with the short (~380 bp) and long (~815 bp) haplotypes described for

the species in the Archie Carr Centre for Sea Turtle Research Database (accstr.ufl.edu). Furthermore, samples bearing mtDNA haplotypes common to Atlantic and Mediterranean nesting beaches or samples that failed to amplify were genotyped for seven nuclear DNA (nDNA) microsatellites previously used in the species: Cm84, Cc117, Cm72 and Ei8 (Fitzsimmons et al. 1995); Cc141 and Cc7 (Fitzsimmons et al. 1996); and Ccar176 (Moore and Ball 2002) the last one modified as described in Carreras et al. (2007).

Origin assessment of individuals

Individual assignments, including that of hatchlings, were done for all individuals using a combination of microsatellites and mtDNA as described in Revelles et al. (2007c) and Carreras et al. (2011). When a mtDNA exclusive haplotype, from either the Atlantic or Mediterranean nesting area, was present in an individual, this individual was assumed to have originated from the corresponding nesting area. All individuals with mtDNA common haplotypes or haplotypes not assigned to any nesting area were assigned using the seven microsatellites and the STRUCTURE version 2.1 software (Pritchard et al. 2000) considering the baseline developed in Carreras et al. (in press). This baseline included microsatellite data from individuals born in Mediterranean nesting beaches sampled in Carreras et al. (2007) and microsatellite data from Atlantic migrants find in western Mediterranean feeding grounds (Carreras et al. in press) and identified by means of mtDNA Atlantic exclusive haplotypes (Carreras et al. 2007). The probability of each individual to be from either the Atlantic or Mediterranean populations was obtained. Assignation of each individual to either group was accepted when probability was higher than 0.7 for that group.

Skeletochronology - LAG interpretation and age estimation

Humeri were selected because of their capability of retaining more periosteal growth marks than other bones (Zug et al. 1986). Sections were cut at diaphyseal level just distal to the deltopectoral crest (Zug et al. 1986). The medial width was measured with digital callipers to the nearest 0.01 mm, prior to cross-sectioning. A preliminary section 8-10 mm thick was prepared using a diamond saw petrographic cutter (Remet Hergon MT60). Preliminary sections of bone were decalcified in 5%

nitric acid (range of decalcification time: 2-71 hours), then washed in tap water to remove any trace of acid. Thin cross-sections 25 μm thick were obtained using a freezing-stage microtome (Reichert-Jung cryocut 1800), then stained with Mayer's hematoxylin and successively mounted with an aqueous medium (Aquovitrex, Erba).

Digital images of stained cross-sections were acquired at a suitable magnification (ranging from 8x to 12.5x, depending on the size of the section) with Leica Application System LAS EZ v.2.3.0 combined with Leica EZ4 D dissecting microscope. When a section was too large for the camera, partial images were acquired and stitched together using Adobe Photoshop (Adobe System Inc.). Lines of arrested growth (LAGs) were counted by two independent readers (SP and RC) using the microscope. Each section was read three times at a minimum of 7 day intervals by each reader. Each LAG was marked on digital images. A consensus on LAGs count and position was reached for each humerus. To compare pair wise LAGs counts between readers the Wilcoxon Signed Rank Test was used (Ramsey and Schafer 2002). High resolution digital images of each cross-section enabled LAGs measurements with the image analysis software ImageJ version 1.43u (rsb.info.nih.gov/ij). Humerus diameter, LAG diameter and resorption core diameter were measured along an axis parallel to the dorsal edge of the bone (Goshe et al. 2010). Resorption core diameter included the medullary cavity and any secondary (endosteal) bone deposited in the area of resorption (Curtin 2009), where LAGs were removed (Castanet and Smirina 1990).

Cyclic annual growth mark deposition has been described for loggerhead sea turtles in the Atlantic Ocean (Klinger and Musick 1992; Coles et al. 2001). Injuries, illness or reduction in food supply may have an influence on growth and may cause the development of accessory lines in the bone (Zug et al. 1986). These lines are usually incomplete or less chromophilic than LAGs. To limit the possibility of overestimating the age, we counted only chromophilic complete lines.

A small number of our samples was from turtles which had experienced, on average, one month in captivity (mean = 31 days, SD = 35, N = 24) in a rescue centre after being found injured. A diffuse mark was detected in the outermost edge of the cross-section of five of those individuals. The outermost edge of the periosteal

bone is where the most recent bone is deposited (Enlow 1969); the outermost LAGs were fully visible and could be discriminated from the border of the cross-sections by June in captive-reared European pond turtles *Emys orbicularis* (Castanet 1985) and in Kemp's ridley sea turtles in the Atlantic ocean (Snover and Hohn 2004). Under the assumption that the same would happen to loggerhead sea turtles in the Mediterranean Sea, the date of recovery for rehabilitation and the date of death of the five individuals were checked and the diffuse outermost mark was counted as an annual growth mark in the three individuals that died in spring, while it was interpreted as a non-annual accessory mark in the two individuals that died in autumn.

A common feature in sea turtles is resorption and remodelling of the innermost part of the humerus (Zug et al. 1986), which destroys the growth marks deposited earliest in life (Castanet and Smirina 1990). In our study, age estimation was obtained by summing the number of measurable LAGs and the estimation of resorbed LAGs through application of a correction factor protocol (Parham and Zug 1997). Strictly speaking, the number and the diameter of LAGs from humeri that retained the first growth mark were used to estimate the number of resorbed LAGs of remodelled humeri. Based upon validation for Kemp's ridley sea turtles (Snover and Hohn 2004, Snover et al. 2007a), a diffuse annulus representing the first year mark was assumed in this study for loggerhead sea turtles. The protocol can be applied only to humeri with a resorption core smaller than the maximum LAG diameter of humeri already aged (Zug et al. 2002). Following Goshe et al. (2010), additional correction factors were developed to extend the protocol and allow age estimation of the whole sample of humeri. Each time, several regression models were assessed to identify the relationship between LAG diameter and LAG number. The best-fitting model was chosen on examination of the residuals and R^2 values. The analysis was performed on turtles of Atlantic and Mediterranean origin separately.

Back-calculation and growth rates

To model the relationship between humerus diameter and individual carapace length, we used the equation proposed by Snover et al. (2007b) after validation on Atlantic loggerhead sea turtles:

$$L = L_{op} + b(D-D_{op})^c \quad (1)$$

where L is the estimated carapace length, L_{op} is the minimum carapace length of a hatchling, D is the medial width of the humerus, D_{op} is the minimum width of a hatchling humerus, b is the slope and c is the coefficient of proportionality.

The back-calculation technique relies on the body proportional hypothesis (Francis 1990) and uses the relationship between marks in hard parts of the body and body length to estimate the length of an individual's body at the time of the formation of the mark.

Growth rates were calculated by subtracting the back-calculated CCL of the inner LAG from that of the outer LAG for each pair of neighbouring LAGs. Growth rates were then assigned to size classes based on the CCL at the beginning of the growth interval (Parham and Zug 1997). Mean growth rate and standard deviation were calculated for each 10 cm size class. Analyses were performed separately for turtles of Atlantic and Mediterranean genotype assignation.

Growth models

Two different approaches were used to model growth. The main approach, based on aging, was carried out as follows: first the estimate of age, obtained by skeletochronology (Zug et al. 1986) and application of the correction factor protocol (Parham and Zug 1997; Goshe et al. 2010); then the estimate of the length at time since the last LAG deposition, obtained from back-calculation (Snover et al. 2007b); finally, the fitting of logistic, Gompertz and von Bertalanffy growth curves to length-at-age data, separately for turtles of Atlantic and Mediterranean origin. The asymptotes were fixed using biological data from the literature on Atlantic and Mediterranean populations (CCL_{max} = 124 cm in Ehrhart and Yoder 1978; CCL_{max} = 99 cm in Margaritoulis et al. 2003, respectively). Akaike's information criterion corrected for small sample sizes (AIC_c) was calculated for each model. The best fitting model was selected on AIC_c scores, ΔAIC_c and Akaike's weights. Additional analyses on nested models were done using the F-test (Ramsey and Schafer 2002).

To support these results, we used a secondary approach that was not based on aging but on the mark-recapture concept. We used back-calculated lengths

(Snover et al. 2007b) and time lapse to fit a Fabens' von Bertalanffy growth interval model (as first applied on sea turtles by Frazer and Ehrhart 1985).

The Fabens' (1965) modified von Bertalanffy equation for mark and recapture data:

$$L_r = A - (A - L_c)e^{-kd} \quad (2)$$

where L_r is the carapace length at recapture, A is the asymptotic carapace length, L_c is the carapace length at first capture, k is the intrinsic growth rate and d is the time between capture and recapture expressed in years. In our case, L_r was the carapace length at the outermost LAG and L_c was the carapace length at the innermost LAG, both estimated using back-calculation, d was the number of measured LAGs and A was fixed using biological data as described above.

Statistical analyses were performed using R version 2.12.1 (R-Development Core Team 2010).

Results

The mtDNA or nDNA markers were amplified successfully from 99 samples, but amplification success was much higher for nDNA markers (77 successfully amplified samples for mtDNA and 99 for nDNA). This differential amplification success was probably because shorter nDNA markers were better preserved in partially degraded samples from dead stranded individuals than the much longer mtDNA marker used in this study. Furthermore, 26 individuals were impossible to allocate due to the presence of the commonly shared haplotype CC-A2.1 and the lack of conclusive microsatellite results (assigning probability lower than 0.7). As a consequence, only 73 samples yielded reliable origin assessments.

Seven different mtDNA haplotypes were found within the study area: CC-A1.1 (2.6% of the samples), CC-A2.1 (79.2%), CC-A2.9 (6.5%), CC-A3.1 (6.5%), CC-A6.1 (1.3%), CC-A10.3 (1.3%) and CC-A20.1 (2.6%). While unique haplotypes from both the Mediterranean (CC-A6.1 and CC-A2.9) and the Atlantic (CC-A1.1 and CC-A10.3) allowed immediate assignment, samples with shared haplotypes were genotyped by microsatellites to increase assignment capability. A high polymorphism degree was found for all loci, these presenting different alleles

ranging from 14 (cc7) to eight (cm72, ccar176, cc117, Ei8) alleles per locus. Overall, eight individuals could be assigned from mtDNA analyses and 65 from microsatellite genotyping, yielding 33 individuals assigned to nesting beaches in the Mediterranean and 40 individuals assigned to nesting beaches in the Atlantic. Skeletochronology was focused on a subset of 65 individuals (30 Mediterranean and 35 Atlantic).

Age estimation

LAGs counts were not statistically different between the two readers (Wilcoxon Signed Rank test: $p = 0.773$); in addition, consensus on LAGs count and position was reached for each humerus. The age was equal to the number of LAGs in six of the Mediterranean origin turtles, which retained all LAGs (range: 2-4 y). The function that best fit the relationship between LAG diameter (dLAG), in mm, and LAG number (nLAG) in this group of turtles was a power function ($R^2 = 0.69$, $N_{LAGs} = 14$):

$$dLAG = 6.3873 \times (nLAG)^{0.3256} \quad (3)$$

Equation 3 was used to estimate the number of resorbed LAGs for 15 humeri with resorption core diameters smaller than 10.08 mm (corresponding to the largest LAG diameter measured in the previous group of turtles). Visible LAGs were renumbered according to the estimated number of resorbed LAGs, then data from the two previous groups were joined together to estimate the number of lost LAGs in humeri with a resorption core diameter smaller than 18.15 mm, which included all the remaining samples. dLAG and nLAG in this group followed a linear relationship ($R^2 = 0.89$, $N_{LAGs} = 101$):

$$dLAG = 5.7177 + 1.2842 \times (nLAG) \quad (4)$$

The same correction protocol was applied to turtles of Atlantic origin. The age was equal to the number of LAGs counted in six humeri (range: 1-5 y). In this case, the function which best fit the relationship between dLAG, in mm, and nLAG was a power function ($R^2 = 0.76$, $N_{LAGs} = 19$):

$$dLAG = 5.7307 \times (nLAG)^{0.3704} \quad (5)$$

Equation 5 was used to estimate the number of resorbed LAGs of 10 humeri with resorption core diameters smaller than 10.61 mm. Visible LAGs were renumbered according to the estimated number of resorbed LAGs, then data from the two previous groups were pooled together to estimate the number of lost LAGs in humeri with a resorption core diameter smaller than 16.65 mm. dLAG and nLAG in this group followed a linear relationship (R^2 = 0.88, N_{LAGs} = 58):

dLAG = 5.2598 + 1.1476 x (nLAG) (6)

Once again, measurable LAGs were renumbered according to the estimated number of resorbed LAGs, then data from the two previous groups were joined together to estimate the number of lost LAGs in humeri with a resorption core diameter smaller than 25.59 mm, which included all the remaining samples. Also in this group, dLAG and nLAG followed a linear relationship (R^2 = 0.91, N_{LAGs} = 130):

dLAG = 6.3655 + 0.7712 x (nLAG) (7)

Growth rates

Measured CCL of turtles assigned to the Mediterranean origin ranged in size from 4.2 cm to 76 cm (mean = 38.1 cm, SD =15.7; Fig. 1), while measured CCL of turtles with an Atlantic origin ranged from 4.5 cm to 80 cm in size (mean = 45.9 cm, SD =19.6; Fig. 1). Despite the small sample of hatchlings available, the length of the two hatchlings from Pelagie Islands previously assigned to a Mediterranean origin (mean = 4.2 cm, SD = 0.4) was consistent with the size of hatchlings from Mediterranean nesting beaches (Dodd 1988; Margaritoulis et al. 2003) and the length of the two hatchlings from Calabria assigned to an Atlantic origin (mean = 4.5 cm, SD = 0.1) was consistent with the size of hatchlings originating from western Atlantic nesting beaches (Dodd 1988).

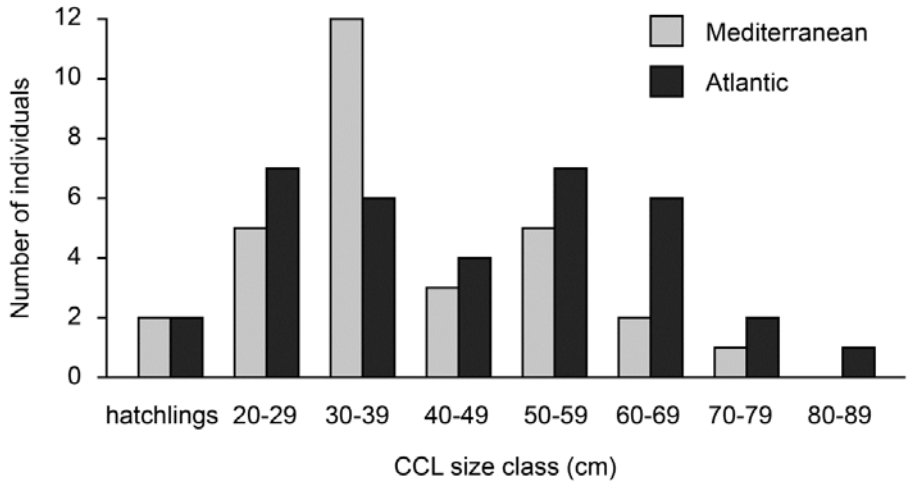

Fig. 1 Frequency distribution of measured CCL class for the subset of loggerhead sea turtles assigned to Mediterranean and Atlantic origin and used for skeletochronological analysis

Parameter estimates of equation 1 were b = 37.5524 and c = 0.8887 with L_{op} = 4.21 and D_{op} = 1.782 for turtles assigned to the Mediterranean origin; b = 30.1919 and c = 0.9678 with L_{op} = 4.54 and D_{op} = 1.794 for turtles with an Atlantic origin. Back-calculated CCLs ranged from 16.5 cm at the innermost LAG to 76.7 cm at the outermost LAG (mean = 35.8 cm, SD =11.0) for turtles with a Mediterranean origin, and from 13.0 cm at innermost LAG to 78.9 cm at outermost LAG (mean = 44.0 cm, SD =16.3) for turtles assigned to the Atlantic origin. Size-specific growth rates and standard deviations were calculated on turtles of Mediterranean and Atlantic origin separately (Table 1).

Table 1 Size-specific growth rates (cm $year^{-1}$) from estimated CCL at all measurable LAG diameters (total pair of neighbouring LAGs = 254), for individuals assigned to Atlantic and Mediterranean origin

CCL size class (cm)	Atlantic assignation					Mediterranean assignation				
	Mean growth rate (cm $year^{-1}$)	SD	Min	Max	n	Mean growth rate (cm $year^{-1}$)	SD	Min	Max	n
13.0-19.9	4.6	1.8	2.3	8.3	12	5.1	0.6	4.4	6.1	6
20.0-29.9	3.2	1.0	2.3	6.1	17	3.5	1.7	1.5	8.6	31
30.0-39.9	3.0	1.3	0.8	5.6	26	2.9	1.5	0.4	8.6	46
40.0-49.9	3.0	1.2	0.9	5.3	32	2.9	1.0	1.3	5.1	29
50.0-59.9	2.1	1.5	0.2	5.6	25	4.1	1.4	2.5	5.0	3
60.0-69.9	2.7	1.1	0.5	4.0	12	4.4	0.4	4.2	4.7	2
70.0-78.9	1.5	0.4	0.5	2.2	11	3.0	0.6	2.6	3.4	2

Growth models

Length-at-age data for turtles of Mediterranean and Atlantic assignation (Table 2) were best fitted by the von Bertalanffy growth model (Table 3; Fig. 2). For the Mediterranean group the von Bertalanffy and the Gompertz growth models were more or less equivalent ($\Delta AIC_c < 2$), while the logistic model was distinguishable; for the Atlantic group the three growth models were clearly distinguishable based on ΔAIC_c values (Table 3).

Table 2 Growth function parameter estimates for Mediterranean and Atlantic origin loggerhead sea turtle length-at-age data

	Logistic $y = a/(1 + e^{((b-x)/c)})$		Gompertz $y = ae(-b \times c^x)$		Von Bertalanffy $y = a(1-e^{(-b(x-c))})$	
Parameter	*b*	*c*	*b*	*c*	*b*	*C*
Atlantic	24.075	18.278	1.649	0.964	0.023	-7.722
Mediterranean	12.077	10.472	1.513	0.936	0.042	-4.848

Parameter "*a*" was fixed at 124 cm for the Atlantic and at 99 cm for the Mediterranean populations (maximum length from Ehrhart and Yoder 1978 and from Margaritoulis et al. 2003, respectively)

The von Bertalanffy models were then the object of further analyses. A full model with two sets of parameters for the Mediterranean and the Atlantic origin data was compared with a reduced model with a common set of parameters for all data. The two models were significantly different (F-test: $p = 0.007$), providing evidence that the two populations display different growth rates over the size range of turtles examined in this study.

Table 3 Growth function fitting criteria for loggerhead sea turtles length-at-age data

Model	AIC_c	ΔAIC_c	Akaike's weight
Atlantic			
von Bertalanffy	360.080	0	0.9880
Gompertz	368.943	8.863	0.0117
Logistic	376.424	16.345	0.0003
Mediterranean			
von Bertalanffy	305.677	0	0.6230
Gompertz	307.345	1.668	0.2706
Logistic	309.210	3.533	0.1064

The lowest AIC_c and greatest Akaike's weight indicate the best fitting model

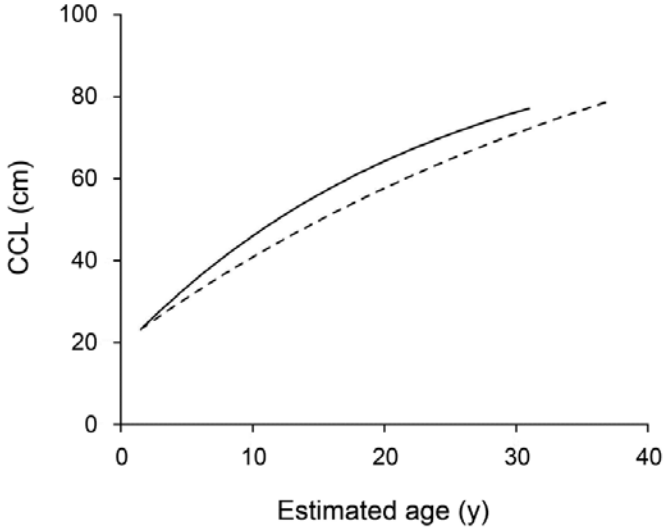

Fig. 2 Length-at-age relationship for loggerhead sea turtles of Mediterranean (*solid line*) and Atlantic (*dashed line*) origin in the Mediterranean Sea as described by the best fitting model, the von Bertalanffy growth model. Curves are limited to the size range of turtles examined in this study. The model predicts that 24 y are required for loggerheads of Mediterranean origin to reach maturation at a size of 69 cm CCL and that 38 y are required for loggerheads of Atlantic origin to grow to 80 cm CCL

This result was obtained using two different asymptotic values, 99 cm for the Mediterranean group and 124 cm for the Atlantic group. The same statistical difference (F-test: p = 0.008) was obtained by using a unique value of 99 cm for both groups, which excluded the possibility that the use of two asymptotic values was the cause of the difference in growth. Both the Mediterranean and the Atlantic growth models, that were characterized by a fixed value (asymptote) and two parameters, were then compared with the respective three parameters full models. In this case we could not discard the null hypothesis (F-test: p = 0.935 for the Mediterranean and p = 0.114 for the Atlantic) so, based on the criterion of parsimony, we chose the reduced models with a fixed value and two parameters.

Using the best fitting model, age at maturation was estimated at 24 years for turtles with a Mediterranean assignation based on the average minimum size of nesting females in the Mediterranean basin (69 cm, in Margaritoulis et al. 2003). A similar result of 23 years was obtained from the Fabens' von Bertalanffy growth interval model. Average size of first-time nesting females from Atlantic populations (98 cm, in Turtle Expert Working Group 2009) was beyond the size range of our sample, thus estimate of age at maturity could not be extrapolated (Bjorndal and Zug 1995).

The Brody growth coefficient resulting from the model fits were different between the two origin groups, with a higher value for the Mediterranean (k = 0.042 y^{-1}, bootstrapped 95% CI: 0.036 – 0.049; Table 2) and lower value for the Atlantic origin group (k = 0.023 y^{-1}, bootstrapped 95% CI: 0.020 – 0.025; Table 2). Differences in intrinsic growth rates were consistent from both the best fitting model (Table 2) and the Fabens' von Bertalanffy growth interval model, with the estimated Mediterranean rate being higher than the estimated Atlantic rate (Fabens' k = 0.051 y^{-1} for turtles with a Mediterranean assignation and 0.036 y^{-1} for turtles with Atlantic assignation).

Discussion

Despite the fact that at least 25 years have passed since the first application of the skeletochronological method to investigate the age of a sea turtle (Zug et al. 1986), and different histological and LAG measurement techniques have been more or less successfully applied over the years (reviewed in Snover et al. 2007b; Goshe et al. 2009), consensus on the use of the same protocol has not been reached yet.

In this study we chose to stain thin cross sections prior to LAG counting, which proved to make LAGs more readable when compared to unstained cross sections (Goshe et al. 2009). The staining technique was preferred in a large portion of skeletochronological literature on other reptiles (Erhert 2007; Curtin et al. 2008; Kolarov et al. 2010) as well as on amphibians (Leclair and Castanet 1987; Guarino et al. 1995; Seglie et al. 2010).

A serious problem with skeletochronology studies in sea turtles is extensive bone remodelling (Zug et al. 1997) and our study was not an exception. The phenomenon results in erosion of the inner periosteal bone of the humeri, deleting a number of innermost LAGs and leaving fragments of not completely resorbed LAGs that were not measurable. Parham and Zug (1997), who first applied correction protocols, stated that among the three protocols they used, the correction factor protocol "matches best the observed pattern of bone growth in *Caretta caretta*". However, their samples lacked small juveniles and wild, aged individuals. Bjorndal et al. (2003) stated that the correction protocol for age estimation was problematic and avoided its use, but the size of the medullary cavity in their sample of humeri allowed them to estimate that a maximum of two LAGs were lost. On

the contrary, our sample was mostly composed of humeri with a medullary cavity larger than the average diameter measured for the first and second LAGs, indicating that in many humeri more than two LAGs had been resorbed. The correction factor protocol was later applied by Zug et al. (2006), Goshe et al. (2010) and Casale et al. (2011a) on sea turtles, but also by Curtin et al. (2008) on tortoises.

We chose to measure LAG diameters, as previously done by Zug et al. (1995, 1997, 2002, 2006), Zug and Glor (1998), and Snover et al. (2007a, 2007b, 2010). If we had chosen to measure the ventral radii instead, as done by Bjorndal et al. (2003), probably we would have been able to measure a few more LAGs, but such a method would have been difficult to perform on our sample and would have left us unconfident in the results. We agree with Snover et al. (2007b), who reported that the position of the medullary cavity was generally asymmetrical, and that the position of the focus differed among individuals. Thus the measurements of the radii would have been highly subjective. Even though fragmented LAGs were not used for age estimation, their count proved to be useful in this study as an additional tool to evaluate the goodness of the estimation of the number of missing LAGs produced by each regression model based on the correction factor protocol.

The model that best fit our length-at-age data was the von Bertalanffy, as in previous studies (Klinger and Musick 1995; Zug et al. 1995, 1997; Parham and Zug 1997; Bjorndal et al. 2000, 2001; Snover 2002; Wallace et al. 2008; Casale et al. 2009a, 2009b), but all models suggested a faster growth rate for loggerhead sea turtles of Mediterranean origin than for those of Atlantic origin. The difference in the growth rate of both groups was remarkable, as turtles came from the same feeding grounds, and might be related to differences in physiology or in the habitat use. For example, previous evidence indicates that juvenile loggerhead sea turtles are primarily oceanic in Mediterranean regions where turtles of Atlantic origin prevail (Cardona et al. 2005; Revelles et al. 2007b), whereas juvenile loggerhead sea turtles within the same size range are primarily neritic in areas where turtles of Mediterranean origin prevail (Casale et al. 2008a; Cardona et al. 2009). Differences in the primary productivity of coastal and oceanic regions in the Mediterranean is very large (Bosc et al. 2004) and hence turtles recruiting earlier to more productive, neritic habitats are expected to grow faster. The reason why turtles of Atlantic origin remain in oceanic environments for an extended time is unknown, but might

be related to the necessity of undertaking the long return, migration across the Atlantic (Bolten and Balazs 1995).

The carapace length of female loggerhead sea turtles nesting on Mediterranean beaches is smaller than that of females nesting on the Pacific and the Atlantic coasts (Margaritoulis et al. 2003). The lowest mean CCL, 66.5 cm (range: 60.0-90.0 cm), is from Cyprus, while the highest mean CCL, 84.7 cm (range: 71.9-93.0 cm, in Margaritoulis et al. 2003) is from Kefalonia, Greece. Loggerhead sea turtles in this range of sizes are usually assigned to the subadult stage in the Atlantic and to the adult stage in the Mediterranean. Predictions based on the von Bertalanffy growth model fitted on our Mediterranean origin turtles suggested that loggerhead sea turtles from the Mediterranean population require an estimated average 24 years (bootstrapped 95% CI: 21-27 y) to reach the average minimum CCL for nesting in the Mediterranean, that is 69 cm (Margaritoulis et al. 2003).

Wallace et al. (2008) estimated that loggerhead sea turtles in the Mediterranean Sea would take 14 years in a fast growth scenario and 25 years in a low growth scenario to reach a size of 70 cm CCL. Genetic origin was not ascertained in their study. Our estimation at the same size of 70 cm was of 25 years (bootstrapped 95% CI: 22-28 y) for the Mediterranean origin group, which is in accordance with the low growth scenario, and of 29 years (bootstrapped 95% CI: 27-32 y) for the Atlantic origin group.

Three previous studies based on different methodologies reported the estimation of age at maturity of loggerhead sea turtles from Mediterranean waters. Estimates of years required to reach the lowest mean CCL size of 66.5 cm of females nesting in the Mediterranean ranged 15-16 years based on skeletochronology (Casale et al. 2011a), 16 years based on capture-mark-recapture (Casale et al. 2009a) and 19-23 years based on length frequency analysis (Casale et al. 2011b). Genetics were not considered, so turtles of Atlantic and Mediterranean nesting grounds were likely to be mixed. However, differences in estimations in those studies could be primarily due to the methodologies applied to obtain the growth curve (see Snover et al. 2007b). Our estimation at the same size of 66.5 cm was of 22 years (bootstrapped 95% CI: 19-24 y) for the Mediterranean origin group and 26 years (bootstrapped 95% CI: 24-29 y) for the Atlantic origin group.

Most of the loggerhead sea turtles of Atlantic origin found in the Mediterranean come from the North-Western Atlantic (Carreras et al. 2006), although a small number from the Cape Verde Islands occur in the western Mediterranean (Monzón-Argüello et al. 2010). Some of the turtles considered for the present study had haplotypes exclusive to the north-western Atlantic and none of them had any of the exclusive haplotypes reported by Monzón-Argüello et al. (2010) for the Cape Verde Islands. As a consequence, most of the turtles assigned to the Atlantic populations are likely to come from the north-western Atlantic. The average size of first-time nester female loggerhead sea turtles nesting along the North-Western Atlantic coasts is 98 cm CCL (range: 87-104, in Turtle Expert Working Group 2009), which is larger than those recorded in our Atlantic group (<80 cm CCL). We did not use our data to estimate the age at maturity for this group because the inference would have required extrapolation of the model beyond the size range of our sample. Despite the different methods used for age estimation, there is increasing evidence that loggerhead sea turtles from the North Atlantic reach sexual maturity around 30 years of age (Frazer and Ehrhart 1985; Crouse et al. 1987; Parham and Zug 1997; Snover 2002), or even later (Bjorndal et al. 2000, 2001; Heppell et al. 2003) and lower projections (Mendonca 1981; Crowder et al. 1994) appear to be underestimates (Braun-McNeill et al. 2008). If this is true, mature loggerhead sea turtles nesting in the Mediterranean are not only smaller than those nesting in the western North Atlantic, but they are also younger.

According to the above reported size at maturity for Atlantic loggerhead sea turtles, the individuals with Atlantic origin analysed in this study should have been assigned to the juvenile or subadult life-stages. However, humeri of the five larger individuals with Atlantic origin, with a CCL ranging from 68 to 80 cm, showed a typical sign of aging, an ectepicondylar foramen, formed due to the gradual closure of the ectepicondylar groove as age increased (Zug et al. 1986). In the western Atlantic, the minimum size of the CCL of an adult is 87 cm (Turtle Expert Working Group 2009) and complete closure of the groove is reached in a turtle of around 90 cm CCL (Zug et al. 1986) and an age close to 30 years (Bjorndal et al. 2000). Predictions based on the von Bertalanffy growth model suggested that, in the Mediterranean, turtles with Atlantic origin and a CCL ranging in size 68-80 cm should be assigned to an age of around 27 years or older (68 cm CCL: mean 27 y,

bootstrapped 95% CI: 25-30 y), which agrees with the closure of the ectepicondylar groove and potential adulthood. Accordingly, these findings suggested that loggerhead sea turtles with Atlantic origin living in Italian waters had a lower rate of growth than loggerhead sea turtles with the same origin but living in the Atlantic Ocean. This might be explained by the much lower productivity of the Mediterranean when compared with the shelf waters along North America (Longhurst 1998). Furthermore, these potentially adult turtles of Atlantic origin have a size similar to that of adult loggerhead sea turtles of Mediterranean origin, which indicates that the turtles of both populations could reach adulthood at the same size if they remain in the Mediterranean long enough. As a consequence, the differences in size at first maturity reported for the Atlantic and the Mediterranean are probably the result of phenotypic plasticity, but should be considered when using demographic models to understand human impacts on these populations.

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3.2. Distribution patterns and foraging ground productivity determine clutch size in Mediterranean loggerhead turtles

Títol: Els patrons de distribució i la productivitat en zones d'alimentació determinen la mida de la posta en tortugues babaues mediterrànies.

Resum: Les tortugues babaues (*Caretta caretta*) presenten una àmplia varietat d'estratègies alimentaries i algunes poblacions utilitzen hàbitats sub-òptims per a alimentar-se. Diferents estratègies d'alimentació poden, però, no ser equivalents en termes de *fitness* i poden donar lloc a diferències entre poblacions pel què fa a la grandària corporal adulta i la mida de la posta. Així, s'ha estudiat si les diferències en la mida de la posta entre zones de nidificació del mar Mediterrani estan relacionades amb l'ús diferencial de zones d'alimentació amb diferents nivells de productivitat. Es va analitzar la composició isotòpica de carboni i nitrogen en nounats de vuit zones de nidificació del mar Mediterrani i es van usar per caracteritzar les zones d'alimentació de les respectives mares. La mida de la posta també va ser analitzada en cada zona de nidificació per tal d'avaluar la relació entre la producció d'ous i la productivitat de les zones d'alimentació utilitzades per les femelles. D'acord amb els resultats obtinguts, la majoria de femelles nidificant a les zones estudiades s'alimentarien al sud del mar Jònic. El mar Adriàtic i el nord del mar Jònic, altament productius, serien majoritàriament usats per femelles nidificants a la Grècia oriental. Aquests patrons de distribució podrien estar relacionats amb els patrons de circulació superficial del mar Mediterrani i amb les migracions a la deriva fetes durant les primeres fases juvenils, ja que aquestes determinen el coneixement individual sobre la localització de les zones més productives. La mida mitjana de la posta en cada zona de nidificació es va correlacionar positivament amb la proporció de femelles amb accés a les zones d'alta productivitat (com el mar Adriàtic i el nord del mar Jònic), fet que té una gran influència en la producció d'ous i, per tant, les femelles amb major accessibilitat a zones altament productives presenten una major mida de la posta.

Title: Distribution patterns and foraging ground productivity determine clutch size in Mediterranean loggerhead turtles.

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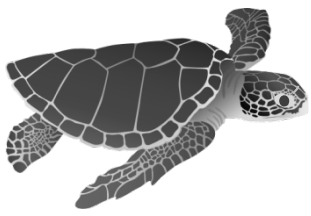

Distribution patterns and foraging ground productivity determine clutch size in Mediterranean loggerhead turtles

L. Cardona[1], M. Clusa[1*], E. Eder[1,2], A. Demetropoulos[3], D. Margaritoulis[4], A.F. Rees[4], A.A. Hamza[5], M. Khalil[6], Y. Levy[7], O. Türkozan[8], I. Marín[1], A. Aguilar[1]

[1] Department of Animal Biology and IRBio, University of Barcelona, Av. Diagonal 643, E-08028 Barcelona, Spain
[2] Centro Nacional Patagónico (CENPAT-CONICET), 9210 Puerto Madryn Chubut, Argentina.
[3] Cyprus Wildlife Society, Emmanuel Xanthou 11, P.O. Box 24281, 1703 Nicosia, Cyprus
[4] ARCHELON, the Sea Turtle Protection Society of Greece, Solomou 57, GR-104 32 Athens, Greece
[5] Libyan Seaturtle Program, Environment General Authority, Alfateh University, P.O. Box 13793, Tripoli, Libya
[6] MEDASSET, P.O. Box 19, Tyre, Lebanon
[7] The Israel Sea Turtle Rescue Centre, Nature Parks Authority, Mevoot Yam, Mikhmoret 40297, Israel
[8] Adnan Menderes University, Faculty of Science and Arts, Department of Biology, Aydin, Turkey
*Email: mclusa@ub.edu

ABSTRACT: Loggerhead turtles (*Caretta caretta*) present a wide variety of foraging strategies and some populations use sub-optimal habitats to forage. Different foraging strategies may not be equivalent in terms of fitness and may result in differences in adult body size and clutch size among populations. Accordingly, we tested whether differences in clutch size among rookeries in the Mediterranean Sea are related to differential use of foraging grounds of contrasting productivity. Stable isotope ratios of carbon and nitrogen of turtle hatchlings from eight Mediterranean rookeries were used to characterise the foraging grounds of their mothers. Clutch size was also studied in each rookery to assess reproductive output linked to foraging ground productivity. According to stable isotope ratios, most of the females nesting in the considered rookeries foraged in the southern Ionian Sea. The highly productive Adriatic/northern Ionian Sea region was mainly used by females nesting in western Greece. The explanation to these patterns might be linked to water circulation patterns and drifting trajectories followed during developmental migrations, which might determine individual knowledge on the location of productive foraging patches. Average clutch size in each rookery was positively correlated to the proportion of females accessing highly productive areas such as the Adriatic/northern Ionian Sea. This has a strong influence on reproductive output and hence females using the most productive foraging grounds had the largest clutch sizes.

KEY WORDS: *Caretta* · Currents · Foraging ground · Primary productivity · Reproductive output · Rookery · Stable isotopes

INTRODUCTION

Habitat quality has a strong influence on survival, fitness and reproductive output in animal species (Halama & Reznick 2001). Free distribution models assume that wild animals exploiting heterogeneous habitats select the most suitable foraging patches based on knowledge of habitat heterogeneity (Stephens & Krebs 1986). However, several wild populations have been recorded foraging in areas that offer lower profitability and reproductive output; i.e. sub-optimal (Pyke 1984). This might have an effect on populations as different foraging strategies may not be equivalent in terms of fitness and may result in differences in adult body size or clutch size among populations (Broderick et al. 2003).

The use of sub-optimal foraging strategies has been recorded in certain populations of large marine vertebrate species such as the South American sea lion (*Otaria flavescens;* Drago et al. 2010), the leatherback turtle (*Dermochelys coriacea*; Shillinger et al. 2008) and the loggerhead turtle (*Caretta caretta*; Zbinden et al. 2011, Eder et al. 2012). The loggerhead turtle is the most abundant sea turtle in subtropical and warm temperate regions of the world and has a complex life cycle characterised by long migrations (Bolten 2003, Plotkin 2003). During juvenile stages, loggerhead turtles undertake developmental migrations in which they disperse thousands of kilometres across the ocean to recruit to adult foraging grounds (Bolten 2003). These juvenile migrations may involve frequent shifts in habitat (McClellan & Read 2007, Casale et al. 2008a, Cardona et al. 2009, Mansfield et al. 2009, McClellan et al. 2010) but, after settlement, adult turtles remain faithful to the same foraging ground throughout most of their life (Broderick et al. 2007, Schofield et al. 2010, Vander Zanden et al. 2010, Hawkes et al. 2011).

However, although adult individual turtles show strong fidelity to foraging grounds and strong philopatry to nesting areas, several authors have suggested that adults nesting in a same rookery may present a wide variety of foraging strategies and destinations (Hatase et al. 2002, Hawkes et al. 2006, Mansfield et al. 2009, Reich et al. 2010, Vander Zander et al. 2010, Hawkes et al. 2011, Zbinden et al. 2011, Arendt et al. 2012a, Ceriani et al. 2012, Eder et al. 2012, Pajuelo et al. 2012). This variety might arise from individual differences in the knowledge on heterogeneity of habitats (Hatase et al. 2002, Hays et al. 2010, Eder et al. 2012).

Because different drifting trajectories are followed during developmental migrations (Wyneken et al. 2008, Hays et al. 2010, Putman et al. 2012a), the habitat patches visited by individuals from the same rookery might stochastically differ (McClellan & Read 2007, McClellan et al. 2010). These differences may influence decisions at the time of recruitment and the most productive habitat patches visited during juvenile stages may be those chosen as adult foraging grounds, as also seen in the leatherback turtle (Fossette et al. 2010, Gaspar et al. 2012).

Productive habitat patches in the Mediterranean Sea are scarce and scattered throughout the basin (Fig. 1). According to the optimal foraging theory (Stephens & Krebs 1986), most of the females nesting in the Mediterranean Sea would be expected to forage in the highly productive Adriatic Sea to maximise reproductive output. However, recent studies have shown many cases departing from this principle.

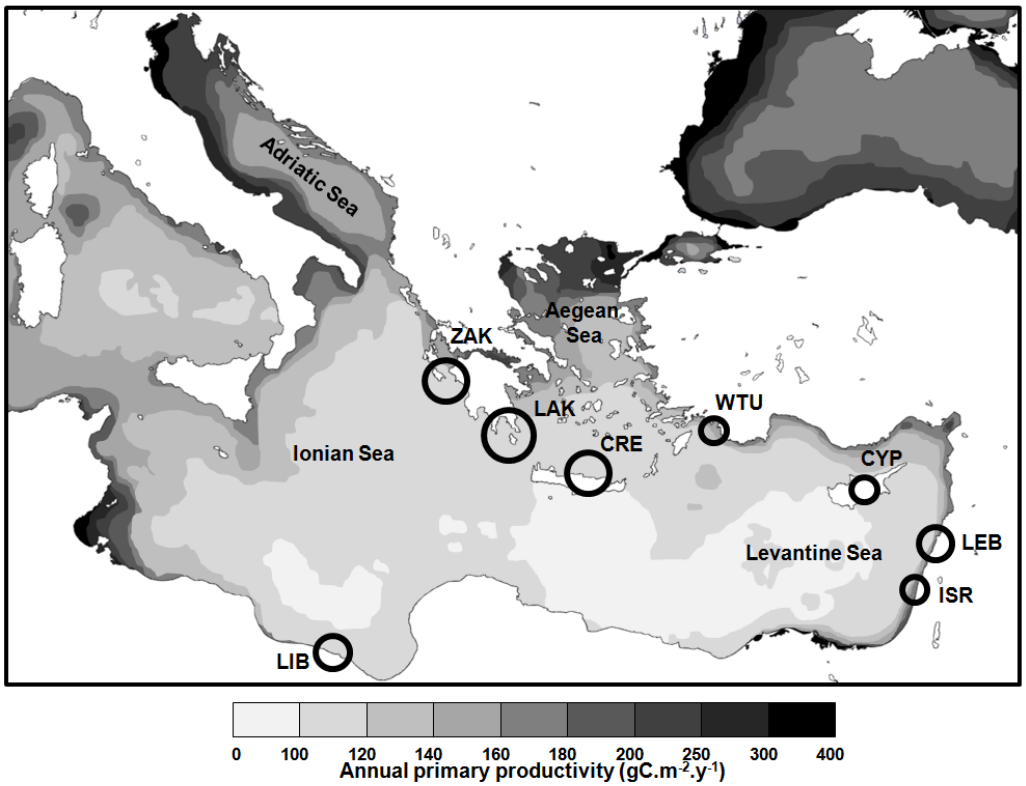

Fig. 1. Sampled rookeries of the loggerhead turtle in the Mediterranean Sea. Circles represented to scale reflecting mean clutch size per rookery (see Table 1). Annual primary production ($gC.m^{-2}.y^{-1}$) over the period 1997-2001 adapted and modified from Bosc et al. (2004). LIB (Libya), ISR (Israel), LEB (Lebanon), CYP (Cyprus), WTU (western Turkey), CRE (Crete), LAK (Lakonikos), ZAK (Zakynthos)

The scant data available from studies based on satellite tracking and passive tag recovery of females indicate that only a very small proportion of turtles nesting in Libya (Hochscheid et al. 2012), Crete (Margaritoulis & Rees 2011, Patel et al. 2012) and Cyprus (Broderick et al. 2007) and only half of the females nesting in western Greece (Margaritoulis et al. 2003, Hays et al. 2010, Zbinden et al. 2011) forage in the Adriatic Sea. The explanation probably lies in the complex pattern of surface circulation in the eastern Mediterranean Sea (Hamad et al. 2006, Hays et al. 2010) which likely hinders access of juveniles from most rookeries in the eastern Mediterranean Sea to the productive waters of the Adriatic Sea, with juveniles from western Greece being the only exception (Hays et al. 2010). Thus, if adult foraging grounds are selected on the basis of knowledge gained during the developmental migration (Hatase et al. 2002, Hays et al. 2010, Eder et al. 2012, Gaspar et al. 2012), only females from rookeries in western Greece would be expected to settle into the Adriatic Sea.

Access to the Adriatic Sea by turtles from western Greece is likely to promote their fitness and may explain why turtles nesting there are larger and lay more eggs than anywhere else in the Mediterranean Sea (Margaritoulis et al. 2003). If this was the case, it would demonstrate that the above mentioned variety of foraging strategies may not be equivalent in terms of fitness (Hatase et al. 2002, Reich et al. 2010, Zbinden et al. 2011, Eder et al. 2012). Accordingly, this paper aims to investigate whether differences in clutch size among rookeries in the eastern Mediterranean Sea are a consequence of differential use by adult females of foraging grounds of contrasting productivity. To do so, we compare the mean clutch size in eight major rookeries with the proportion of females from each rookery that forage in highly productive habitat patches, as characterised by stable isotopes of carbon and nitrogen.

MATERIALS AND METHODS

Sampling

Previous research of sea turtles has demonstrated that stable isotope ratios in females and hatchlings are highly correlated (Frankel et al. 2012). Accordingly,

stable isotope ratios in eggs and hatchlings offer a good alternative to reconstruct the foraging habitats of females without disturbing them during the nesting process.

Samples of muscle were taken from 152 dead hatchlings from a selection of rookeries in the Mediterranean Sea (Fig. 1, Table 1). Nest sampling (2003-2006) included central Libya (west of Sirte), Israel (scattered sites along the whole coastline), Lebanon (El Mansouri), Cyprus (Alagadi and Akamas), western Turkey (Fethiye) and Greece (Rethymno on the Island of Crete, Lakonikos Bay and Zakynthos). Nests were excavated after hatchling emergence and samples were collected from one fresh-dead hatchling per nest. Through this methodology, no hatchling was sacrificed for the present experiment. No differences in stable isotope composition between live and fresh-dead hatchlings were expected as decomposition was not obvious and even if so, Payo-Payo et al. (2013) found that stable isotope ratios in the muscle of loggerhead turtles do not change over time due to decomposition. Samples were stored in 95% ethanol, known not to modify stable isotope ratios of muscle tissue (Hobson et al. 1997).

Independency between samples can be assumed as sampling protocol was designed to avoid pseudoreplication, e.g. female flipper tagging and samples taken from clutches laid within a 15-day window to avoid hatchlings from the same individual turtle as females rarely nest at intervals shorter than this period (Dutton 1995). Clutch size was calculated from the excavated nests remains, including both unhatched eggs and empty egg shells.

Stable isotope analysis

The analysis of stable isotope signatures in animal tissues provides information on diet but also can be used to track foraging ground locations, as tissue signatures reflect those of the specific food webs present in a certain area (Hobson 1999; Fry 2006).

Although the isotopic landscape, or isoscape, of the central and eastern Mediterranean Sea is poorly known, Zbinden et al. (2011) reported differences between the average $\delta^{15}N$ values in female turtles foraging in the Adriatic/northern Ionian Sea and in those foraging in the southern Ionian Sea. To gain a further insight into the spatial variation in isotopic ratios we collected samples of the

benthic crab *Liocarcinus depurator*, a widespread species that constitutes a major component in the diet of adult loggerhead turtles (Tomás et al. 2001, Casale et al. 2008a, Travaglini & Bentivegna 2011). A sample of five benthic crabs was collected in seven locations spread over the central and eastern Mediterranean Sea (Fig. 2): Port Said (southern Levantine Sea), Limassol (northern Levantine Sea), Chania (southern Aegean Sea), Zakynthos (north-eastern Ionian Sea), Trieste (north Adriatic Sea), Catania (north-western Ionian Sea) and Lampedusa (south-western Ionian Sea).

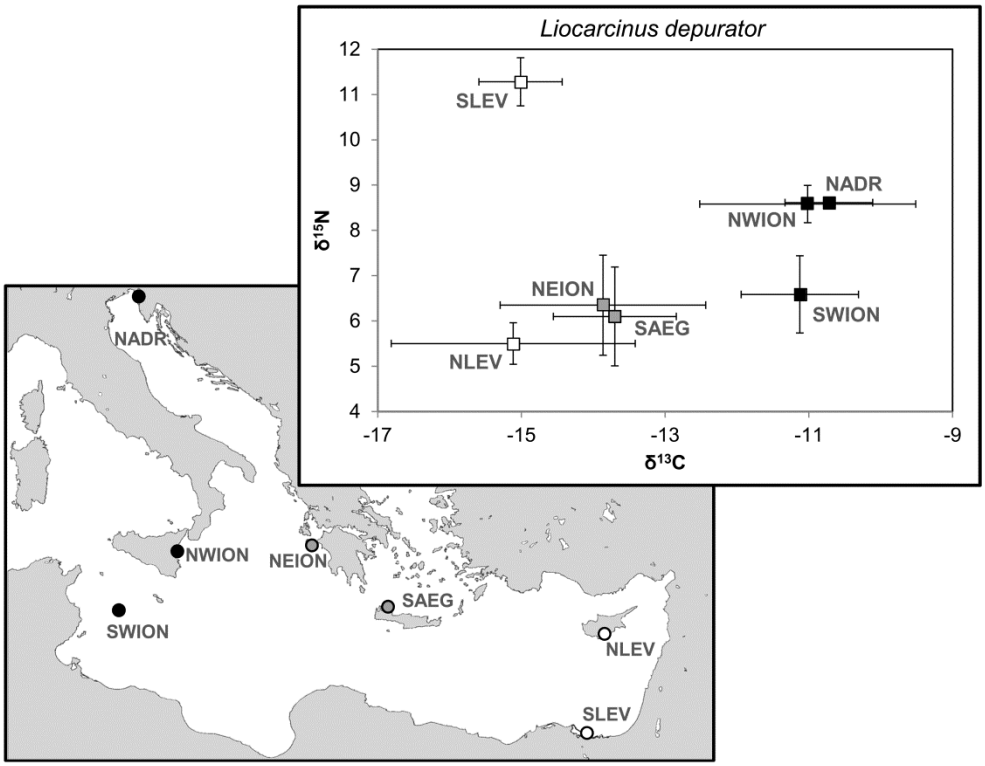

Fig. 2. *Liocarcinus depurator*. Sampling locations and stable isotope ratios (‰) from seven different areas of the Mediterranean Sea: SLEV (southern Levantine Sea), NLEV (northern Levantine Sea), SAEG (southern Aegean Sea), NEION (north-eastern Ionian Sea), NADR (northern Adriatic Sea), NWION (north-western Ionian Sea), SWION (south-western Ionian Sea). Standard deviation bars included. Graded colour scale reflects position of the sampled areas within the Mediterranean Sea from westernmost (black) to easternmost areas (white)

Muscle tissue samples from crabs and hatchlings were oven-dried at 60 ºC for 48-72h and then ground into fine powder. Only one hatchling per nest was analysed because little variation had been previously found among individuals from a given nest (Frankel et al. 2012). Lipids were extracted from all tissues with a chloroform-methanol (2:1) solution. Approximately 0.3 mg of powdered sample were weighed into tin cups, combusted at 1000 ºC, and analysed in a continuous

flow isotope ratio mass spectrometer (Flash 112 IRMS Delta C Series EA Thermo Finningan) at the Scientific and Technological Centre of the University of Barcelona. Stable isotope ratios were expressed in parts per thousand (‰) according to the equation $\delta X = [(Rsample/Rstandard)-1] \times 1000$, where X is ^{13}C or ^{15}N and R is the corresponding ratio of the heavier to the lighter isotope (i.e. $^{13}C/^{12}C$ or $^{15}N/^{14}N$). International isotope standards of known $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios were used to a precision of 0.2‰: IAEA CH6 ($\delta^{13}C$ = -10.3‰), USGS 40 ($\delta^{13}C$ = -25.8‰) and IAEA CH7 ($\delta^{13}C$ = -31.6‰) for carbon and USGS 40 ($\delta^{15}N$ = -4.3‰), IAEA N1 ($\delta^{15}N$ = +0.8‰), IAEA 600 ($\delta^{15}N$ = +1.0‰) and IAEA N2 ($\delta^{15}N$ = +20.4‰) for nitrogen.

Data analysis

Differences in the isotope composition among populations of *L. depurator* and loggerhead hatchlings were assessed independently for carbon and nitrogen through ANOVA tests with SPSS v15.0.

Zbinden et al. (2011) analysed egg yolk whereas in the present paper we analyse muscle from hatchlings. To our knowledge, studies have not focused on the isotopic correlation and discrimination factors between egg yolk and muscle of hatchlings, although the relative abundance of the heavy isotopes is expected to increase during embryonic development due to the preferential excretion of light isotopes due to animal metabolism (Martínez del Rio et al. 2009). To assess the relevance of such a potential source of bias, the stable isotope ratios provided by Zbinden et al. (2011) for egg yolk from females nesting in Zakynthos were compared with the stable isotope ratios of hatchlings for the same beach (see below) using a Student's t-test with SPSS v15.0. As differences were not statistically significant (see results), the values from Zbinden et al. (2011) were considered good proxies to classify the foraging ground of the females whose hatchlings were analysed in the present study. Individuals that fell in the overlapping range between the Adriatic/northern Ionian Sea and the southern Ionian Sea were assigned to the area with the closest values for subsequent calculations. Values of $\delta^{15}N$ or $\delta^{13}C$ beyond the range reported by Zbinden et al. (2011) were considered to reveal foraging in other areas and hatchlings were classified accordingly. A Pearson's correlation test carried out with SPSS v15.0 was used to assess the relation between

the proportion of hatchlings from females foraging in the Adriatic/northern Ionian Sea in each rookery and the distance to that region. The test was performed twice with minimum linear and coastal distances to test for possible differences.

The relationship between reproductive output and putative foraging ground was assessed by comparing the average clutch size of nests likely to have been laid by mothers foraging in the Adriatic/northern Ionian Sea ($\delta^{15}N > 11.5$ ‰, according to Zbinden et al. 2011) with those with hatchlings presenting a value of $\delta^{15}N$ lower than 11‰ thus likely to have been laid by mothers foraging in the southern Ionian Sea. A Student's t-test was performed to analyse the statistical significance of such difference.

A data set of 158 nests from Israel was used to asses whether the protocol used here resulted in any bias in clutch size estimation. A Student's t-test was used to compare the average clutch size of nests with at least one dead hatchling and those without any dead hatchling.

RESULTS

Stable isotope analysis

The $\delta^{13}C$ ratios of *L. depurator* varied significantly among sampling locations ($F_{6,28} = 14.041$, $p < 0.001$) and revealed a longitudinal gradient along the eastern Mediterranean Sea, with the highest values in the Adriatic Sea and the western Ionian Sea and the lowest values in the Levantine Sea (Fig. 2). Differences in the $\delta^{15}N$ values of *L. depurator* were also statistically significant among sampling locations ($F_{6,28} = 37.938$, $p < 0.001$), with the highest values in the southern Levantine, the Adriatic and the north-western Ionian seas and the lowest values in the northern Levantine Sea.

Values of $\delta^{15}N$ (Table 1) for turtle hatchlings were significantly different among rookeries ($F_{7,145} = 1.553$, $p = 0.037$) although no significant differences were detected for $\delta^{13}C$ values ($F_{7,145} = 9.092$, $p = 0.151$). The post-hoc Tukey test revealed significant differences of $\delta^{15}N$ values only between Zakynthos and Israel.

Differences between the stable isotope ratios reported by Zbinden et al. (2011) for egg yolk from Zakynthos and the corresponding ratios here reported for

hatchling muscle from the same beach were not statistically significant, either for $\delta^{15}N$ (yolk = 11.0 ± 2.3; muscle = 11.4 ± 2.3; t_{30} = 0.436, p = 0.666) or $\delta^{13}C$ (yolk = -17.7 ± 1.6; muscle = -16.7 ± 1.6; t_{30} = 1.717, p = 0.096). Accordingly, the stable isotope ratios reported by Zbinden et al. (2011) were used as a benchmark to identify the foraging grounds of the females laying the nests here considered.

When considered individually, 106 hatchlings fell within the range of $\delta^{15}N$ values previously reported for the eggs from females foraging in the southern Ionian Sea, 21 within the range of $\delta^{15}N$ values corresponding to foraging in the Adriatic/northern Ionian Sea and 20 hatchlings to the range in between the two areas (Fig. 3).

Table 1. *Caretta caretta*. Mean stable isotope ratios of hatchlings muscle (‰) and mean and range clutch sizes (number of eggs) of nests with at least one dead hatchling from the major loggerhead rookeries in the Mediterranean Sea. Number of analysed individuals (n) and standard deviations (±) included. LIB (Libya), ISR (Israel), LEB (Lebanon), CYP (Cyprus), WTU (western Turkey), CRE (Crete), LAK (Lakonikos), ZAK (Zakynthos)

Rookeries	Stable Isotopes Ratios			Clutch Size	
	n	$\delta^{15}N$	$\delta^{13}C$	Mean	Range
LIB	25	9.8±0.9	-16.5±1.8	91.1±14.3	81-101
ISR	18	9.3±1.9	-17.0±1.8	78.4±22.9	35-123
LEB	18	9.9±1.3	-16.2±1.9	91.5±51.6	57-122
CYP	27	9.9±2.3	-16.4±2.0	79.0±16.9	38-113
WTU	18	10.1±2.1	-15.1±2.2	80.5±37.5	55-105
CRE	14	9.6±1.5	-16.1±2.0	102.0±25.2	52-149
LAK	13	10.7±2.4	-16.2±1.9	129.1±24.9	99-171
ZAK	20	11.4±2.3	-16.7±1.6	111.8±21.9	71-136

The $\delta^{15}N$ values of the remaining 5 hatchlings fell outside that range and their mothers likely foraged in other areas. Although females foraging in the Adriatic/northern Ionian Sea and in the southern Ionian Sea were not expected to differ in $\delta^{13}C$ values, 17 hatchlings felt beyond the $\delta^{13}C$ range values reported for both areas, thus indicating the use of other foraging grounds (Fig. 4). According to the isoscape revealed by *L. depurator*, the two hatchling samples from Israel, characterised by very low $\delta^{13}C$ values, could correspond to females foraging in the Levantine Sea. The remaining 15 hatchlings were too enriched in ^{13}C to correspond

to females using the Ionian Sea or the Adriatic Sea according to the values provided by Zbinden et al. (2011) and, according to the eastward decrease in $\delta^{13}C$ revealed by the *L. depurator* isoscape, they might have foraged somewhere to the west.

The proportion of females foraging in the Adriatic/northern Ionian Sea increased downstream the main current, from 4% in Libya to 45% in Zakynthos (Fig. 5). A significant correlation was observed between the shortest geographical distance (Lat/Long distance) from each rookery to the Adriatic Sea and the proportion of females foraging in the Adriatic/northern Ionian Sea (Pearson's correlation test, r = -0.769, p = 0.026). When the distance from the beach to the Adriatic Sea was computed along the coastline (coastal distance) the correlation was still significant (Pearson's correlation test, r = -0.784, p = 0.021).

Clutch size

The average clutch size per rookery ranged from 78 to 129 eggs per nest and the individual clutch size from 35 to 171 eggs (Table 1). These recordings correspond to the average clutch size of nests with at least one dead hatchling, which is slightly larger than the average clutch size of nests without dead hatchlings in the data set from Israel used as reference (clutch size $_{\text{no dead}}$= 70.5 ± 20.7 eggs; clutch size $_{\text{one dead}}$ = 78.1 ± 24.9 eggs; t_{151}=2.069, p=0.040).

Clutch size was significantly larger when the $\delta^{15}N$ values of the hatchlings was >11.5‰ (mean clutch size = 104.6 ± 29.2 eggs, n = 40) than when the $\delta^{15}N$ values of the hatchlings was <11‰ (mean clutch size = 89.4 ± 29.1 eggs; n = 74) (t_{112} = 2.229, p = 0.028). Accordingly, the two rookeries with the highest proportion of females likely to forage in the Adriatic/northern Ionian Sea (Zakynthos and Lakonikos) had the largest average clutch size (Fig. 1; Table 1).

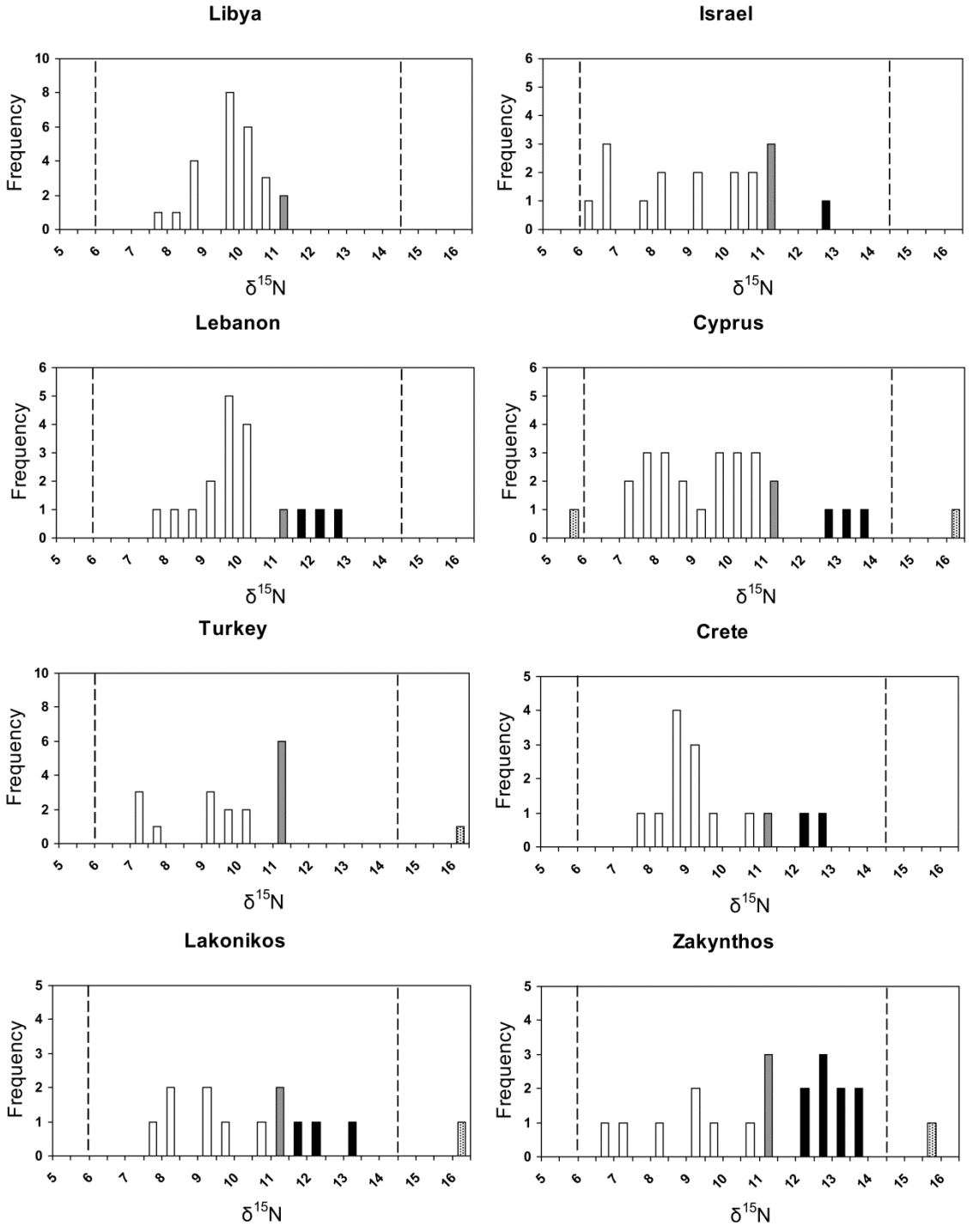

Fig. 3. *Caretta caretta*. Individual $\delta^{15}N$ values (‰) from loggerhead hatchlings sampled in the studied rookeries. Dashed lines show the range reported by Zbinden et al. (2011) for the Ionian Sea and the Adriatic Sea combined. White bars denote hatchlings from females likely to have foraged in the southern Ionian Sea, black bars denote hatchlings from females likely to have foraged in the Adriatic/northern Ionian Sea and grey bars show hatchlings with intermediate values. The dotted bars represent hatchlings corresponding to females foraging somewhere else

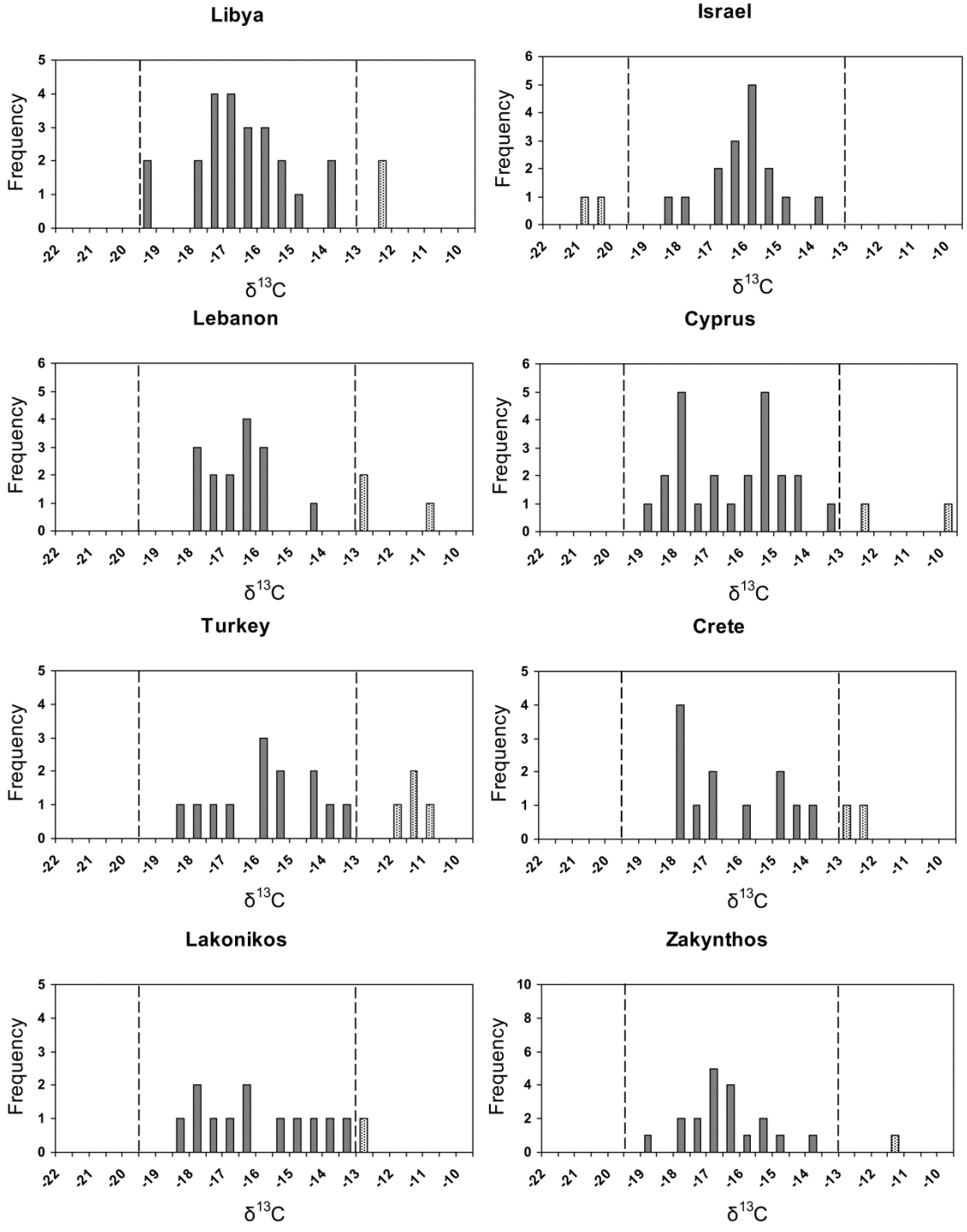

Fig. 4. *Caretta caretta*. Individual $\delta^{13}C$ values (‰) from loggerhead hatchlings sampled in the studied rookeries. Dashed lines show the range reported by Zbinden et al. (2011) for the Ionian Sea and the Adriatic Sea combined. Grey bars denote hatchlings from females likely to have foraged either in the Ionian Sea or the Adriatic Sea and dotted bars denote hatchlings corresponding to females foraging somewhere else

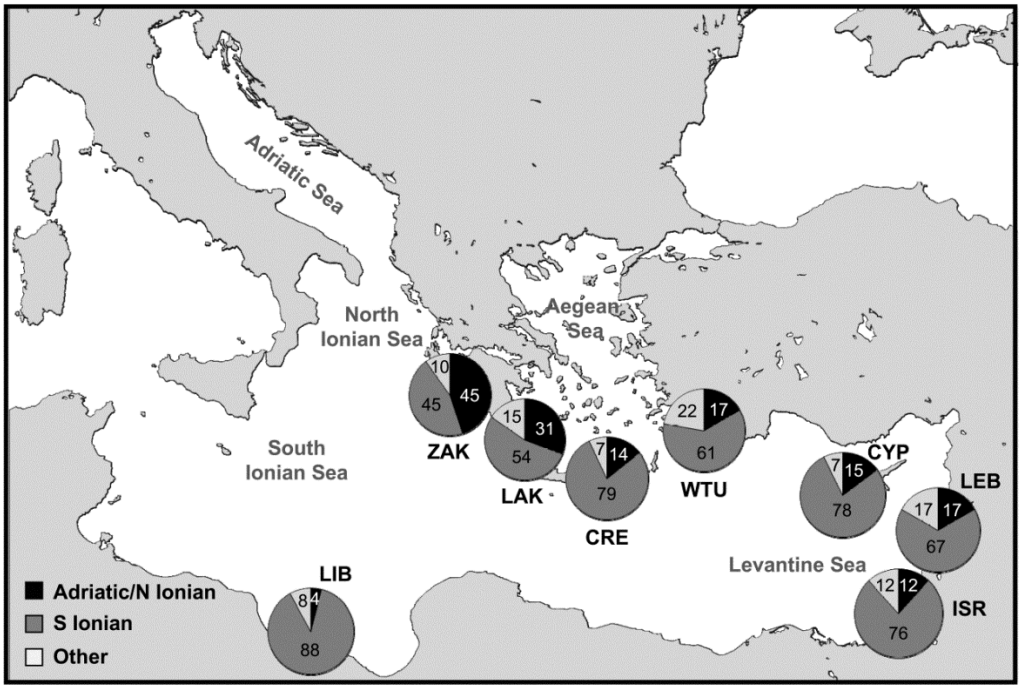

Fig. 5. *Caretta caretta*. Proportion of nesting females potentially foraging in each of the three different areas, as derived from stable isotope analyses. Percentages included in the pies

DISCUSSION

The stable isotope results reported here identify the southern Ionian Sea as the major foraging ground for most of the rookeries analysed and indicate that the Adriatic/northern Ionian Sea region is used by a high proportion of females nesting in western Greece. This is supported by the reconstruction of the regional isoscape through values obtained for *L. depurator*, which revealed a previously un-described decline in the $\delta^{13}C$ values in the Mediterranean Sea moving from west to east. This pattern is probably caused by the decline of planktonic primary productivity from west to east in the region (Bosc et al. 2004; Fig. 1) and hence reflects a decreasing reliance of primary producers on ^{13}C. On the other hand, the highest $\delta^{15}N$ values were observed in the Adriatic Sea, the north-western Ionian Sea and the southern Levantine Sea. These high $\delta^{15}N$ values observed could be the result of ^{15}N-enriched freshwater run-off (Oczkowski et al. 2009), particularly in the Adriatic Sea and north-western Ionian Sea, where its waters receive 60-70% of the inorganic nutrient load from the Po River discharge (Degobbis & Gilmartin 1990; Voss et al. 2011). Contrarily, the lower $\delta^{15}N$ values found elsewhere were characteristic of Mediterranean oceanic waters (Pantoja et al. 2002).

Direct comparison between stable isotope ratios in *L. depurator* and in turtles is not possible because discrimination factors are not known to accurately compare the two species isotopically (Vander Zanden & Rasmussen 2001). However, the isoscape derived from *L. depurator* reflects the relative enrichment in heavy isotopes expected for turtles foraging in productive regions. The usefulness of this approach is demonstrated by the concordance between the results previously reported by Zbinden et al. (2011) on stable isotope ratios in the eggs laid in Zakynthos and the regional isoscape here obtained from *L. depurator*; as both concur in indicating that samples from the Adriatic Sea are more enriched in ^{15}N than those from the southern Ionian Sea.

Previous studies with tagging and satellite tracking had identified the southern Ionian Sea as a main foraging ground for adult loggerhead turtles nesting in western Greece (Margaritoulis et al. 2003, Hays et al. 2010, Zbinden et al. 2011), Libya (Hochscheid et al. 2012), Crete (Margaritoulis & Rees 2011, Patel et al. 2012) and Cyprus (Broderick et al. 2007), but nothing was known about the foraging destinations of turtles nesting in western Turkey or Israel. The isoscape derived from *L. depurator* confirms the southern Ionian Sea as an isotopically distinct region and the stable isotope ratios from turtle hatchlings confirm that the southern Ionian Sea is a major foraging ground for adult females from the studied rookeries. It should be kept in mind that the protocol used did not consider nests without dead hatchlings, which are in turn characterised by a slightly lower clutch size than those with at least one dead hatchling. As females foraging in the southern Ionian Sea are characterized by a smaller clutch size than those foraging in the Adriatic/northern Ionian Sea, this means that the proportion of females foraging in the southern Ionian Sea has actually been slightly underestimated in this study. In any case, the widespread utilisation of the southern Ionian Sea by adult females is hardly surprising, as the area is easily accessible following the main cyclonic current of the eastern Mediterranean (Fig. 6) during developmental migrations according to virtual particle tracking models (Hays et al. 2010; Putman & Naro-Maciel 2013).

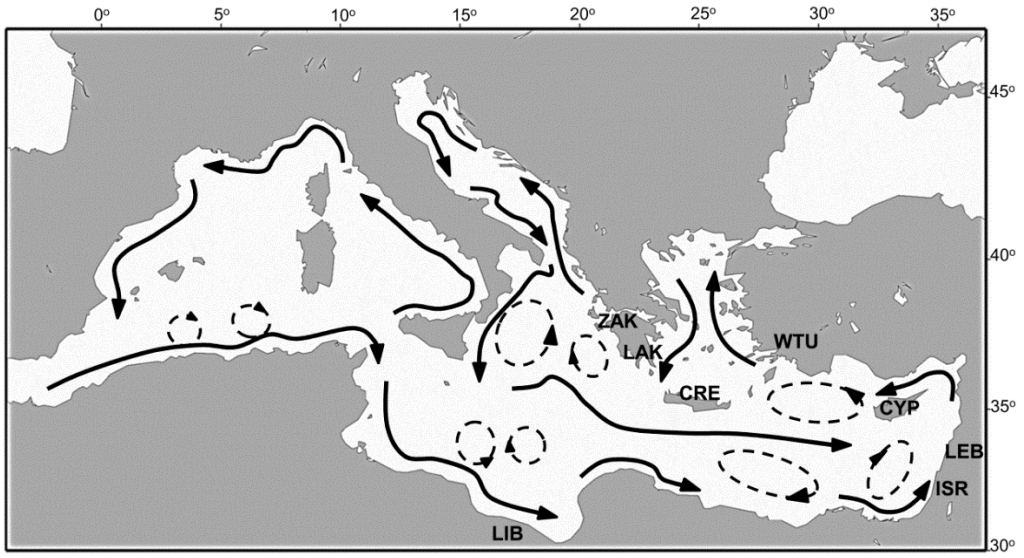

Fig. 6. Main surface circulation patterns of the Mediterranean Sea. Thin dashed lines show transient gyres and eddies. Adapted and modified after Robinson et al. (2001) and Millot & Taupier-Letage (2004)

On the contrary, stable isotope data indicate that the highly productive Adriatic Sea (Bosc et al. 2004) and the adjoining northern Ionian Sea are largely used by females nesting in western Greece (this study, Zbinden et al. 2011), but seldom used by females from other regions (this study, Margaritoulis & Rees 2011, Patel et al. 2012, Hochscheid et al. 2012). We have found a decreasing proportion of turtles with stable isotope ratios consistent with foraging in the Adriatic/northern Ionian Sea as we moved upstream from Zakynthos to Libya. These results are consistent with the peripheral position of the Adriatic and northern Ionian Seas within the general current system of the eastern Mediterranean Sea (Hamad et al. 2006; Fig. 6) in contrast to the central position of the southern Ionian Sea and the results of the virtual particle tracking models (Hays et al. 2010; Putman & Naro-Maciel 2013). It should be noted, however, that some parts of the northern Ionian Sea cannot be properly differentiated from the southern Aegean Sea in the isoscape derived from *L. depurator* and that the Aegean Sea is used at least by turtles nesting in Crete according to tag recovery data (Margaritoulis & Rees 2011). As a consequence, some of the turtles classified as foraging in the Adriatic/northern Ionian Sea might actually forage in the southern Aegean Sea. Further research is needed to clarify the origin of turtles foraging there.

The limited use of the Adriatic/northern Ionian Sea by females from rookeries other than Zakynthos and Lakonikos is intriguing because the Adriatic

Sea is indeed the most productive area of the western Mediterranean Sea (Bosc et al. 2004) and females foraging there are larger (Margaritoulis et al. 2003) and lay more eggs than females foraging elsewhere (this study, Zbinden et al. 2011). If turtle distribution was only dependent on a balance between food availability and distance to rookery, turtles from western Turkey, Cyprus, Israel and Lebanon would be expected to use the Adriatic/northern Ionian Sea and the southern Ionian Sea in equal proportions as these two foraging grounds are equidistant from rookeries in the eastern Mediterranean Sea. However, this has only been observed in Zakynthos (western Greece), where turtles present a strong dichotomy between these two equidistant foraging grounds (this study; Zbinden et al. 2011). Thus, settlement at the shortest distance to their natal areas, as proposed by Bowen et al. (2005), might not be enough to explain selection of foraging grounds used by turtles nesting in the eastern Mediterranean Sea. Rather the contrary, differences in knowledge of the location of productive foraging grounds due to limited dispersal during the developmental migration could also explain why adult turtles from other rookeries do not massively use the peripheral Adriatic/northern Ionian Sea as a foraging ground. Future research should focus on hatchling and juvenile tracking to test both hypotheses, as none can be excluded with the current data.

Another highly productive area in the eastern Mediterranean Sea is the coastal fringe situated east of the Nile delta, in the southern Levantine Sea (Bosc et al. 2004, Oczkowski et al. 2009). Satellite tracking has revealed the presence of females from Cyprus in the area (Broderick et al. 2007), but did not clarify whether these turtles were in transit towards the Ionian Sea because transmission ceased after the individuals reached the area. The absence of individuals simultaneously enriched in ^{15}N and depleted in ^{13}C in comparison with those foraging in the southern Ionian Sea indicates, according to the isoscape derived from *L. depurator*, limited foraging in the southern Levantine Sea, an intriguing result because this area is located downstream from the nesting beaches in Libya (Fig. 6). However, mesoscale eddies in the southern Ionian basin might retain Libyan hatchling drifters within that sub-basin (Hays et al. 2010) and hence might limit the eastward dispersal of turtle hatchlings to the Levantine Sea. This, in turn, might limit their knowledge on habitat heterogeneity and restrict adult females to use the area as a foraging ground (McClellan & Read 2007, McClellan et al. 2010). Finally, the

stable isotope ratios also showed that the northern Levantine Sea might be used as a foraging ground only by a few females from Israel, a finding in accordance with previous studies using satellite tracking (Broderick et al. 2007). This is hardly surprising, considering the low primary productivity of most of the area (Bosc et al. 2004).

The distribution patterns here described demonstrate the existence of a strong link between the foraging grounds used by adults and the location of their rookeries. This has consequences on the reproductive output since we found a strong correlation between the specific foraging grounds used and the average clutch size of its nesting females. Thus, females foraging in the Adriatic/northern Ionian Sea had larger clutch sizes than females from the same rookery that forage in less productive areas such as the southern Ionian Sea. Differences between both groups may actually be even larger, because the protocol used for this study did not consider nests without dead hatchlings and hence might have slightly overestimated the average clutch size of individuals foraging in areas of low productivity.

Although female size was not assessed in this study, differences in clutch size are likely related to differences in body size (Frazer & Richardson 1986; Miller 1997; Zbinden et al. 2011). Sea turtle females foraging in highly productive foraging grounds grow larger, improving their reproductive output and hence laying a larger number of eggs (Broderick et al. 2003; Plot et al. 2013). Accordingly, the largest clutch sizes were found in western Greece, where the largest nesting loggerhead females in the Mediterranean Sea have been recorded (Zakynthos = 82.7-83.8cm CCL; Lakonikos = 84.1-84.6cm CCL; Margaritoulis et al. 2003). On the contrary, clutch size and female body size (Margaritoulis et al. 2003) were smaller in rookeries that hosted a large proportion of females foraging in the southern Ionian Sea, although turtles nesting there might present behavioural adaptations to increase fitness such as the reduced remigration interval recorded in Cyprus (Broderick et al. 2003). However, data on remigration intervals were not available for the studied beaches and hence solid conclusions on this issue could not be drawn in the current study.

Importantly, the reported results suggest that differences between rookeries are not shown at population level but at individual level. Turtles foraging in highly

productive foraging grounds nest at the same rookeries as turtles foraging in less productive grounds and those individual differences might be stochastically driven by water circulation patterns that determine the drifting trajectories followed during developmental stages (Wyneken et al. 2008, Hays et al. 2010, Putman et al. 2012a); which might modulate the knowledge on productive habitat patches available in the area to be used as adult foraging grounds (McClellan & Read 2007, McClellan et al. 2010). Actually, the populations with a larger proportion of females foraging into the highly productive habitats of the Adriatic/northern Ionian Sea are the largest in the region, whereas females foraging in the southern Ionian Sea dominate in some of the smallest populations (Casale & Margaritoulis 2010). The Israeli and Lebanese populations are particularly interesting, as thousands of turtles were slaughtered annually during the 1920s (Sella 1982), but only a few tens of adult turtles survive currently. The regional decrease in oceanic productivity caused by the regulation of the Nile (Oczkowski et al. 2009) might have played a role in preventing the recovery of those populations, according to the results provided here.

In addition, the foraging grounds used not only can have an effect on fitness of loggerhead populations nesting the Mediterranean Sea but also can alter their probability of survival. Bycatch rates are highly variable within the basin (Casale 2011) and the impact of fisheries interactions on foraging populations will depend on the overlap between fishing and turtle distribution (Wallace et al. 2008, 2013) and also on the birth rate of the populations involved. In this context, populations with a large number of individuals foraging in the southern Ionian Sea are more vulnerable to on-sea mortality than those foraging in more productive habitats. Thus, the results here presented highlight the need for further research on turtle distribution and foraging ground use to ensure survival of this species.

In summary, the observed differences in reproductive output were a result of differential use of foraging grounds of contrasting productivity. Previous research showed that access to foraging grounds in the eastern Mediterranean Sea is likely dependent on rookery location within the surface current system, which might explain the observed differences in reproductive output both at an individual and at a rookery level. This is relevant from a conservation point of view because foraging ground selection plays an important role on the species fitness and hence, females

feeding in less productive foraging grounds will have smaller clutch sizes and will therefore be more vulnerable to anthropogenic and natural threats.

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CHAPTER 4. Interaction with fishing activities

4.1. Population make-up of turtle bycatch in the Mediterranean Sea: relevance of fishing ground and fishing gear

Títol: Composició de tortugues capturades accidentalment al mar Mediterrani: rellevància de zona i art de pesca.

Resum: Les interaccions pesqueres representen una important amenaça per a les tortugues marines, fet que comporta una creixent necessitat de comprendre els efectes que té la captura accidental en les seves poblacions. Diferents tipus d'arts de pesca s'usen generalment en una mateixa zona i poden diferir en les seves taxes de captura i mortalitat associada. A més, els arts de pesca utilitzats en zones d'alimentació per a tortugues poden tenir efectes diferents degut a variacions en la composició d'aquestes zones, tal i com s'ha suggerit prèviament al mar Mediterrani, on tortugues babaues d'origen atlàntic comparteixen zones d'alimentació amb tortugues d'origen mediterrani. En aquest estudi hem utilitzat marcadors intrínsecs (isòtops estables) i marcadors genètics (ADN mitocondrial i nuclear) per analitzar els patrons d'ús de l'hàbitat i la composició genètica de les tortugues capturades accidentalment amb palangres de superfície i xarxes d'arrossegament/tremall en tres regions mediterrànies diferents (el sud-est d'Espanya continental, el sud de les Illes Balears i el sud d'Itàlia). No s'han trobat diferències isotòpiques ni genètiques entre les tortugues capturades amb palangres de superfície i xarxes d'arrossegament/tresmall en cap de les tres regions però si entre regions. En conseqüència, la composició de les captures accidentals a les zones d'alimentació mediterrànies depèn de la zona en la qual es duen a terme les operacions pesqueres més que no pas de l'art de pesca utilitzat. Aquests resultats posen de manifest la necessitat de conèixer detalladament la distribució de la tortuga babaua per reduir l'impacte de la captura accidental en les poblacions més petites i vulnerables.

Title: Population make-up of turtle bycatch in the Mediterranean Sea: relevance of fishing ground and fishing gear.

Authors: Clusa M, Carreras C, Pascual M, Gaughran J, Piovano S, Avolio D, Ollano G, Fernández G, Levy Y, Tomás J, Raga JA, Aguilar A, Cardona L.

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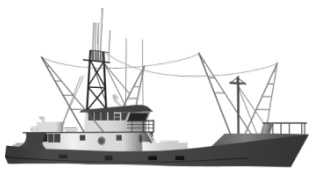

Population make-up of turtle bycatch in the Mediterranean Sea: relevance of fishing ground and fishing gear

Marcel Clusa[a], Carlos Carreras[a,b], Marta Pascual[c], Stephen J. Gaughran[a,d], Susanna Piovano[e*], Diego Avolio[f], Gepi Ollano[g], Gloria Fernández[h], Jesús Tomás[i], Juan Antonio Raga[i], Alex Aguilar[a], Luis Cardona[a]

[a] Department of Animal Biology and IRBio, University of Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain
[b] Centre for Ecology and Conservation, Penryn Campus, University of Exeter, Penryn, TR10 9EZ, United Kingdom.
[c] Department of Genetics and IRBio, University of Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain
[d] Sackler Institute for Comparative Genomics, American Museum of Natural History, Central Park West at 79th Street, New York, NY 10024 USA
[e] Department of Life Sciences and Systems Biology, University of Torino, Via Accademia Albertina 13, 10123 Turin, Italy
[f] Sea Turte Rescue Centre CTS, Brancaleone, Italy
[g] Cetaceans and Sea Turtles Rescue Centre Laguna di Nora, Nora, 09010 Pula, Italy
[h] Fundación Aspro Natura, c/ Garcilaso de la Vega 9, E-07181, Costa d'en Blanes, Calvià , Spain
[i] Cavanilles Institute of Biodiversity and Evolutionary Biology, University of Valencia, Aptdo. 22085, 46071 Valencia, Spain
*S.Piovano current address: School of Biological and Chemical Sciences, Laucala Campus, University of the South Pacific, Suva, Fiji Islands

ABSTRACT

Fisheries interactions represent an important threat for sea turtles and there is a growing need to understand the effects of bycatch on their populations. Different types of fishing gear are usually used in a same area but may differ in bycatch and mortality rates associated. Furthermore, fishing gears may differ in the population make-up of caught turtles in mixed foraging grounds as previously suggested to be the case in some areas of the Mediterranean Sea, where loggerhead turtles of Atlantic and Mediterranean origin share common foraging grounds. To assess whether this observation can be generalised we have analysed the patterns of habitat use and the genetic make-up of turtle bycatch from drifting longlines and bottom trawling/trammel nets in three different regions (eastern mainland Spain, southern Balearic Islands and southern Italy). We have analysed 176 incidentally caught juvenile loggerhead turtles in these three areas with intrinsic (stable isotope ratios) and genetic (mitochondrial and nuclear DNA) markers. No isotopic or genetic differences were found between turtles caught with drifting longlines and bottom trawling/trammel nets within any of the three regions. Nevertheless, differences were detected among regions with both markers. Accordingly, the population make-up of turtle bycatch depends on the area where the fishing operations are conducted but not on the fishing gear used. This highlights the need for detailed knowledge on turtle distribution in the ocean to reduce the impact of bycatch on the smaller and more vulnerable populations.

Keywords: Bycatch; *Caretta*; longline; microsatellites, mtDNA; nDNA; trawling, stable isotopes.

Introduction

Fishing is responsible for the decline of many marine species because of a combination of overfishing and habitat disturbance (Cushing, 1988; Jackson et al., 2001; Pauly et al., 2005). Bycatch, the unintentional capture of non-targeted species during fishing operations (Hall et al., 2000), has been described as one of the most important threats causing the decline of marine species worldwide, specially of large marine vertebrates: birds (Tasker et al., 2000), sharks (Dulvy et al., 2008), marine mammals (Read et al., 2006) and sea turtles (Wallace et al., 2013). This remarkable decline of large marine vertebrate populations is mainly because of their high vulnerability due to long lifespan, late age at maturity and low reproductive output (Heppell et al., 1999; Lewison et al., 2004a).

As most sea turtles are listed as endangered under the IUCN Red List of Threatened Species, sea turtle bycatch has been of strong concern among scientists for the past decade (Lewison et al., 2013). Drifting longline fisheries that target large pelagic fish (Lewison et al., 2004b; Lewison and Crowder, 2007) have been the focus of most of the research on turtle bycatch, but recent research has demonstrated that bottom trawling and set nets also capture a relevant number of turtles in neritic areas (Wallace et al., 2013).

Sea turtles present complex life cycles involving several habitat shifts (Plotkin, 2003). Loggerhead turtles (*Caretta caretta*) spend several years in the open ocean during early live and recruit to neritic habitats as late juveniles or immatures (Bolten 2003) although, in some populations, a number of adults may remain oceanic through their entire life (Hatase et al. 2002; Eder et al. 2012). The foraging grounds in the Mediterranean Sea are used by juvenile loggerhead turtles of Atlantic and Mediterranean origin (Laurent et al., 1993; Bowen et al., 2003; Carreras et al., 2006, 2011; Chaieb et al. 2012; Clusa et al., *in press*) and hence bycatch in the Mediterranean may have broad implications across the North-Atlantic.

Laurent et al. (1998) reported a contrasting population make-up for the turtle bycatch of drifting longliners and that of bottom trawlers. In the same study, it was suggested that drifting longlines captured a mixture of turtles of Atlantic and Mediterranean origin, whereas bottom trawling captured only turtles of

Mediterranean origin. Laurent et al. (1998) assumed no regional differences in the distribution of loggerhead turtles of Atlantic and Mediterranean origin and compared the longline bycatch composition from the western and central Mediterranean with that of bottom trawling from the central and eastern Mediterranean. Recent research has revealed complex distribution patterns of loggerhead turtles within the Mediterranean Sea, with a prevalence of turtles of Atlantic origin in some areas of the western Mediterranean and the prevalence of turtles of Mediterranean origin in the eastern Mediterranean (Carreras et al., 2006, 2011; Maffucci et al., 2006; Clusa et al., *in press*). Furthermore, the existence of turtles of Atlantic origin within the bycatch of bottom trawlers has been recently reported at least in some regions (Casale et al. 2008b). Accordingly, the differences observed by Laurent et al. (1998) could be also attributed to differences in the distribution of the loggerhead turtles of Atlantic and Mediterranean origin and not to contrasting patterns of habitat use. However, turtles of Mediterranean origin are thought to recruit to neritic habitats at a younger age and smaller size than those of Atlantic origin present in the Mediterranean (Casale et al. 2008a; Piovano et al. 2011). This, combined with differences between gears in regards to bycatch rates (Casale 2011) and mortality rates (Carreras et al. 2004, Casale et al. 2004; Alvarez de Quevedo et al. 2013), generate a complex scenario that makes difficult to allocate the impact of bycatch to the populations involved.

To disentangle whether fishing ground or/and fishing gear have an impact on population bycatch we collected samples of loggerheads caught by drifting longlines and bottom trawling/trammel nets within three major Mediterranean regions (eastern mainland Spain, southern Balearic Islands and southern Italy). More specifically, we asses with stable isotope ratios and genetic mitochondrial and nuclear DNA markers: i) whether juvenile loggerhead turtles caught with different gears in a same region consistently differ in their patterns of habitat use and ii) whether juvenile loggerhead turtles caught with different fishing gears in a same region differ in haplotype frequencies and natal origin. With this approach we wish to assess the impact that different fishing gears on different foraging grounds might have on Atlantic and Mediterranean rookeries.

Material and methods

Bycatch sampling

Tissue samples were analysed from 176 juvenile loggerhead turtles incidentally caught from 2002 to 2012 in three major regions of the western and central Mediterranean (Fig. 1): 55 from eastern mainland Spain (SPA), 85 from southern Balearic Islands (BAL) and 36 from southern Italy (SIT). Turtles were classified in two groups for each region, according to bycatch source: 1) turtles showing evidence of direct interaction with drifting longlines (DLL) and 2) turtles showing evidence of direct interaction with trawling and trammel fisheries (TWL).

Muscle samples were collected from dead animals and stored in 95% ethanol whilst blood samples were taken from live animals and stored frozen. Live animals were tagged to avoid pseudoreplication. Only turtles smaller than 69cm curved carapace length (CCL) were analysed in this study because this is the mean minimum size of nesting females in the Mediterranean (Margaritoulis et al., 2003). Turtles of Atlantic origin visiting the Mediterranean become adult at a much larger size (Piovano et al., 2011).

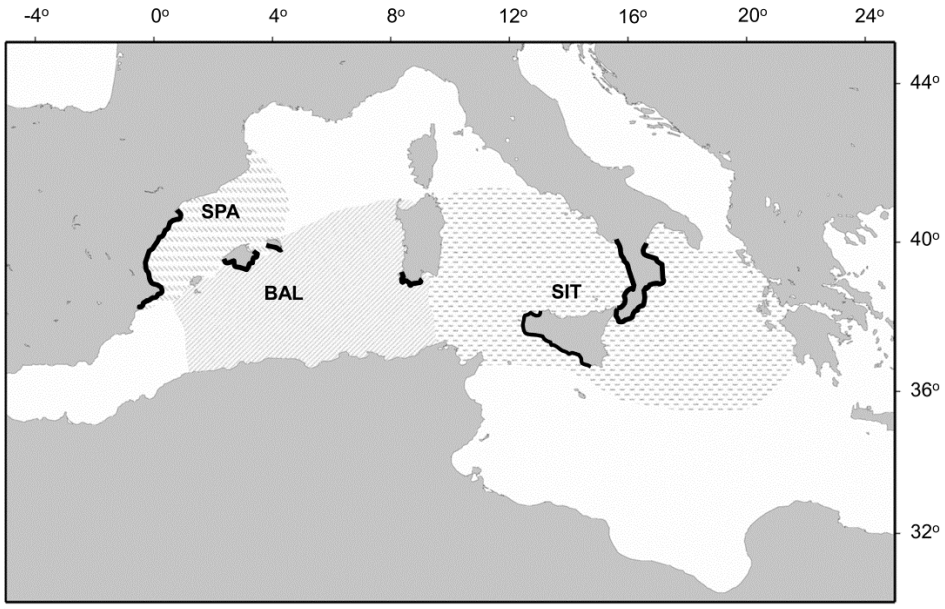

Fig. 1. Study area with shaded zones representing the analysed regions used by juvenile loggerhead turtles: SPA (eastern mainland Spain), BAL (southern Balearic Islands) and SIT (southern Italy). Black lines are the surveyed coastlines.

Stable isotope characterisation of bycatch

Muscle samples from 11 individuals from each fishing gear and region were oven-dried at 60 ºC for 48-72h. They were ground into fine powder and lipids were extracted from all tissues with a chloroform-methanol (2:1) solution. Approximately 0.3 mg of each dry, powdered sample were weighed into tin cups. Subsequent combustion at 1,000 ºC and analysis in a continuous flow isotope ratio mass spectrometer (Flash 112 IRMS Delta C Series EA Thermo Finningan) was undertaken at *Serveis Científics i Tecnològics* at the University of Barcelona. Stable isotope ratios were expressed in the following delta notation (δ) in parts per thousand (‰):

$$\delta X = [(R\text{sample}/R\text{standard})\text{-}1] \times 1000$$

where X is ^{13}C or ^{15}N and R is the corresponding ratio of the heavier to the lighter isotope (i.e. $^{13}C/^{12}C$ or $^{15}N/^{14}N$). International isotope standards of known $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios were used to a precision of 0.2‰: IAEA CH6 ($\delta^{13}C$ = -10.3‰), USGS 40 ($\delta^{13}C$ = -25.8‰) and IAEA CH7 ($\delta^{13}C$ = -31.6‰) for carbon and USGS 40 ($\delta^{15}N$ = -4.3‰), IAEA N1 ($\delta^{15}N$ = +0.8‰), IAEA 600 ($\delta^{15}N$ = +1.0‰) and IAEA N2 ($\delta^{15}N$ = +20.4‰) for nitrogen.

Two-way ANOVA (gear x region) was used to test for statistical differences in the nitrogen and carbon stable isotope ratios of turtles caught by the different fishing gears in the three regions analysed. ANOVAs were undertaken independently for carbon and nitrogen with SPSS v15 (SPSS Inc., 2006).

Genetic characterisation of bycatch

DNA from the 176 samples was extracted with the QIAamp extraction kit (QIAGEN®) to assign the natal origin (Atlantic or Mediterranean) of the bycaught turtles. A fragment of the mtDNA control region was amplified by polymerase chain reaction (PCR) using the primer pairs TCR1-TCR2 (short fragment; Norman et al., 1994) and LCM15382-H950 (long fragment; Abreu-Grobois et al., 2006) following the protocols described in Carreras et al. (2006) and Clusa et al. (2013), respectively. All samples were sequenced in forward and reverse directions to confirm variable sites on both strands of DNA on an ABI 3730 automated DNA Analyser at *Serveis Científics i Tecnològics* at the University of Barcelona.

Sequences were manually aligned with BioEdit v7.1.6 (Hall, 1999) and compared to the short (380bp) and long (815bp) haplotypes previously described for this species; compiled by the Archie Carr Center for Sea Turtle Research of the University of Florida (ACCSTR; http://accstr.ufl.edu).

Individual assignments to natal origin were undertaken following the sequential method described in Revelles et al. (2007c) and Carreras et al. (2011). When an individual carried an mtDNA haplotype exclusive to an Atlantic or Mediterranean nesting area, this individual was assumed to have originated from the corresponding nesting area without any further analysis. Individuals carrying shared haplotypes, orphan haplotypes (not described in any nesting area to date) or individuals that failed to amplify for the long mtDNA fragment were genotyped for seven nuclear DNA (nDNA) microsatellites previously used for *C.caretta*: Cc117, Cm72, Cm84 and Ei8 (FitzSimmons et al., 1995); Cc7 and Cc141 (FitzSimmons et al., 1996); and Ccar176 (Moore and Ball, 2002; modified by Carreras et al., 2007). These individuals were assigned with STRUCTURE v2.1 (Pritchard et al., 2000) considering the baseline developed in Carreras et al. (2007, 2011). Origin assignments were only considered when the probability of belonging to the Atlantic or Mediterranean population was higher than 0.7 for one of the two groups.

Overall differences in origin frequencies (Atlantic or Mediterranean) between fishing gears among regions were assessed with an Analysis of Molecular Variance (AMOVA) considering region as a grouping factor in ARLEQUIN v3.1 (Excoffier et al., 2005). Signification of the pairwise genetic differences (F_{ST}) based on origin frequencies between juveniles from these groups was assessed with an Exact test using the same programme.

Results

Stable isotope characterisation of bycatch

The values of $\delta^{13}C$ did not differ among fishing gears or regions (Two-way ANOVA; model: $F_{5,66} = 2.296$, $p = 0.056$; Fig. 2). Conversely, the $\delta^{15}N$ values were significantly different (Two-way ANOVA; model: $F_{5,66} = 5.496$, $p < 0.001$), because of significant differences among regions ($F_{2,66} = 12.690$, $p < 0.001$; Fig. 2), but not among fishing gears ($F_{1,66} = 0.1258$, $p = 0.724$; Fig. 2). The post-hoc Tukey test

revealed much lower $\delta^{15}N$ values for the turtles caught in the central Mediterranean than for those captured in the western Mediterranean, without differences between eastern mainland Spain and southern Balearic Islands (Fig. 2). Nevertheless, there was a significant interaction term ($F_{1,66} = 5237$, $p < 0.001$) because in southern Italy turtles bycaught with drifting longlines were more enriched in ^{15}N than turtles bycaught with bottom trawling/trammel nets whereas the opposite was true off eastern mainland Spain and southern Balearic Islands.

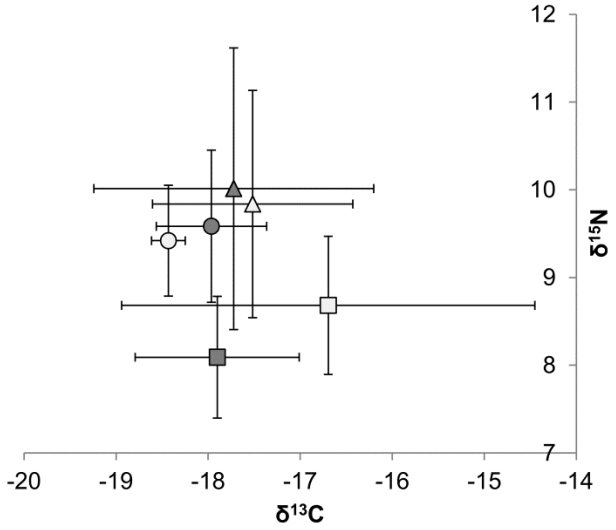

Fig. 2. Stable isotope composition of juvenile loggerhead turtles from the regions analysed. Samples pooled by fishing gear (*light grey*: drifting longline; *dark grey*: trawling/trammel net) and region (*triangles*: eastern mainland Spain; *circles*: southern Balearic Islands; *squares*: southern Italy). Sample size is 11 turtles for each category.

Genetic characterisation of bycatch

Individual assignment through the amplification of mtDNA and nDNA allowed us to assign 153 individuals (87.0%) to their natal populations. Of these, short mtDNA haplotypes allowed assigning the origin of 47 individuals, long mtDNA haplotypes assigned another 12 individuals and 94 individuals were assigned with microsatellite markers. The remaining 23 individuals (13.1%) could not be assigned due to amplification failure or low assigning probabilities.

Individuals of Atlantic and Mediterranean origin were not homogeneously distributed among groups (Fig. 3), but there were no statistically significant differences between fishing gears within each region (AMOVA; percentage of variation = 2.91%, $p = 0.181$).

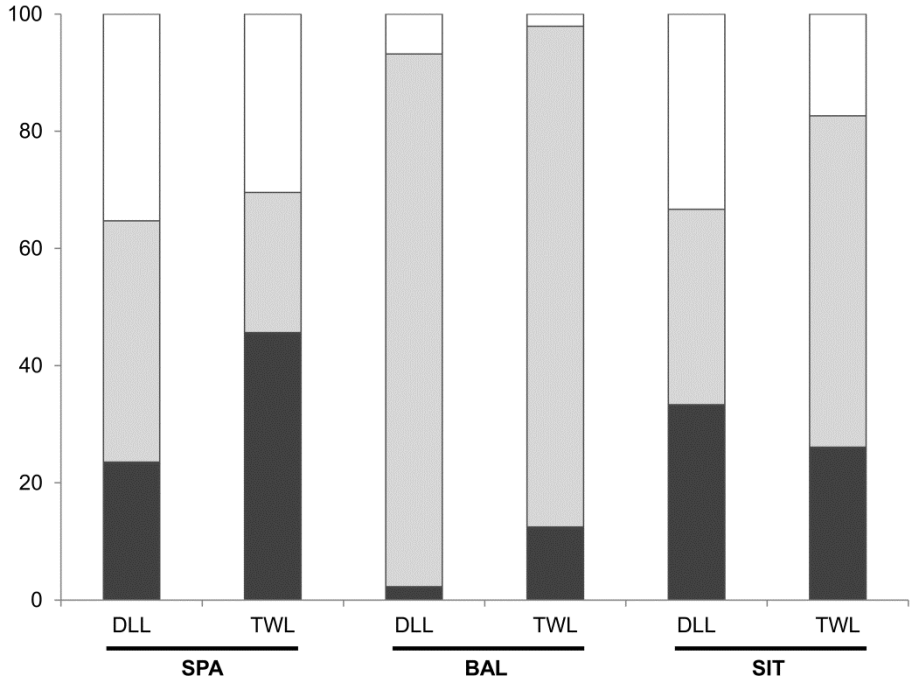

Fig. 3. Individual assignment results showing the percentage of individuals incidentally caught in the studied regions by drifting longlines (DLL) and trawling/trammel nets (TWL) from Mediterranean (*dark grey*) and Atlantic (*light grey*) rookeries. The proportion of unassigned individuals is shown in white. Region acronyms shown in Fig. 1.

Differences among regions were only detected at the edge of statistical significance (AMOVA; percentage of variation = 33.54%, $p = 0.062$) but the Exact test revealed that pairwise differences were significant between southern Balearic Islands and the other two regions (all $p < 0.05$; Table 1). Thus, turtles of Atlantic origin caught in southern Balearic Islands represented 87.8% of the turtle bycatch of drifting longlines and 81.8% of the turtle bycatch of bottom trawling/trammel in the same area.

Table 1
Genetic differentiation (F_{ST}) based on origin frequencies between juvenile loggerhead turtles from the three Mediterranean regions analysed: SPA (eastern mainland Spain), BAL (southern Balearic Islands) and SIT (southern Italy). Fishing gears studied in each region: DLL (drifting longline), TWL (trawling/trammel net).

F_{ST} between fishing gears among regions							
		SPA		BAL		SIT	
		DLL	TWL	DLL	TWL	DLL	TWL
SPA	DLL						
	TWL	0.029					
BAL	DLL	**0.463**	**0.581**				
	TWL	**0.248**	**0.456**	0.017			
SIT	DLL	-0.011	-0.050	**0.636**	**0.466**		
	TWL	-0.067	0.097	**0.345**	**0.162**	0.056	

Bold values show significant pairwise differences ($p < 0.05$).

Contrarily, the proportion of turtles of Atlantic origin was always lower than 65% both for drifting longlines and trammel nets/bottom trawling off eastern mainland Spain and southern Italy (Fig. 3).

Discussion

Our results revealed no genetic or isotopic differences between the loggerhead turtles caught with drifting longlines and bottom trawling/trammel nets within any particular region. Instead, differences were detected both for genetic structuring and stable isotope ratios at a regional level. These results suggest that the differences in the genetic characterisation of longline and trawling bycatch previously reported by Laurent et al. (1998) emerged because fishing gears were regionally nested and hence the gear and region factors were confounded. Conversely, differences vanished as fishing gears from the same region were compared.

The absence of differences in the stable isotope ratios of loggerhead turtles caught by drifting longlines and neritic gears off southern Balearic Islands and southern Italy is highly consistent with previous satellite telemetry data. Juvenile turtles inhabiting the southern Balearic Islands spend most of the time in oceanic waters and only occasionally visit the continental shelf (Cardona et al., 2005; Revelles et al., 2007b). Accordingly, we can assume that a single pool of turtles exists there. The same is true in southern Italy, where juvenile turtles regularly move between oceanic and neritic habitats (Bentivegna 2002; Casale et al., 2007, 2012a). Only off eastern mainland Spain two groups of primarily oceanic and neritic juveniles may exist (Cardona et al., 2009, 2012), although the results here reported suggest more frequent habitat exchanges than previously thought, not only because of similar stable isotope ratios but also because of the large standard deviation values within each group of turtles.

The significantly lower values of $\delta^{15}N$ in turtles from southern Italy are consistent with the eastward pattern of particulate organic matter within the Mediterranean Sea described by Pantoja et al. (2002). This difference, combined with the turnover rate of stable isotopes in muscle (Reich et al., 2008), is congruent with the limited exchange of turtles between adjoining basins on a monthly scale

previously suggested by tagging (Revelles et al., 2008) and satellite telemetry (Bentivegna, 2002; Cardona et al., 2005; Revelles et al., 2007b; Eckert et al., 2008).

Genetic results demonstrated that not only longliners affect Atlantic populations but also neritic fisheries do, as individual assignments highlighted the contribution of juveniles of Atlantic origin in the three regions studied. Even if turtles from Atlantic and Mediterranean populations were caught in both fisheries, differential contribution of each area was found between the turtles caught off eastern mainland Spain/southern Italy and southern Balearic Islands. This difference is driven by the heterogeneous composition of the Mediterranean Sea, with the southern Balearic Islands region presenting a remarkably higher proportion of turtles assigned to Atlantic rookeries in comparison to the other two. This is not surprising as the Algerian basin had been described as a hot-spot for Atlantic juveniles (Laurent et al., 1993; Carreras et al., 2006, 2011; Monzón-Argüello et al., 2009, 2010; Clusa et al. *in press*), with a decreasing relative abundance from the Strait of Gibraltar to the Adriatic Sea (Carreras et al., 2006; Maffucci et al., 2006; Clusa et al. *in press*).

Relatively low proportions of turtles assigned to Mediterranean nesting areas were found in the sample sets of eastern mainland Spain and southern Italy. Mixed stock analyses from the same area estimated a presence of 80-90% of turtles of Mediterranean origin (Carreras et al., 2006) against the 30-40% found in the current and posterior studies (Carreras et al., 2011) through individual assignment. This is likely a consequence of the high proportion of unassigned individuals in both regions and the lower probability of assignment of turtles of Mediterranean origin as compared with those of Atlantic origin. Firstly, haplotype CC-A1.1 is the most frequent haplotype in Atlantic rookeries and is also exclusive from that area (Shamblin et al., 2012). Conversely, haplotype CC-A2.1 might be the most common in Mediterranean rookeries (Clusa et al., 2013; Garofalo et al., 2013) but is also shared with Atlantic rookeries. Thus, assignment power is higher for turtles carrying CC-A1.1 than for turtles carrying CC-A2.1 (the majority in eastern mainland Spain and southern Italy). Secondly, microsatellites correctly assign all the turtles of Atlantic origin, but failed to assign some turtles of Mediterranean origin (Carreras et al. 2011). As a consequence, a higher proportion of unassigned individuals is expected where turtles of Mediterranean origin prevail. This

hypothesis is supported by congruence of direct assignation and mixed stock analysis about the proportion of turtles of Atlantic origin.

Overall, the two approaches demonstrate that the fishing gear used and the foraging ground exploited are not two factors independently affecting turtle populations of contrasting origin as previously suggested (Laurent et al., 1998). Accordingly, comparison between fishing gears from different regions should be avoided to eliminate bias. Laurent et al. (1998) concluded that only juvenile turtles of Mediterranean origin were present in the neritic zone. However, our results found Atlantic juveniles in all neritic fisheries and hence, the presence of Mediterranean turtles in neritic zones should no longer be considered exclusive. This is in accordance with the flexible amphi-stage life cycle proposed by Casale et al. (2008a) as juveniles belonging to different populations were found in both oceanic and neritic zones.

Implications for future conservation

Fisheries interactions can represent an important threat for sea turtle populations (Lewison et al., 2004a; Lewison and Crowder, 2007) and there is a growing need to understand the effects that bycatch have on wild populations. However, this can become a difficult subject as reliable data on fishing effort and bycatch quantification in some areas is irregular or scarce (Davies et al., 2009). Furthermore, proper identification of the populations affected is essential, as populations with contrasting origin and conservation status may share foraging grounds.

The heterogenic presence of Atlantic and Mediterranean populations in Mediterranean bycatch highlights that bycatch impacts will strongly depend on turtle distribution, spatio-temporal overlap with fishing activities and mortality associated with each fishing gear. However, distribution patterns are unknown for some populations foraging in the Mediterranean Sea and estimates on the number of sea turtles killed every year due to direct interaction with fishing gears in the basin are still unclear. Certainly, Casale (2011) estimated that over 132,000 sea turtles are caught every year in the Mediterranean Sea, of which 44,000 (the majority loggerhead turtles) are killed due to fatal interactions with fisheries but

further research is needed to increase the resolution of such estimates at a fine-scale level.

Drifting longlines have been defined as the most threatening of all fishing gears for juvenile loggerhead turtles in the Mediterranean Sea (Gerosa and Casale, 1999; Deflorio et al., 2005), mainly impacting in areas off Spain, Morocco, Tunisia, Italy, Greece and Libya (Laurent, 1990; Jribi et al., 2008; Casale 2011). The estimated bycatch rate is of approximately 60,000 turtles caught per year (Lewison et al., 2004b; Casale 2011) and the mortality rate is approximately 35% (Álvarez de Quevedo et al., 2013). Of all the fishing countries involved, Spain used to be the one with the highest rate of loggerhead bycatch (Camiñas et al., 2006) and the current study reveals that Spain captures a large proportion of turtles of Atlantic origin, thus potentially impacting primarily the populations nesting in the Atlantic. Conversely, the Italian fleet captures a larger proportion of turtles of Mediterranean origin, as this fleet operates primarily in the Ionian Sea (Deflorio et al., 2005; Casale 2011).

In regards to trawling activities, the countries involved are Spain, Italy, Tunisia, Croatia, Greece, Turkey, Egypt and Libya (Laurent, 1996; Lazar and Tvrtkovic, 1995; Oruç, 2001; Cardona et al., 2009; Álvarez de Quevedo et al., 2010; Casale 2011) with a particular incidence in the northern Adriatic Sea (Lazar and Tvrtkovic, 1995; Casale et al., 2004; Lazar et al., 2004). Even if bycatch derived from trawling activities has been considered less relevant than that coming from longlines because of its lower catching rates (39,000 captures per year; Casale 2011), it should not be ignored. Mortality rates associated with trawling and set nets are significantly higher than in longlines (Carreras et al., 2004; Wallace et al., 2013) although rates vary depending on soak times and fishing depth.

In the current study, even if the analysed fishing gears caught turtles of both Atlantic and Mediterranean origin, we can conclude that the impact that fisheries may have on wild populations will differ depending on the foraging grounds used by each of these populations. This is shown by the lack of differentiation between turtles caught with drifting longlines and trawling/trammel nets within each region but the existence of composition differentiation among regions. Accordingly, the type of fishing gear used in each region determines the mortality rate of the caught

turtles and the susceptibility of being caught but does not determine the origin of its individuals. This is tightly related to the fishing grounds used and the overlap with the different turtle populations.

With hatchlings and juvenile turtles using foraging grounds located in faraway areas from their rookeries, insufficiency of focusing all the conservation measures in nesting beaches comes to light. Further research should focus on determining the composition of each foraging ground and the distribution patterns of turtle populations, but also should accurately assess fishing fleet distribution to ensure conservation of all the sea turtle populations inhabiting the Mediterranean Sea.

Acknowledgements

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Global Discussion

GLOBAL DISCUSSION

The results presented in this thesis have revealed deeper structuring in nesting and foraging grounds for loggerhead turtles than previously thought. An earlier colonisation of the Mediterranean has been discovered, fine-scale rookery contributions to Mediterranean foraging grounds have been unveiled, the habitat use of nesting females has been discovered and new management units have been described within the Mediterranean Sea. The effects that foraging ground use have on loggerheads biology has also been approached showing that individual patterns of habitat use may have a remarkable effect on fitness (clutch size) and growth rates. This, in turn, may be driven by surface water circulation patterns of the basin and the trajectory followed by juvenile turtles during their developmental migration. Finally, this thesis has highlighted the importance of regional studies to understand the consequences of fisheries bycatch as the actual impact will depend on the origin of the turtles incidentally caught.

THE STRUCTURING OF LOGGERHEAD ROOKERIES IN THE MEDITERRANEAN SEA IS MARKER DEPENDENT

The genetic structure of loggerhead turtles nesting in the Mediterranean Sea had been widely studied before (Encalada et al. 1998; Laurent et al. 1998; Carreras et al. 2007; Garofalo et al. 2009; Yilmaz et al. 2011; Saied et al. 2012). Management units had already been defined by Carreras et al. (2007) and Yilmaz et al. (2011), but the presence of larger sample sets from unsampled (Libya) or previously poorly sampled rookeries (Lebanon) in this thesis allowed a truly global analysis of the whole basin in *Chapter 1.1* and *Chapter 1.2*.

Molecular genetics is certainly a powerful tool for conservation but the results here presented underline a strong reliance on the type and number of markers used. Four management units have been identified with the analysis of long fragments of mtDNA in *Chapter 1.1*: Dalyan and Dalaman (Turkey), Libya, Calabria (Italy), and the rest of eastern rookeries (Israel, Lebanon, Cyprus, eastern Turkey, middle Turkey, western Turkey, Crete and western Greece).

The use of longer mtDNA fragments was crucial as the short haplotype CC-A2 was split in long haplotypes, some of them exclusive to a specific rookery (CC-A2.8 is only present in Crete and CC-A2.9 only in Israel), which allowed to unveil deeper structuring in the basin.

In the case for nDNA, the number of microsatellite markers used in *Chapter 1.2* also upgraded what was previously known. Carreras et al. (2007) used seven markers and although some structuring was detected within the Mediterranean Sea, high degrees of male-mediated gene flow were suggested. Similarly, previous studies with five or less markers failed to detect any structuring in loggerhead populations in the Atlantic (Bowen et al. 2005). However, by using 15 microsatellite markers (*Chapter 1.2*) a much deeper structuring was detected. Microsatellite markers presented a higher power of fine-scale differentiation than the single mtDNA marker and hence allowed the detection of five previously unknown units: Libya and Cyprus, Israel, Lebanon, western Turkey and Greece. The improved performance of genetic analyses based on the use of multiple markers was also highlighted in *Chapter 3.1* and *4.1*, where assignment power to a natal origin remarkably increased with an increase in the number of markers used. Accordingly, only eight turtles could be directly assigned in *Chapter 3.1* with long fragments of mtDNA whilst this number increased to 65 when combined with seven microsatellite markers. Thus, the results presented in the present thesis corroborate the need for larger sets of markers in studies on loggerhead population structure.

POPULATION GENETIC DIFFERENTIATION AMONG LOGGERHEAD ROOKERIES IN THE MEDITERRANEAN SEA AND REPRODUCTIVE BEHAVIOUR

By combining the results obtained for mtDNA and nDNA, a strong philopatry not only for females but also for males could be detected in the Mediterranean in *Chapter 1.2*. Thus, the use of genetics unveiled that males are remarkably philopatric and that isolation by distance between rookeries exists due to highly-restricted gene flow. There is increasing evidence that adult male loggerhead turtles behave similarly to adult females and use the same foraging grounds (Hatase et al. 2002; Schofield et al. 2009, 2013; Arendt et al. 2012a,b; Casale et al. 2013; Varo-Cruz et al. 2013). However, even if the existence of male

philopatry was generally agreed (Miller 1997), it was still unclear whether loggerhead mating occurred in foraging grounds, in breeding grounds close to nesting beaches or en-route to nesting areas. The levels of genetic isolation among rookeries described in this thesis suggest that mating would be occurring in breeding grounds close to nesting beaches in the majority of Mediterranean rookeries even if some previous mating in foraging grounds could also be occurring (e.g. females nesting in Cyprus but mating in Libyan grounds). This similarity in the behaviour of both sexes is not surprising as philopatry has been described as a successful strategy for female sea turtles to ensure viability of nesting beaches but also as a convenient behaviour for males as philopatry increases the chances of finding available females to mate with (Schofield et al. 2009).

Even if male-mediated gene flow was generally restricted and both sexes were philopatric, sex-biased behaviour was observed in Greece. Females showed strong philopatry and fine-scale fidelity (Crete and western Greece were differentiated with mtDNA) whilst males facilitated gene flow among Greek rookeries; hence showing no differentiation with nDNA.

THE IMPORTANCE OF WATER CIRCULATION IN FORAGING GROUND STRUCTURING

Water circulation patterns have been traditionally underlined as important physical factors affecting hatchling and early juvenile dispersal (Carr and Meylan 1980; Bolten et al. 1992) due to the positive buoyancy and limited swimming abilities of small individuals (Milsom 1975). The results presented in *Chapter 2.1* showed that turtles do not distribute homogeneously within the Mediterranean Sea and that differences exist not only in the distribution of juveniles from Atlantic and Mediterranean nesting areas but also among those from Mediterranean rookeries. This heterogeneous distribution of juvenile turtles was consistent with the main water current patterns both at a large and fine scale.

Mixed stock analyses (MSAs) showed that juveniles of Atlantic origin found within the Mediterranean basin were mainly from North-American rookeries, which would be expected considering that Florida hosts the world's largest nesting aggregation of this species and that the Gulf Stream System joins the American coast with Europe. However, almost no juveniles from Cape Verde were found in

the Mediterranean and this might seem surprising as Cape Verde hosts the second largest nesting aggregation (Marco et al. 2012) with 14,000 nests laid every year on its beaches (Laurent et al. 1999). Even if hatchlings and juveniles from Cape Verde also inhabit the north Atlantic, these are not found in the Mediterranean Sea because the archipelago is connected with the American continent by the North Equatorial Current rather than with the Mediterranean Sea (Mansfield and Putman 2013), underlining the relevance of currents in juvenile distribution.

At a fine-scale level, MSAs results revealed the composition of some of the largest Mediterranean foraging grounds and also corroborated the importance of currents on juvenile distribution. Accordingly, the prevalence in the Adriatic Sea of turtles from western Greece might be explained by the pattern of water entering the Adriatic Sea having previously flowed past the coast of western Greece (Millot and Taupier-Letage 2004). Likewise, the prevalence of turtles from Libyan beaches in the Ionian Sea may be linked to the mesoscale eddies present in the Ionian Sea (Robinson et al. 2001; Hamad et al. 2006; Hays et al. 2010), which might trap the hatchlings and juveniles swimming off Libya in the sub-basin and prevent dispersal across the eastern Mediterranean.

These results are in accordance with the fact that young juveniles are distributed depending on current patterns on which they passively drift. However, the individuals sampled in *Chapter 2.1* ranged from 30 to 69cm CCL and hence would be capable of dispersing independently of prevailing currents within the Mediterranean Sea (except in the Strait of Gibraltar, the Alboran Sea and the Algerian Stream; Revelles et al. 2007c). In accordance, together with the fact that the genetic structuring in foraging grounds is highly consistent with the distribution of water masses and the pattern of surface currents, other mechanisms might exist that perpetuate hatchling and early juvenile distributions to older stages.

Recent studies have pointed out that adult distribution might be linked to current patterns as a result of habitat imprinting. There is increasing evidence that young turtles become imprinted by the habitats they visit during their developmental migration (determined by currents), which in turn determine the habitats where they will settle and forage as adults (Hatase et al. 2002; Hays et al. 2010; Fossette et al. 2010; Eder et al. 2012). Turtles of Mediterranean origin begin

settlement at approximately 40cm CCL (Casale et al. 2008a), which suggests that the genetic structuring reported in *Chapter 2.1* might emerge from such a process as imprinting. This however, might not apply to turtles of Atlantic origin, as their natal rookeries are more than 6,000km away from the Mediterranean foraging grounds they used as juveniles. This results in a remarkable trade-off between philopatry and habitat knowledge, that finally leads them to leave the Mediterranean once they are large enough to overcome the currents in the Alboran Sea and the Strait of Gibraltar and settle in the western Atlantic (Bowen et al. 2005). Accordingly, adult turtles of Atlantic origin are highly scarce in the Mediterranean Sea.

Water currents and the consequences of distribution and habitat use

Not only the results here presented supported the link between water circulation patterns and turtle distribution but also allowed a deeper analysis of the consequences that this variability in turtle distribution might have on Atlantic and Mediterranean populations. The combination of skeletochronology and genetic analyses in *Chapter 3.1* revealed different growth rates between turtles of contrasting natal origin. Thus, turtles of Atlantic origin feeding in the Mediterranean Sea presented lower growth rates not only in comparison to turtles of Mediterranean origin but also in comparison to turtles of Atlantic origin that do not enter the Mediterranean Sea. This could be explained by habitat use and productivity of the foraging grounds used. Turtles of Atlantic origin are usually oceanic in the Mediterranean Sea (Carreras et al. 2006, 2011) whilst turtles of Mediterranean origin of the same size may be already settled in neritic zones (more productive than oceanic zones; Bosc et al. 2004). Accordingly, as turtles of Mediterranean origin recruit earlier to more productive, neritic habitats, these are expected to grow faster. Differences in productivity could also explain why turtles of Atlantic origin inhabiting the Mediterranean Sea grow slower than those that do not enter the basin. Because the Mediterranean Sea is highly oligotrophic in comparison to Atlantic neritic waters (Longhurst 1998), this could be affecting the growth rates and time of residence of Atlantic turtles that enter the basin. This, in turn, may have important consequences as turtles foraging in the Mediterranean face high rates of bycatch which might have a remarkable negative impact on Atlantic populations (see below).

In regards to the distribution of turtles of Mediterranean origin within the Mediterranean Sea, stable isotope analyses allowed to track foraging grounds for nesting females through the analysis of dead hatchlings in *Chapter 3.2*. Stable isotope analyses identified the southern Ionian Sea as the major female foraging ground for most of the studied rookeries (even if presenting some of the lowest productive patches of the basin; Bosc et al. 2004). Conversely, the highly productive Adriatic/northern Ionian Sea was mainly used by turtles from Greek rookeries (as also in Zbinden et al. 2011) and rarely from elsewhere (as in Margaritoulis and Rees 2011; Patel et al. 2012; Hochscheid et al. 2012). The limited use of the Adriatic/northern Ionian Sea by females from rookeries other than Zakynthos and Lakonikos is intriguing because the Adriatic Sea is indeed the most productive area of the western Mediterranean Sea and females foraging there are larger and lay more eggs than females foraging elsewhere (Margaritoulis et al. 2003). If turtle distribution was only dependent on a balance between food availability and distance to rookery, turtles from the easternmost rookeries would be expected to feed in the Adriatic/northern Ionian Sea and in the southern Ionian Sea in equal proportions. However, this seems to be the case only in western Greece. The explanation to this particular distribution might also lie in the hypothesis of habitat imprinting.

Differences among populations distribution were found to be highly congruent with the current patterns described for the Mediterranean Sea. Accordingly, the majority of turtles might feed in the southern Ionian Sea, even if less productive, because the Adriatic Sea is in a peripheral position within the main surface currents of the basin (Hamad et al. 2006); hence unknown for most of the turtles from the easternmost rookeries. Contrarily, the Adriatic Sea is easily accessible for hatchlings swimming off the Greek rookeries as these encounter a water current bifurcation, with one current flowing northwards into the Adriatic Sea and another one flowing south-eastwards (Hays et al. 2010). Thus, half of the adult turtles departing from western Greece migrate to the Ionian Sea after nesting and the other half to the Adriatic Sea (Zbinden et al. 2011; Schofield et al. 2013).

Importantly, if the habitat imprinting hypothesis is true, individual tracks followed during the early stages as passive drifters might explain the observed distribution variability between individuals from a same population seen in *Chapter*

3.2. The reported results suggest that differences between rookeries are not shown at population level but at individual level, with turtles that forage in highly productive foraging grounds nesting at the same rookeries as turtles foraging in less productive grounds. Depending on the currents encountered and the stochasticity of natural phenomena, hatchlings from the same rookery might follow different developmental migrations (Wyneken et al. 2008; Hays et al. 2010; Putman et al. 2012a). This variability results in different habitat patches visited during this period (McClellan and Read 2007; McClellan et al. 2010) and may influence decisions at the time of recruitment following individual knowledge on habitat heterogeneity.

The distribution patterns described in *Chapter 3.2* (and also in *Chapter 2.1*) thus demonstrate the existence of a strong link between the foraging grounds used by turtles and the location of their rookeries. This has consequences on the reproductive output since a strong correlation was found between average clutch size and the stable isotope ratios females which, in turn, depend on the foraging ground used. Accordingly, females foraging in the Adriatic/northern Ionian Sea had larger clutch sizes than females from the same rookery that forage in less productive areas such as the southern Ionian Sea. In addition, the foraging grounds used not only may have an effect on fitness of loggerhead populations nesting the Mediterranean Sea but also can alter their probability of survival.

LOGGERHEAD TURTLES AND MEDITERRANEAN FISHERIES

Bycatch rates are highly variable within the basin (Casale 2011) and the impact of fisheries interactions on foraging populations will depend on the overlap between fishing and turtle distribution but also on the birth rate of the populations involved (Wallace et al. 2008, 2013). The last chapter of the current thesis (*Chapter 4.1*) allowed a deeper look at the population make-up of turtle bycatch in the Mediterranean Sea. By analysing stable isotope signatures and genetic composition through individual assignments, the habitat use and the natal origin of bycaught turtles was revealed.

The two approaches demonstrated that oceanic (drifting longlines) and neritic (bottom trawling and set nets) fishing gears used within a same region capture turtles from the same populations. Thus, differences in bycatch composition

may exist among regions but not between fishing gears within each region. These results suggest that the differences in the genetic characterisation of longline and trawling bycatch previously reported by Laurent et al. (1998) emerged because fishing gears were regionally nested and hence the gear and region factors were confounded. Conversely, because differences vanished as fishing gears from the same region were compared in *Chapter 4.1*, comparison between fishing gears from different regions should be avoided to eliminate bias in future studies.

The bycatch composition found in the current thesis shows that fisheries impacts are highly dependent on the overlap between fishing grounds and loggerhead foraging grounds. Thus, turtle distributions, led by currents and the knowledge acquired during developmental migrations (as seen in *Chapter 2.1* and *3.2*), may determine the susceptibility to bycatch depending on the foraging area used. Accordingly, turtles of Atlantic origin represented a remarkably large proportion of turtle bycatch in southern Balearic Islands because the Algerian basin has been described as a hot-spot for Atlantic juveniles in *Chapter 2.1* and in previous studies (Laurent et al., 1993; Carreras et al., 2006, 2011; Monzón-Argüello et al., 2009, 2010) due to specific patterns of water mass circulation (Revelles et al. 2007c). Likewise, the proportion of turtles of Atlantic origin in turtle bycatch decreased in the other studied areas as the relative abundance of Atlantic individuals declines downwards the main cyclonic current from the Strait of Gibraltar to the Adriatic Sea (Carreras et al. 2006; Maffucci et al. 2006).

The fact that oceanic and neritic fishing gears are accidentally catching juveniles of both Atlantic and Mediterranean origin also suggests that not only turtles of Mediterranean origin occur in Mediterranean neritic habitats; opposite to what was previously thought (Laurent et al. 1998). Accordingly, with Atlantic individuals being caught by both oceanic and neritic fisheries, the impact that Mediterranean fisheries might have on Atlantic populations may be higher than previously thought. The number of females nesting in southern Florida has declined 43% since 1998 although the numbers of green and leatherback turtles nesting in the same beaches have increased (Witherington et al. 2009). As a consequence, because the reason for the decline is not to be found in the nesting beaches, Mediterranean bycatch (among other threats affecting turtles in other foraging areas) might account for part of this remarkable decline. The western Mediterranean is the area

with the highest longline fishing pressure (Casale 2011) and this is where the highest Atlantic contributions of loggerhead turtles are found (*Chapter 2.1* and *Chapter 4.1*). As fishing effort in the western Mediterranean peaked in the early 1990 (Farrugio et al. 1993), we believe the steep decline observed since 1998 in the number of females nesting in Florida could be due in part to the high rates of incidental bycatch in the western Mediterranean.

In addition, turtles of Atlantic origin foraging in the Mediterranean Sea are expected to move to neritic habitats in the north-western Atlantic at a much higher age than those that remain in Atlantic waters, as seen in *Chapter 3.1*, due to their incapability to swim off the basin due to strong currents at the Strait of Gibraltar (Revelles et al. 2007d). Thus, loggerhead turtles of Atlantic origin entering the Mediterranean Sea are exposed to high levels of incidental mortality for a much longer time (Álvarez de Quevedo et al. 2013), potentially increasing bycatch negative effects on the population. However, the relevance of this mortality to the American management units will depend on the proportion of loggerhead turtles that enter the Mediterranean Sea and that is still unknown to date.

In regards to the impact that fisheries might have on Mediterranean populations, fine-scale MSA results in *Chapter 2.1* suggested that will depend on the specific contribution of each nesting population to the shared foraging grounds and the bycatch rate in each foraging ground. Thus, the western Mediterranean might not only be a threat for populations nesting in North-America but also in Libya and most particularly in Misurata. Likewise, bycatch in the Adriatic Sea (consisting of mainly trawling; Casale 2011) might primarily affect the population nesting in western Greece, whereas bycatch in the Levantine Sea might affect primarily the populations nesting in Turkey, Lebanon and Israel. Even if bycatch might have a negative impact on these nesting populations, the magnitude of this impact might differ as fishing effort, soak times, depth of sets and type of gear may vary among regions. Even if turtles of both natal origins are caught by oceanic and neritic fishing gears, all these factors have their own catchability and mortality rates associated (Lewison et al. 2004a) and hence the impact that fisheries might have on nesting populations may vary depending on the fisheries nature. Moreover, population size is also a relevant factor determining fisheries impacts. Stable isotope results presented in *Chapter 3.2* showed that clutch size is highly correlated to

foraging ground productivity. In rookeries where a large proportion of females feed in low productive grounds, such as Israel or Cyprus, clutch sizes are small and hence the demographic relevance of bycatch may be larger for these rookeries. Nonetheless, similar rates of bycatch might not be detrimental for other populations (e.g. Greece). Consequently, future studies should attempt to calculate the mortality rates of turtles from each management unit at each fishing ground and model their demographic consequences on each management unit.

In order to reduce bycatch in the Mediterranean Sea, fishing fleets should take specific precautions to try to reduce accidental catches but also local governments should implement stronger legal regulations to control and minimise bycatch impacts. Some bycatch reduction measures include modification of gear, bait types, set locations and timing and depth of sets (Gilman et al. 2006). In the case for drifting longline fisheries, switching the traditionally used J hooks to larger, circle hooks decreases the probability of ingestion whilst not affecting fishing catch rates (Watson et al. 2005; Swimmer et al. 2011). Bait type may also be relevant in this context as the use of squid bait implies higher bycatch probabilities than fish bait due to the elasticity and strength of squid tissues (Gilman et al. 2006, 2010). Because of these characteristics, the use of squid as bait pushes individual turtles to bite the bait several times while increasing the cumulative risk of injury and ingestion (Gilman et al. 2006); something that does not occur with the softer fish bait. The depth in which the hooks are set is also relevant in the catchability and mortality associated with drifting longlines. Thus, longlines set less than 50m deep have higher bycatch rates than deeper sets as turtles spend the majority of their time within the first 40m (Polovina et al. 2003). Finally, hauling on board all bycaught turtles and removing the hooks would help to dramatically reduce post-release mortality rates (Álvarez de Quevedo et al. 2013).

In regards to bottom trawling in neritic zones, the most renowned bycatch mitigation measure is the use of turtle excluder devices (TEDs), which allow turtles to escape from trawl nets in case of entrapment. Because mortality associated with bottom trawling is usually caused by suffocation, although it highly depends on soak times (Robins-Troeger et al. 1995), TEDs are successful management tools as turtles can escape the net through a window and emerge to the surface to breath. TEDs have been widely used in U.S. waters by law and, with full compliance and

proper implementation, turtle bycatch and mortality dramatically decreased over the past two decades in the area (Finkbeiner et al. 2011). However, TEDs may result in a serious reduction of fish landings as Mediterranean bottom trawlers target large species. Accordingly, limiting the tow duration rather than using TEDs might be a better regulation in some regions of the basin (Álvarez de Quevedo et al. 2010).

Even if proven successful in other areas over the globe, these mitigation measures are still not taken into consideration in many Mediterranean countries. If both the Atlantic and Mediterranean loggerhead turtle populations are to be preserved in the Mediterranean Sea, strong implementation should be undertaken at a legal and social level with the aim to ensure sustainable fishing. Reducing fleet numbers, restricting fishing seasons, decreasing soak times or promoting the use of turtle-friendly bait/hooks are highly recommended in the Mediterranean to decrease turtle bycatch. Only with this and a deeper knowledge on turtle distribution patterns and habitat use will turtle bycatch be sufficiently reduced in the basin. However, the need to also protect and enhance artisanal fisheries at the same time must not be forgotten if regulations are to be properly followed.

POTENTIAL CLIMATE CHANGE EFFECTS IN THE MEDITERRANEAN SEA

Global warming and its collateral effects have been a matter of concern during the past decade not only for the impacts that it might have on the planet's wildlife but on humankind itself. Air temperature has been predicted to increase 1.1-2.9 ºC by 2099 (IPCC 2007) and, with it, sea temperature and sand temperature of nesting beaches for loggerhead turtles will also increase.

Marine turtles have adapted to previous climate fluctuations (Dutton et al. 1999; Encalada et al. 1996; Reece et al. 2005) and genetic results in *Chapter 1.1* suggested that this has also been the case in the Mediterranean Sea, where loggerhead turtles could have survived Pleistocenic glacial eras in warm refugia off the North-African coast. However, the speed of the climate fluctuation and the levels of human pressure have remarkably changed since. Because turtle nesting is highly dependent on temperature, some loggerhead populations would be expected to expand northwards as temperature increases, colonising areas currently too cold

for reproduction. However, most of the coastline in the northern shore of the Mediterranean Sea has been intensely developed by the tourism industry and few beaches remain suitable for turtle nesting nowadays (Mazaris et al. 2009). Furthermore, total beach surface might decrease as the sea level rises and buildings, roads and other infrastructures will impede beaches to move inland. In this context, competition between the tourism industry and nesting loggerhead turtles is expected to increase, with uncertain results for loggerhead turtles.

With an increase in sand temperature, sex ratios might be also highly affected because of the temperature-dependant sexual determination of this species (Hawkes et al. 2009). Higher temperatures could lead to large shifts in female-biased sex ratios and, with a decrease in male production, a loss of genetic differentiation among rookeries could occur as suggested to happen in Cyprus in *Chapter 1.2*. As the number of males decreases, opportunistic mating in foraging grounds might homogenise the genetic diversity of certain populations. The relevance of these effects will be potentially stronger in small nesting populations such as those present in the Levantine rookeries (Israel and Lebanon; Margaritoulis et al. 2003); populations that have been already severely reduced due to direct exploitation during the early 1920s (Sella 1982).

Apart from these impacts, climate change might also strongly affect loggerhead populations through a remarkable variation of water circulation patterns. As suggested throughout the current thesis, loggerhead distribution and consequently fitness and survival probabilities are tightly linked to current patterns and a warming climate might lead to changes in global wind patterns, large-scale ocean-atmosphere patterns and the strength, direction and behaviour of major current systems (Hoegh-Guldberg 2011). Accordingly, the biology, migratory behaviour and reproductive output of Mediterranean (and also Atlantic) loggerheads might be severely affected by these changes. However, the low predictability of these environmental changes and the limited knowledge on how turtles respond to permanent variations in water currents make it impossible to predict the effects of such global warming impacts.

FUTURE RESEARCH DIRECTIONS

The current thesis has revealed previously unknown details on loggerhead populations inhabiting the Mediterranean Sea but further research is still needed to bring light to some of the hypotheses drawn.

In regards to the genetic structuring of loggerhead populations, the results here presented have highlighted the need to use large numbers of markers in future studies. Even if the use of microsatellite markers in sea turtle research has been slowly increasing during this decade (Carreras et al. 2007, 2011; Monzón-Argüello et al. 2008; Garofalo et al. 2013), there is still an over-dominance of studies that only focus on mtDNA analyses. In addition, the majority of them use short fragments of mtDNA and thus limit the resolution of such studies as seen in *Chapter 1.1*. Accordingly, the re-analysis of turtles from certain areas with primers that amplify for longer fragments would be highly recommendable. This, together with the use of multiple microsatellite markers, would help increase the resolution of genetic differentiation not only among rookeries but also among foraging grounds. In addition, genetic characterisation of unsampled rookeries with long fragments of mtDNA could also potentially allow the discovery of new exclusive haplotypes and thus improve MSAs by decreasing the number of orphan haplotypes (i.e. found in foraging grounds but not described in any nesting area to date). Only by having a complete knowledge on the genetic structuring of nesting areas will individual assignments to natal origin and MSAs be successful.

Regarding population structure at sea, tracking hatchlings as they set off Mediterranean rookeries could directly prove the relationship between water circulation patterns and hatchlings/early juveniles distribution in the basin. In regards to the hypothesis of foraging ground settlement based on previous knowledge on habitat heterogeneity presented in *Chapter 2.1* and *Chapter 3.2*, this could also be corroborated with hatchling/juvenile turtle tracking by recapturing individuals in older stages and comparing tracked migrations followed during early stages with their current distributions. From a conservation point of view, this could be used to predict future distributions of specific populations and design specific management plans; of important relevance if current patterns are about to change due to global warming as seen above.

Even if all these questions were answered, conservation would still not be possible without a proper assessment of direct human impacts affecting loggerhead turtles in the Mediterranean Sea. Reliable estimates on catch rates, fleet distribution, number of hooks used and soak times are still scarce in the majority of Mediterranean countries and in some are even inexistent (Casale and Margaritoulis 2010). As the impact that fisheries might have on populations of contrasting origin depends on the distribution of these populations and the overlap between fishing and foraging grounds (*Chapter 4.1*), deeper control and regulation should be implemented from governing parties to allow further understanding of these interactions.

Moreover, future research should also focus on modelling the effects of bycatch mortality on the dynamics of loggerhead populations. To do so, variables such as mortality associated to each fishing gear, natural mortality rates, duration and abundance of each life-cycle stage and their distribution must be previously unveiled. This is already known for some populations and fishing gears used in the Mediterranean (Casale et al. 2007; Casale et al. 2008a; Álvarez de Quevedo et al. 2013) but to unveil whether Mediterranean fisheries are causing the abrupt decline recorded in nesting areas of northern America, the number of turtles entering the basin every year must be defined. This, together with the estimated Atlantic contributions to different Mediterranean foraging grounds found in *Chapter 2.1* and the bycatch rates previously published (Casale 2011) will allow reliable predictions by using population models. The same should be also applied to Mediterranean populations, much less abundant than the Atlantic not only because of human impacts affecting them but also because of the presence of smaller nesting populations in the basin.

Overall, because this is a highly migratory species, different threats can affect loggerhead populations at very distant locations, each one of these with its own threats associated. In order to obtain a reliable scenario of the whole Mediterranean, international cooperation is crucial and thus, the development of this thesis has been tightly linked to numerous international co-authors that collaborated in sampling and discussing the results. Only by doing so, future conservation plans at a local and larger scale may succeed and the survival of this fascinating species ensured.

CONCLUSIONS

CONCLUSIONS

- Loggerhead turtles colonised the Mediterranean Sea from the Atlantic ca. 65,000 years ago (20,000-200,000), during the Pleistocene, and survived cold phases in warm refugia off the North-African coast.

- The current genetic structure of *Caretta caretta* rookeries in the Mediterranean Sea reflects colonisation processes and is the result of local extinctions during Pleistocenic glaciations and posterior re-colonisations from warm refugia.

- Male-mediated gene flow is highly restricted among the majority of Mediterranean nesting areas, which reflects strong philopatry for both males and females in the basin.

- Mating seems to be occurring close to nesting areas although sporadic mating in foraging grounds or en-route to breeding areas could also occur. The traceability of this opportunistic mating might depend on sperm competition in breeding areas, influenced by the number of males present.

- Distribution of juvenile turtles of Atlantic and Mediterranean origin is not homogeneous within the Mediterranean basin: there is a higher proportion of juvenile individuals of Atlantic origin in the Algerian basin, from Libya in central and western Mediterranean foraging grounds, from western Greece in the Adriatic Sea and from the eastern Mediterranean rookeries (Turkey, Lebanon and Israel) in the southern Levantine Sea.

- The results presented are congruent with the hypothesis that young turtles become imprinted by the habitats they visit during their developmental migration (determined by currents), which in turn determine the habitats where they will settle as adults. Accordingly, the strong genetic structuring found in foraging grounds reflects the main currents present in the Mediterranean basin.

- Differences in distribution and habitat use between turtles of contrasting origin lead to differences in growth rates and reproductive output.

- Turtles of Atlantic origin present lower growth rates not only in comparison to turtles of Mediterranean origin but also in comparison to turtles of Atlantic origin that do not enter the Mediterranean Sea. Differences in habitat productivity would explain such intra- and inter-population variation.

- Among turtles of Mediterranean origin, there is a strong correlation between productivity of foraging grounds used and reproductive output (clutch size). Turtles feeding in highly productive foraging grounds such as the Adriatic/northern Ionian Sea present larger clutch sizes.

- Even if the Adriatic/northern Ionian Sea is the most productive area, it is only used by mainly Greek turtles. Contrarily, the southern Ionian Sea (less productive) is the major female foraging ground for most of the studied rookeries. The explanation to this particular distribution might also lie in the hypothesis of habitat imprinting during developmental migrations.

- Stable isotope signatures and genetic characterisation of bycaught turtles demonstrated that the fishing gear used and the foraging grounds exploited are not two factors independently affecting turtles of contrasting origin. Thus, differences in bycatch composition are found among regions but not between fishing gears within each region.

- Turtles of Atlantic and Mediterranean origin were caught in both neritic and oceanic zones. This reveals that neritic zones are not exclusively used by turtles of the Mediterranean population, as traditionally thought.

- The heterogenic presence of Atlantic and Mediterranean populations in Mediterranean bycatch highlights that bycatch impacts will strongly depend on turtle distribution, habitat use, spatio-temporal overlap with fishing activities and mortality associated with each fishing gear.

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APPENDICES

Appendix A1

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Mitochondrial DNA reveals Pleistocenic colonisation of the Mediterranean by loggerhead turtles (*Caretta caretta*)

Marcel Clusa [a,*], Carlos Carreras [a], Marta Pascual [b], Andreas Demetropoulos [c], Dimitris Margaritoulis [d], Alan F. Rees [d], Abdulmaula A. Hamza [e], Mona Khalil [f], Monica Aureggi [g], Yaniv Levy [h], Ogüz Türkozan [i], Adolfo Marco [j], Alex Aguilar [a], Luis Cardona [a]

[a] Department of Animal Biology and IRBio, Faculty of Biology, University of Barcelona, Av. Diagonal 643, E-08028 Barcelona, Spain
[b] Department of Genetics and IRBio, Faculty of Biology, University of Barcelona, Av. Diagonal 643, E-08028 Barcelona, Spain
[c] Cyprus Wildlife Society, Emmanuel Xanthou 11, P.O. Box 24281, 1703 Nicosia, Cyprus
[d] ARCHELON, the Sea Turtle Protection Society of Greece, Solomou 57, GR-10432 Athens, Greece
[e] Libyan Seaturtle Program, Environment General Authority, Alfateh University, P.O. Box 13793, Tripoli, Libya
[f] MEDASSET, P.O. Box 19, Tyre, Lebanon
[g] Naucrates, Via Corbetta 11, 22063 Cantù, CO, Italy
[h] The Israel Sea Turtle Rescue Centre, Nature Parks Authority, Mevoot Yam, Mikhmoret 40297, Israel
[i] Adnan Menderes University, Faculty of Science and Arts, Department of Biology, Aydin, Turkey
[j] Biological Station of Doñana-CSIC, Apdo. 1056, E-41013 Seville, Spain

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ABSTRACT

As the loggerhead turtle (*Caretta caretta*) is a philopatric species with a strong genetic structure, the analysis of mtDNA can be used to track evolutionary and colonisation events. In this study we use a genetic approach to understand the population structure of *C. caretta* in the Mediterranean Sea and to test whether loggerheads could have colonised the Mediterranean during the Pleistocene and survived the cold phases in warm refugia. We amplified a long mtDNA D-loop fragment (815 bp) from 168 dead hatchlings sampled from a selection of rookeries in the Eastern Mediterranean: Libya, Israel, Lebanon, Cyprus and Greece. Previously published data from Turkey and Calabria (Southern Italy) were also included in the analyses. The population nesting in Libya emerged as the oldest population in the Mediterranean, dating from the Pleistocene ca. 65,000 years ago (20,000–200,000). This reveals that the Libyan population might have settled in the Mediterranean basin before the end of the last glacial period. The remaining nesting sites, except Calabria, were subsequently colonised as the population expanded. The populations nesting in Eastern Turkey and Western Greece settled ca. 30,000 years ago (10,000–100,000), whereas the remaining populations originated as a result of a more recent Holocenic expansion. As Calabria presented a unique Atlantic haplotype, found nowhere else in the Mediterranean, we consider this nesting site as the result of an independent colonisation event from the Atlantic and not the recent spread of Mediterranean populations. This reveals that the current genetic structure of *C. caretta* rookeries in the Mediterranean would be the result of at least two colonisation events from the Atlantic, the oldest one in Libya and a most recent in Calabria, combined with local extinctions during Pleistocenic glaciations and re-colonisations from glacial refugia in Libya, Eastern Turkey and Western Greece.

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1. Introduction

The Pleistocene extended from 2.5 mya to 12 kya and was characterised by multiple glacial–interglacial cycles that caused dramatic changes in the distribution of organisms (Taberlet et al., 1998; Wilson and Eigenmann Veraguth, 2010). As ice sheets spread during glacial cycles, species often retreated towards the Equator although some populations survived in areas that acted as refugia (Haffer, 1982). Furthermore, a dryer climate and lower sea levels during glacial periods caused dramatic changes in species distribution even in areas that were not covered by ice (Hewitt, 1996; Maggs et al., 2008). When ice retreated due to post-glacial temperature rises, species re-expanded their distribution polewards, occupying previously inhospitable areas (Hewitt, 2000). These patterns are well established for terrestrial organisms, but the response to Pleistocenic glacial–interglacial cycles is still unclear for many marine species.

After the Messinian Salinity Crisis (5.33–5.59 mya), the Mediterranean basin was colonised by subtropical biota of Atlantic origin

* Corresponding author at: Department of Animal Biology, Faculty of Biology, University of Barcelona, Av. Diagonal 643, E-08028 Barcelona, Spain. Tel.: +34 934035370.
E-mail address: mclusa@ub.edu (M. Clusa).

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(Pérès, 1985). During the following climatic fluctuations, species distributions were affected by changes in the sea level, water temperature and salinity (Grant and Bowen, 1998). According to the fossil records, the most thermophilic groups became extinct during the first cold period of the Pleistocene and waves of extinction and invasion changed the composition of the Mediterranean biota in every climatic phase (Pérès, 1985). Nevertheless, recent molecular evidence has suggested that at least some of the subtropical species currently found in the Mediterranean are not recent Holocenic invaders, but have a pre-glacial origin and survived the glacial peaks in warmer refugia within the Mediterranean (Almada et al., 2001; Domingues et al., 2007; Wilson and Eigenmann Veraguth, 2010). Molecular data indicate that the southern parts of the Mediterranean, being warmer than northern areas during the Pleistocene (Thiede, 1978), acted as refugia for sea grasses (e.g. *Posidonia oceanica*, Arnaud-Haond et al., 2007; *Cymodocea nodosa*, Alberto et al., 2008) and that the Ionian and Aegean Sea, acted in the same way for some fish species (Bahri-Sfar et al., 2000; Magoulas et al., 1996).

Marine turtles have tropical affinities and females are highly philopatric, returning to specific geographical locations to nest (Carr and Ogren, 1960; FitzSimmons et al., 1997; Meylan et al., 1990). This results in strong genetic structuring when mtDNA is considered (Bowen and Karl, 2007; Lee, 2008), allowing evolutionary and colonisation events to be traced (Garofalo et al., 2009). The loggerhead turtle (*Caretta caretta* L.) is the least thermophilic cheloniid and regularly nests in subtropical and warm temperate regions where sand temperature is higher than 24 °C for a sufficiently long period of time (Miller et al., 2003). Paleoclimatic reconstructions of sea surface temperatures indicate that loggerhead turtles could not use the Western Mediterranean even as a foraging ground due to low sea surface temperatures during the last glacial peak (summer surface temperature<17 °C; Thiede, 1978). Only the Eastern Mediterranean was warm enough to allow turtle nesting, as summer sea surface temperatures were usually higher than 22 °C (Thiede, 1978); the minimum threshold for loggerhead turtle nesting (Miller et al., 2003). Thus, in the case that *C. caretta* had already colonised the Mediterranean prior to glaciation events, these Eastern regions could have acted as refugia for loggerhead turtles through the cold phases of the Pleistocene. Nevertheless, Bowen et al. (1993a) proposed a recent Holocenic origin for loggerhead turtles currently nesting in the Mediterranean. However, their conclusion was based on the analysis of just one nesting ground from the Ionian Sea (Bay of Kyparissia), the only rookery sampled at that time. New genetic data on the Mediterranean populations have come to light since (Carreras et al., 2007; Chaieb et al., 2010; Encalada et al., 1998; Garofalo et al., 2009; Laurent et al., 1998; Saied et al., 2012; Yilmaz et al., 2011).

To track the colonisation history of the Mediterranean by loggerhead turtles and to test the possible existence of warm refugia during the cold phases we have analysed mtDNA sequences from multiple nesting grounds in the Eastern Mediterranean, including previously poorly sampled locations.

2. Material and methods

2.1. Sample collection

Samples of skin and/or muscle were taken from 168 dead hatchlings and embryos from unhatched eggs during post-hatch nest excavations of nesting grounds in the Mediterranean Sea between 2003 and 2006 (Fig. 1, Table 1). These included Libya (west of Sirte), Israel (scattered sites along the whole coastline), Lebanon (El Mansouri), Cyprus (Alagadi and Akamas) and Greece, with samples from Western Greece (Zakynthos and Lakonikos Bay) and Crete (Rethymno). Samples were stored in 95% ethanol and samples from Greece, Israel and Lebanon previously analysed by Carreras et al. (2007) were also used for this study. Independency among samples can be assumed because sampling included protocols to avoid pseudoreplication. These included female tagging and samples taken from clutches laid within a 15-day window to avoid hatchlings from the same individual turtle, as females rarely nest at intervals shorter than this period (Dutton, 1995). However, the new samples from Lebanon were collected in different years from those from Carreras et al. (2007) and hence, additional pseudoreplication tests were undertaken to ensure independency between samples. Pseudoreplication was assessed by amplifying the new samples with seven microsatellite loci (Carreras et al., 2007) and comparing them with the Lebanon samples in Carreras et al. (2007). A pairwise relatedness analysis implemented in GenAlEx v6.4 (Peakall and Smouse, 2006) was used for the comparison.

2.2. DNA extraction and amplification

DNA was extracted with the QIAamp extraction kit (QIAGEN®) and 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′-GTCTCGGATTTAGGG GTTT-3′) (Abreu-Grobois et al., 2006). The analysis of longer sequences has been proven to improve the genetic resolution in *C. caretta* populations (Monzón-Argüello et al., 2010; Saied et al., 2012). The resulting fragment contains the 380 bp fragment traditionally used for population studies on this species (Carreras et al., 2006; Encalada et al., 1998; Norman et al., 1994). PCR cycling parameters were 94 °C for 5 min followed by 35 cycles at 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 90 s, and a final extension period of 72 °C for 10 min. Resulting products were purified by enzymatic reaction (ExoSAP) and sequencing reactions undertaken with fluorescent dye terminators (BigDye v3.1®). All samples were sequenced in both forward and reverse directions on an ABI 3730 automated DNA Analyser (Applied Biosystems®) to confirm variable sites on both strands of DNA.

2.3. Data analysis

Alignment was conducted using BioEdit v5.0.9 (Hall, 1999) and sequences were compared to short and long 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). New haplotypes identified were named following ACCSTR standardised nomenclature and submitted to GenBank (Accession nos. JF837821–JF837824).

To understand the genetic relationships between the sampled rookeries, pairwise genetic distances (γ_{st}) were calculated by the DnaSP v5 software package (Librado and Rozas, 2009). The significance of genetic differentiation among these regions was assessed using Hudson's nearest neighbour statistics (S_{NN}) with 1000 permutations in DnaSP. Published long sequence data from Southern Italy (Calabria; Garofalo et al., 2009) and Turkey (Yilmaz et al., 2011, which includes Turkish samples from Carreras et al., 2007) were also used in the analyses. Five nesting groups were considered in Turkey as suggested by the authors' conclusions (Yilmaz et al., 2011): Dalyan, Dalaman, Western Turkey (Fethiye, Patara, Kale, Kumluca and Çirali), middle Turkey (Gazipaşa, Kizilot, Tekirova and Belek) and Eastern Turkey (Anamur, Göksu Deltasi, Alata, Kazanli, Akyatan, Ağyatan and Samandağ). Recently published data from Libya (Saied et al., 2012) were not added to our dataset to avoid pseudoreplication as samples from both datasets were collected from the same location (Sirte) within a three year window. However, genetic differentiation analyses were undertaken with both datasets separately to look for possible differences. Following Narum (2006), modified false discovery rate (FDR) was used to evaluate statistical significance instead of the sequential Bonferroni correction when analysing multiple comparisons. Haplotype diversity (h; Nei, 1987) and nucleotide diversity (π; Nei, 1987) were estimated using ARLEQUIN v3.1 (Excoffier et al., 2005) and Fu's *Fs* values for each nesting region were calculated with DnaSP.

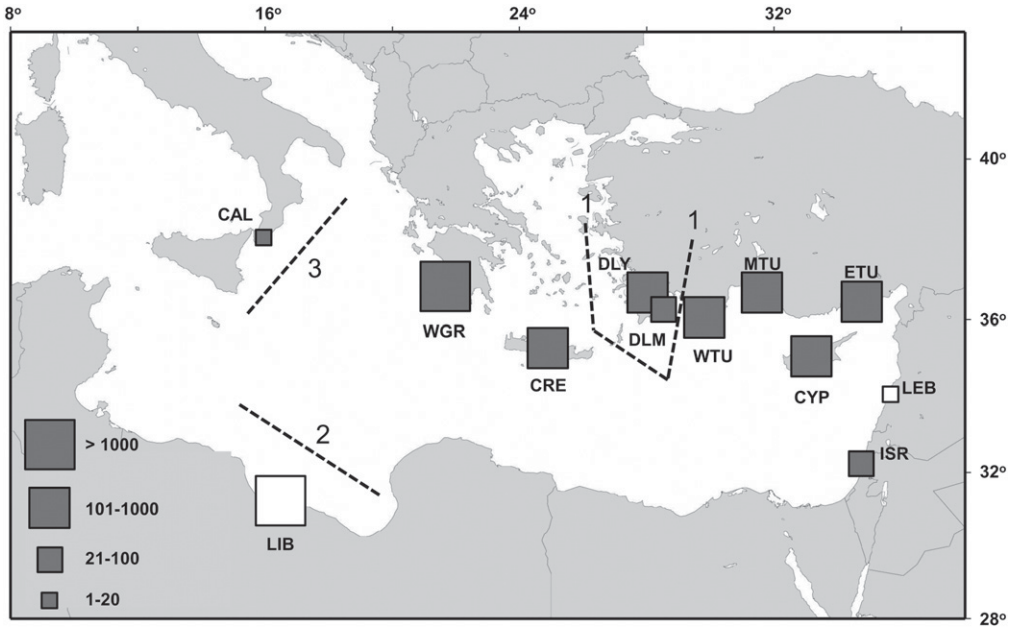

Fig. 1. Sampled nesting areas of loggerhead turtles in the Mediterranean Sea. Nesting areas: Libya (LIB), Israel (ISR), Lebanon (LEB), Cyprus (CYP), Eastern Turkey (ETU), middle Turkey (MTU), Western Turkey (WTU), Dalaman (DLM), Dalyan (DLY), Crete (CRE), Western Greece (WGR: Zakynthos and Lakonikos Bay), Calabria (CAL). Data for Calabria and Turkey are from Garofalo et al. (2009) and Yilmaz et al. (2011), respectively. Grey squares feature the average values of nests per season derived from monitoring projects and white squares are estimates (adapted from Casale and Margaritoulis, 2010; Margaritoulis et al., 2003). Dashed lines represent the location of the three strongest genetic breaks revealed by BARRIER. The lowest number indentifies the strongest barrier.

Fs detects deviation from neutrality and tends to be negative under an excess of recent mutations (Fu, 1997), which can result from population expansion. A partial correlation test between nucleotide diversity, mean width of the continental shelf (calculated with the ArcGIS software; ESRI, 2011) and the sea surface palaeotemperature (Thiede, 1978) in each nesting area was also carried out with SPSS v15 (SPSS Inc., 2006). The test was used to relate genetic diversities with environmental factors that could have affected nesting patterns. When necessary, variables were log-transformed or arcsine-transformed to satisfy the normality criterion (Zar, 1984).

Genetic structuring on a geographical scale was analysed with a Mantel test using genepop v 4.1 (Rousset, 2008). This analysis was conducted with minimum linear (Lat/Long positions) and coastal distances (following the coastline) between locations, calculated using the ArcGIS software (ESRI, 2011). Subsequently, based on a γ_{st} distance matrix, BARRIER v 2.2 (Manni et al., 2004) was used to assess the relative order of importance of genetic breaks that could limit gene flow between populations. Previous studies based on mtDNA and microsatellite markers suggested that four is the most likely number of populations present in the Eastern Mediterranean (Carreras et al., 2007), which would imply the existence of three putative barriers. In consequence, we chose a priori to show four barriers since we used additional populations. In order to assess the proportion of genetic variation that explained the differences among nesting grounds, an analysis of molecular variance (AMOVA) was undertaken with ARLEQUIN considering the four groups identified by the three strongest barriers.

To graphically relate pairwise genetic distances (γ_{st}) between areas, a Principal Coordinate Analysis (PCA) was performed with GenAlEx v6.4 (Peakall and Smouse, 2006). Relationships between haplotypes were obtained by the calculation of a haplotype network with the network v4.5.1.6 software (Bandelt et al., 1999) using a Median Joining method. Less likely events were weighted differently from likely events, changing deletion (double weight) and transversion weights (3×) according to user guidelines.

Finally, a molecular clock was applied to date the different colonisation events, using two different approaches. In the first one, the substitution rate for the 815 bp mtDNA fragment was calibrated assuming that the divergence between the two major branches of the Atlantic/Mediterranean haplotype tree occurred as a consequence of the rise of the Isthmus of Panama (Bowen, 2003). The Isthmus started rising 15 mya and did not become a complete marine barrier until ca. 3 mya (Lessios, 2008). Consequently, we rooted our molecular clock at 3 mya for conservative purposes. The substitution rate was obtained following the methodology previously used for testudines by Avise et al. (1992) considering the 39 fixed mutations existing between the closest related haplotypes (CC-A1.6 and CC-A31.1) of the two major branches of the Atlantic/Mediterranean haplotype tree resulting in a substitution rate of ~0.8%My^{-1}. However, it has been recently pointed out that the molecular evolutionary rate of mitochondrial DNA may be time-dependent (Crandall et al., 2012; Ho et al., 2011; Karl et al., 2012). Consequently, the substitution rate likely overestimates divergence times (Crandall et al., 2012), as calibration was done with an old event (3 mya). No recent calibration points or well-known pedigrees exist to estimate accurate divergence rates for this species. Thus, a second, more conservative approach using the mutation rate to date haplotype coalescence times was used following Emerson (2007). The mutation rate has been described to be 3–10 times faster than the substitution rate in other species (Howell et al., 2003; Lambert et al., 2002). In addition, the mean rate of change for mtDNA genes in three marine invertebrate species calibrated with radiometric dates for sea-level rise yielded values 3 times faster than those estimated from fossils and vicariant events (Crandall et al., 2012). Thus, we estimated the divergence time between haplotypes of *C. caretta* using a mutation rate three times faster than the substitution rate and obtained lower and upper estimates by also dating coalescence times using the substitution rate and a mutation rate ten times faster than the substitution rate. A Bayesian relaxed-clock model was subsequently applied as implemented in beast v1.6.2 (Drummond and Rambaut, 2007). Four unique Atlantic haplotypes (CC-A1.1, CC-A1.3, CC-A1.4 and CC-A1.6),

Table 1
Absolute frequencies of haplotypes per sampling location for long mtDNA sequences. Short sequence equivalents and total number of individuals (n) per sampling location included. Locations: Libya (LIB), Israel (ISR), Lebanon (LEB), Cyprus (CYP), eastern Turkey (ETU), middle Turkey (MTU), western Turkey (WTU), Dalaman (DLM), Dalyan (DLY), Crete (CRE), Lakonikos (LAK), Zakynthos (ZAK), Calabria (CAL).

Short	CC-A2			CC-A3		CC-A6	CC-A13	CC-A20	CC-A26	CC-A29	CC-A31	CC-A32	CC-A43	CC-A50	CC-A52	CC-A53	CC-A65	n
Long	CC-A2.1	CC-A2.8	CC-A2.9	CC-A3.1	CC-A3.2	CC-A6.1	CC-A13.1	CC-A20.1	CC-A26.1	CC-A29.1	CC-A31.1	CC-A32.1	CC-A43.1	CC-A50.1	CC-A52.1	CC-A53.1	CC-A65.1	
LIB	11		10	3					1								2	27 (0)
ISR	15		2							2								19 (19)
LEB	17			2														19 (9)
CYP	44													1				45 (0)
ETU[a]	60			8	1								1		1	1		72
MTU[a]	46						1											48
WTU[a]	60			16														76
DLM[a]	5			15														20
DLY[a]	25			15														40
CRE	16	4																20 (19)
LAK	18					1												19 (19)
ZAK	16					2						1						19 (19)
CAL[b]	22							14			2							38

Re-sequenced samples (previously analysed in Carreras et al., 2007 with the short fragment) are given in brackets.
[a] Data from Yilmaz et al. (2011).
[b] Data from Garofalo et al. (2009).

were chosen as outgroups to root our Mediterranean haplotype tree. Markov-Chain Monte Carlo (MCMC) simulations were run for 10,000,000 generations, with the first 10% discarded as burn-in.

3. Results

A total of 17 haplotypes were found among the analysed Mediterranean rookeries (Table 1). In Lebanon, the comparison of the microsatellite genotypes (data not shown) of the new and old samples from Carreras et al. (2007) indicated that pseudoreplication did not occur in this population (mean LRM $= -0.062 \pm 0.110$). Thus, all samples from Lebanon rookeries were pooled for further analyses. Most long haplotypes found in the current study were concurrent with the short ones previously described for the Mediterranean Sea (Carreras et al., 2007; Encalada et al., 1998; Laurent et al., 1998), as the new fragments include the old 380 bp fragments (Abreu-Grobois et al., 2006). However, some haplotypes identified with the 380 bp sequence were split into additional haplotypes, due to further polymorphism in the additional fragment of the longer sequences (e.g. Table 1; CC-A2 split into CC-A2.1, CC-A2.8, CC-A2.9). Three new haplotypes were described because of an increase in sequence length that could be directly related to the 380 bp haplotypes: CC-A29.1 in Israel, CC-A32.1 in Zakynthos and CC-A50.1 in Cyprus (Table 1; GenBank accession nos. JF837821–JF837823). Furthermore, a new haplotype, not previously described for either long or short sequences, was found in Libya (CC-A65.1; GenBank accession no. JF837824); an unsampled or low sampled region in previous studies with short sequences (Carreras et al., 2007; Encalada et al., 1998; Laurent et al., 1993, 1998; but see Saied et al., 2012).

CC-A2.1 was the most frequent haplotype in the dataset (77.33%), followed by CC-A3.1 (12.50%). Of the remaining haplotypes, 13 were unique to a specific nesting beach and two were shared between Mediterranean nesting sites, although they did not occur at high frequencies. The haplotype network showed a divergent sub-group with two unique haplotypes in Libya (CC-A26.1 and CC-A65.1) and one haplotype also shared with Israel (CC-A2.9) (Fig. 2). Eastern Turkey also presented a sub-group with unique related haplotypes (CC-A3.2 and CC-A52.1). However, Eastern Turkey's unique haplotypes had fewer mutation changes from the ancestral haplotype (CC-A2.1) than haplotypes from Libya. An ambiguity in the haplotype tree was found (am, Fig. 2) between CC-A3.1 and the unshared haplotypes from Western Greece (CC-A6.1 and CC-A32.1). It was resolved as indicated by Carreras et al. (2007) for short fragments based on geographical location similarities, as CC-A32.1 is only present in Western Greece and CC-A3.1 has not been found on these rookeries. On the other hand, CC-A32.1 and CC-A6.1 share a gap but differ by a transition whilst CC-A32.1 and CC-A3.1 differ by that gap but share the transition. Thus, the most parsimonious explanation to this ambiguity is that the transition independently arose twice, as previously suggested (Carreras et al., 2007).

Haplotype ($h = 0.04–0.70$) and nucleotide ($\pi = 0.000–0.002$) diversities were highly variable (Table 2) due to the high number of haplotypes present in Eastern Turkey, Western Greece, Libya and Calabria in comparison to Cyprus, where very low variability was detected. Significant pairwise genetic differences were found in the majority of comparisons including Libya, Calabria and Dalaman (Table 3), thus revealing genetic structure within the basin (Global $\gamma_{st} = 0.262$, $P < 0.001$). Global γ_{st} values of all the Mediterranean rookeries did not differ when changing our dataset from Libya with the data from Saied et al. (2012) (Global $\gamma_{st} = 0.264$, $P < 0.001$). Furthermore, the two sets from Libya did not differ statistically ($\gamma_{st} = 0.001$, $P = 0.215$) despite some unshared haplotypes. Thus, both datasets agree in identifying Libya as the most diverse nesting area in the Mediterranean (Table 2).

Due to the lack of statistically significant divergence between Lakonikos and Zakynthos ($\gamma_{st} = 0.027$, $P = 0.99$) they were considered as subsamples of the same population, pooled for further analyses and

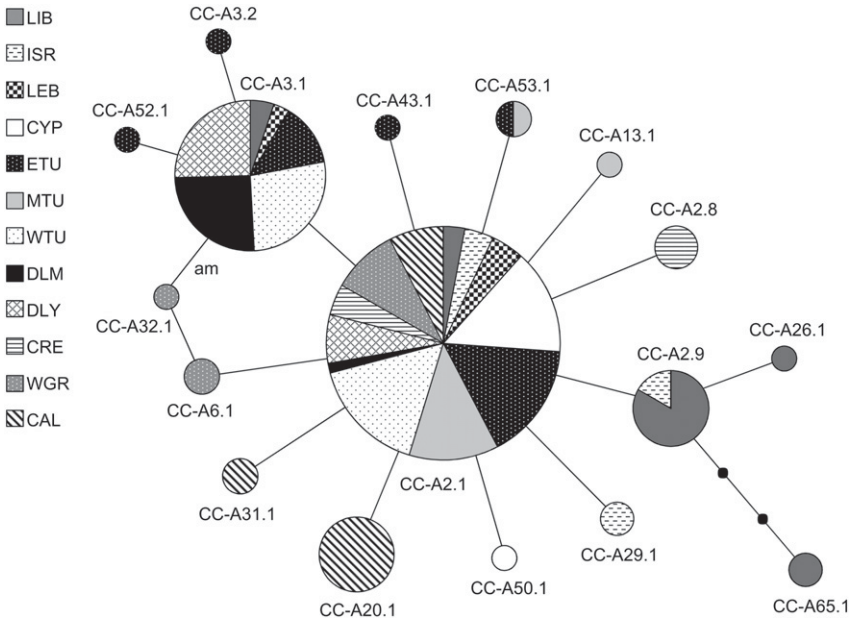

Fig. 2. Unrooted parsimony haplotype network of mtDNA for *Caretta caretta* in the Mediterranean Sea. Connecting lines represent single mutational changes between haplotypes with a probability higher than 95%. Unsampled intermediate haplotypes are indicated by dots and pie graphs represented to scale reflecting haplotype frequencies. [am] *Ambiguity resolved in text.*

referred to as Western Greece (WGR). This grouping was supported by the presence of the unique CC-A6.1 haplotype in both nesting areas and the evidence of female exchanges between Aegean Greece and the Ionian islands found in previous tagging studies (Margaritoulis, 1998) and microsatellite analyses (Carreras et al., 2007).

The global Fu's *Fs* test ($Fs = -15.459$, $P<0.01$) was significant, indicating deviation from neutrality and a possible recent population expansion in this area, although for each location separately only Eastern Turkey presented a significantly negative Fu's *Fs* value ($Fs = -4.119$, $P<0.01$; Table 2). The arcsine-transformed nucleotide diversity estimates were strongly correlated (partial correlation $r = 0.847$, $P = 0.002$) with the log-transformed mean width of the continental shelf and the sea surface temperature values during the last glacial period.

Geographic and genetic distances were uncorrelated both when using Lat/Long positions (Mantel test, $P = 0.160$) and minimum coastal distances (Mantel test, $P = 0.165$). BARRIER indicated that the strongest genetic barrier detected by the Monmonier's maximum difference algorithm (Fig. 1) was found between Dalaman and Dalyan and the remaining populations (Barrier 1, $\gamma_{st} = 0.582$). The second barrier separated Libya (Barrier 2, $\gamma_{st} = 0.227$) and the third, Calabria from the rest (Barrier 3, $\gamma_{st} = 0.184$). The fourth was found between Dalaman and Dalyan (Barrier 4, $\gamma_{st} = 0.125$). The four groups (Libya, Dalaman and Dalyan, Calabria and the rest of the populations) identified by the three strongest barriers (Fig. 1) were subsequently used for the AMOVA analysis (Table 4). Under this analysis, the highest percentage of variation was found within populations (66.57%) although the percentage of variation between groups was also significant and high (28.68%). PCA based on genetic distances (γ_{st}) between locations (Table 3) identified Dalaman, Dalyan, Libya and Calabria as highly distinct rookeries, with too small an amount of differentiation among the remaining rookeries to be classified as separate units (Fig. 3).

Finally, based on the haplotype network and the number of mutations between haplotypes, a molecular clock was applied to date haplotype divergences. Dates were estimated with a mutation rate three times faster than the substitution rate. We used the inferred substitution rate calculated for this species (~0.8%My^{-1}) as a lower bound and a mutation rate 10 times faster than the substitution rate as an upper bound. Haplotype CC-A65.1 (exclusive to Libya), with four changes from the ancestral CC-A2.1, revealed Libya as the oldest population while haplotypes CC-A32.1 (exclusive to Western Greece) and CC-A3.2 and CC-A52.1 (exclusive to Eastern Turkey), with two changes from the Atlantic ancestor, suggested that these areas would have been more recent. Thus, Libya could have been colonised ca. 65,000 years ago (20,000–200,000) and Western Greece and Eastern Turkey ca. 30,000 years ago (10,000–100,000). The remaining populations originated as a result of a more recent, Holocenic expansion. All results were supported by the relaxed-clock model tree implemented in beast (Fig. 4), with haplotypes unique to Libya, Eastern Turkey and Western Greece diverging before the rest, thus revealing these as the oldest populations of the Mediterranean.

Table 2
Haplotype and nucleotide diversities including standard deviations (±), results of Fu's Fs test and sample sizes per sampling location. The latitude (Lat.) and longitude (Long.) positions refer to a central point per nesting area, not to the specific position of the beach sampled, as samples came from wide areas pooled under one single location. Population abbreviations as in Table 1. Western Greece (WGR) groups individuals from LAK and ZAK.

	Haplotype diversity	Nucleotide diversity	Fu's *Fs*	n	Lat.	Long.
LIB	0.704 ± 0.054	0.0017 ± 0.0012	−0.909	27	30°59′19″N	17°34′50″E
ISR	0.374 ± 0.130	0.0005 ± 0.0005	−0.671	19	32°02′37″N	34°44′45″E
LEB	0.199 ± 0.112	0.0002 ± 0.0004	−0.055	19	33°16′32″N	35°11′33″E
CYP	0.044 ± 0.042	0.0001 ± 0.0002	−1.548	45	35°04′09″N	33°19′33″E
ETU	0.297 ± 0.067	0.0004 ± 0.0005	**−4.119**	72	36°45′50″N	34°52′37″E
MTU	0.082 ± 0.054	0.0001 ± 0.0002	−2.976	48	36°42′24″N	31°34′16″E
WTU	0.337 ± 0.054	0.0004 ± 0.0005	1.338	76	36°12′31″N	29°34′17″E
DLM	0.395 ± 0.101	0.0005 ± 0.005	0.976	20	36°41′51″N	28°45′33″E
DLY	0.481 ± 0.042	0.0006 ± 0.0006	1.728	40	36°47′28″N	28°37′16″E
CRE	0.337 ± 0.110	0.0004 ± 0.0005	0.721	20	35°21′51″N	24°27′29″E
WGR	0.198 ± 0.083	0.0003 ± 0.0004	−1.407	38	35°59′00″N	21°39′15″E
CAL	0.541 ± 0.049	0.0007 ± 0.0007	0.522	38	37°55′06″N	15°58′45″E

In bold significant values (Fu's *Fs*, $P<0.01$).

4. Discussion

The study of molecular genetic differentiation between populations of endangered species has been described as a powerful tool for conservation planning (Crandall et al., 2000; Moritz, 1994). However, the markers selected and the length of the DNA sequences analysed can

Table 3
Pairwise genetic distances between Mediterranean nesting populations (γ_{st}) (below diagonal) and S_{NN} significance (P) values (above diagonal).

	LIB	ISR	LEB	CYP	ETU	MTU	WTU	DLM	DLY	CRE	WGR	CAL
LIB	–	**0.001**	**~0.000**	**~0.000**	**~0.000**	**~0.000**	**~0.000**	**~0.000**	**~0.000**	**~0.000**	**~0.000**	**~0.000**
ISR	0.108	–	0.083	**~0.000**	**0.001**	**0.006**	**~0.000**	**~0.000**	**~0.000**	0.024	**0.011**	**~0.000**
LEB	0.160	0.056	–	0.087	0.913	0.061	0.329	**~0.000**	0.033	0.046	0.538	**~0.000**
CYP	0.243	0.062	0.053	–	**0.006**	0.873	**~0.000**	**~0.000**	**~0.000**	**0.007**	0.718	**~0.000**
ETU	0.160	0.038	0.002	0.040	–	0.039	0.189	**~0.000**	**0.003**	**0.001**	0.136	**0.001**
MTU	0.235	0.054	0.042	0.011	0.039	–	**~0.000**	**~0.000**	**~0.000**	**0.003**	0.690	**~0.000**
WTU	0.166	0.059	0.012	0.085	0.009	0.084	–	**~0.000**	0.075	**~0.000**	**0.007**	**~0.000**
DLM	0.318	0.437	0.422	0.622	0.264	0.582	0.220	–	**~0.000**	**~0.000**	**~0.000**	**~0.000**
DLY	0.187	0.139	0.077	0.224	0.062	0.214	0.031	0.125	–	**~0.000**	**~0.000**	**~0.000**
CRE	0.178	0.082	0.091	0.118	0.056	0.101	0.078	0.464	0.161	–	**0.010**	**~0.000**
WGR	0.227	0.060	0.028	0.012	0.024	0.011	0.059	0.592	0.186	0.113	–	**~0.000**
CAL	0.211	0.124	0.131	0.196	0.145	0.189	0.165	0.388	0.213	0.144	0.184	–

Bold values were significant after FDR correction for a threshold of $\alpha = 0.05$ (S_{NN}, $P<0.0105$).

significantly alter the results (Monzón-Argüello et al., 2010; this study). For loggerhead turtles, the existence of genetic structure within the Mediterranean was previously detected with short sequences (380 bp) of the mtDNA control region (Carreras et al., 2007; Chaieb et al., 2010; Encalada et al., 1998; Laurent et al., 1998). Nonetheless, the higher nucleotide diversity present in the longer mtDNA fragment (815 bp), along with the analysis of individuals from previously poorly sampled populations, allowed us to unveil a deeper structuring within the Mediterranean Sea.

4.1. Genetic structuring

The use of longer sequences allowed the splitting of the short CC-A2 and CC-A3 haplotypes into long haplotypes (CC-A2.1, CC-A2.8, CC-A2.9 and CC-A3.1 and CC-A3.2), which in turn revealed further structuring within Crete and Israel (CC-A2.8, CC-A2.9) and Eastern Turkey (CC-A3.2). Furthermore, the inclusion of Calabria (Garofalo et al., 2009) and Libya in the analyses revealed high levels of structuring previously undescribed (Bowen et al., 1993a; Carreras et al., 2007), as these two regions emerged as the most genetically diverse. This is because of the presence of two unique haplotypes in each of the two regions (CC-A26.1 and CC-A65.1 in Libya, and CC-A20.1 and CC-A31.1 in Calabria) and also because of a higher degree of divergence between these haplotypes and the other Mediterranean haplotypes. However, even though Libya and Calabria were found to be the rookeries with the highest diversity indexes, PCA and BARRIER analyses identified Dalaman and Dalyan as a higher differentiated unit. This is because of the high occurrence of CC-A3.1 in these two regions and in particular in Dalaman, where the proportion of CC-A3.1 was even higher than that of CC-A2.1 (Yilmaz et al., 2011), something not observed in any other rookery of the basin. The nesting area of Eastern Turkey hosted three unique haplotypes (CC-A3.2, CC-A43.1 and CC-A52.1) and one only shared with middle Turkey (CC-A53.1). Nonetheless, their frequencies were remarkably low and thus Eastern Turkey did not emerge as a major genetic unit. Cyprus was confirmed as a region with low genetic variability despite the large increase in sample size in relation to previous studies (Carreras et al., 2007; Encalada et al., 1998). However, could be slightly differentiated from most of the other nesting areas by the overwhelming dominance of the CC-A2.1 haplotype. In conclusion, we identify four major clusters of nesting grounds: Libya, Dalaman and Dalyan, Calabria and the rest of the Eastern Mediterranean, although some genetic differentiation exists within the latter cluster (Table 3).

Table 4
Analysis of molecular variance (AMOVA) for four Mediterranean genetic groups (Libya, Dalaman and Dalyan, Calabria and the rest of the sampled Mediterranean rookeries) based on the main three breaks inferred by BARRIER.

Source of variation	d.f.	Percentage of variation	F-statistic	P
Among groups	3	28.68	FCT: 0.28681	<0.005
Among populations within groups	8	4.74	FSC: 0.06653	~0.000
Within populations	450	66.57	FST: 0.33426	~0.000

4.2. Evolutionary history

The short (380 bp) haplotypes CC-A2, CC-A3 and CC-A20 are shared by Mediterranean and Atlantic rookeries (Bowen et al., 2004; Carreras et al., 2007; Garofalo et al., 2009; Monzón-Argüello et al., 2010; Shamblin et al., 2011), indicating that this could have probably been the minimal ancestral haplotypic composition of the stock of loggerhead turtles that colonised the Mediterranean from the Northern Atlantic. Nevertheless, Carreras et al. (2007) also suggested an alternative hypothesis in which the origin of CC-A3 in the Mediterranean could have been independent from the Atlantic, in a clear case of homoplasy. Only a future long sequence screening of the variants of the CC-A3 and CC-A20 short haplotypes present in the Atlantic nesting beaches will clarify which hypothesis is correct. Nevertheless, at least two different CC-A3 variants have already been detected in the Mediterranean (Table 1 from Yilmaz et al., 2011).

Regarding the species history within the Mediterranean Sea, the analysis of individuals from previously poorly sampled nesting grounds (Libya, Turkey) revealed an earlier colonisation of the basin than previously suggested (Bowen et al., 1993a). This dating relies not only on the new haplotypes found in these nesting grounds, but also on the divergence rates applied. The substitution rate estimated (~0.8%My^{-1}) is higher than previously published estimates for other testudines (0.2 to 0.4%, Avise et al., 1992; Bowen et al., 1993b) probably due to the use of different markers and the length of the sequences analysed. Bowen et al. (1993b) analysed the cytochrome b region, which presents a lower substitution rate than the control region of the mtDNA (Dutton et al., 1996). Furthermore, differences in nucleotide diversity along the control region can alter the estimates depending on the length and region sequenced (Monzón-Argüello et al., 2010). As a consequence, the long sequences of the control region presented here had higher nucleotide diversity than the shorter fragments and thus, the substitution rate estimated in the present study is higher. Nevertheless, this makes our estimates of the substitution rate among Mediterranean haplotypes more conservative and thus, the older coalescence times inferred are solely due to the presence of previously unsampled haplotypes from Libya and Turkey. The time estimates changed when using mutation rates 3 and 10 times higher than the phylogeographically calibrated substitution rate (Crandall et al., 2012; Emerson, 2007). The presence of four mutations in a Libyan haplotype (CC A65.1) from its Atlantic ancestor haplotype (likely to be CC-A2.1) places the oldest colonisation of the Mediterranean as a pre-Holocenic event occurring ca. 65,000 years ago (20,000–200,000). Thus, regardless of the molecular rate used, *C. caretta* seems to have been present in the Mediterranean before the end of the last glacial period (~18,000 years ago; Thunell, 1979). According to this 3× molecular rate, turtles could have survived several cold periods in the Mediterranean (Cacho et al.,

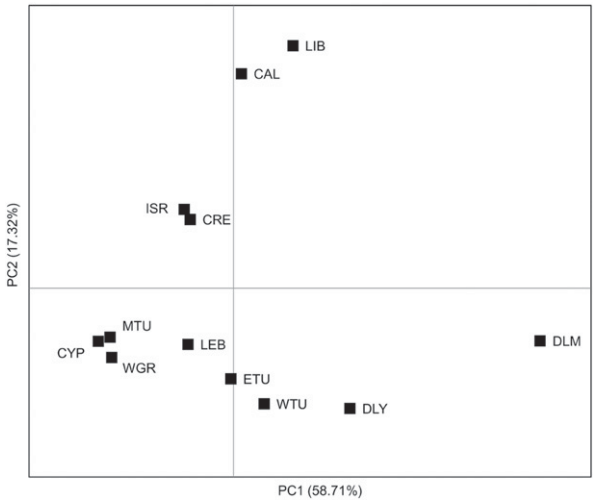

Fig. 3. Principal Coordinate Analysis for pairwise genetic distances (γ_{st}) between nesting colonies in the Mediterranean. First two Principal Coordinates (PC1 and PC2) and the percentage of variation explained by the 2 axes included.

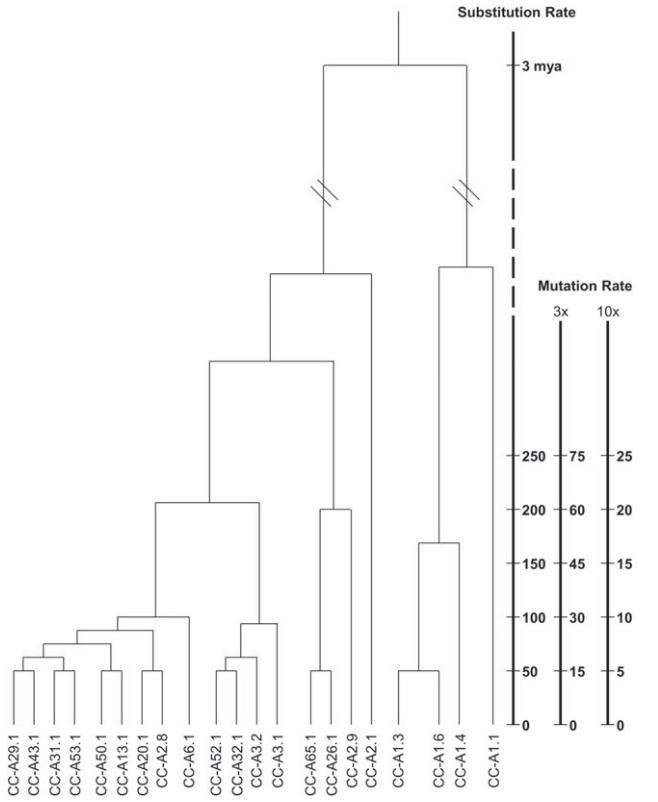

Fig. 4. Haplotype tree adapted from the Bayesian relaxed-clock model results inferred by beast. Time bars show different estimated dates (kya) for haplotype coalescence under a substitution rate of ~0.8%My^{-1} (left) and mutation rates 3 and 10 times faster (centre and right, respectively) following Emerson (2007).

2000). The nesting grounds in Western Greece also present a haplotype (CC-A32.1) that is separated from its Atlantic ancestor by two changes, indicating that the population in that area has been stable for a long period of time. The presence of this haplotype dates the colonisation of Western Greece at ca. 30,000 years ago (10,000–100,000). This might also be true for Eastern Turkey, as haplotypes CC-A3.2 and CC-A52.1 are also separated by two mutations from the Atlantic ancestor, if it is CC-A2.1, or by one mutation if CC-A3.1 was already present in the colonisers. In the latter, the colonisation of Eastern Turkey would be more recent, 15,000 years ago (5000–50,000), but discriminating between these two scenarios is dependent of future long sequences analyses of individuals from the Western Atlantic rookeries. The possible pre-Holocenic colonisation was not suggested by Bowen et al. (1993a) because they only considered palaeoclimatic evidence for a more restricted genetic sampling area. Thus, the presence of cold temperatures off Greece 18–12 kya, which could not have allowed nesting success on its beaches, brought Bowen et al. (1993a) to hypothesise a much more recent colonisation. However, the analysis of genetic markers locates this origin earlier than previously thought, suggesting that loggerhead turtles colonised the Mediterranean ca. 65,000 years ago (20,000–200,000) and that might have survived glacial periods by nesting at least in Libya and perhaps in Western Greece and Eastern Turkey as well. Thus, the first colonisation event would have happened during the upper Pleistocene and hence before the last glacial maximum.

The star-like shape of the haplotype network is a strong indication of recent expansions such as those related to post-glacial colonisation events (Kaiser et al., 2010; Maggs et al., 2008). This is corroborated by the global Fu's *Fs* although signal of expansion was only found significant for Eastern Turkey. Furthermore, as geographic and genetic distances were uncorrelated both when using Lat/Long positions and minimum coastal distances, we can discard isolation by distance as an explanation for the overall differentiation pattern. The higher diversity and haplotype divergences found in Libya (Saied et al., 2012; this study), and to a lesser extent in Western Greece and Eastern Turkey, suggest that these three areas could have acted as refugia during cold events maintaining stable population sizes with mild or null bottlenecks. The glacial phase that affected the area from ca. 120 to 20 kya (Woodward and Hughes, 2011) probably caused the extinction of most of the populations in the basin leading to the disappearance of some ancestral haplotypes. However, some populations present in the warmer parts of Northern Africa would have survived during these glacial events. During the ensuing interglacial periods, loggerhead turtles might have recolonised the Eastern Mediterranean, only to become extinct in most of the new nesting grounds with the last glacial maximum. Nevertheless, the presence of haplotype CC-A6.1 in Western Greece and haplotypes CC-A3.2 and CC-A52.1 in Eastern Turkey indicates that these populations might have survived at least the most recent glacial peak. Consequently, the northern part of the Eastern Mediterranean and Western Peloponnese seems to have acted as warm refugia for marine species at that time, as has already been suggested for fishes (Domingues et al., 2008). This hypothesis could explain the genetic structure currently seen in Turkey, with a strong westward decline in haplotype diversity and a high variability in the frequency of CC-A3.1 between adjoining sites.

The existence of the highest frequencies of unique haplotypes in Libya and Eastern Turkey suggests that Western Greece probably was less suitable than the Libyan and Turkish coasts as a refugium. This may be explained by Libya and Eastern Turkey presenting a wider continental shelf which allowed a gentle progression of nesting beaches when the sea level decreased during glacial periods (Patarnello et al., 2007). Conversely, off the coast of Greece (Peloponnese), the continental shelf is much narrower which resulted in major redistribution of beaches and loss of many suitable nesting sites due to sea level fluctuations. This can be corroborated by the results found in our study, showing a strong correlation between the nucleotide diversity, width of the continental shelf and sea surface temperature in each of these refugia. Thus, the presence of warmer temperatures and wider continental shelves off Libya and Eastern Turkey could explain the high genetic variability found in these two areas. According to this correlation, Egypt could also be a potential refugium, but its population was depleted during the first half of the 20th century due to direct exploitation (Nada and Casale, 2010; Sella, 1982). It is worth noting that currently, the largest rookeries in the Mediterranean are found at these potential refugia (Libya, Turkey and Western Greece; Fig. 1). However, this could be an artefact since population sizes in the easternmost

Mediterranean rookeries (Israel and Lebanon) have notably changed in the past centuries due to human impacts such as fishing, direct exploitation and beach excavations (Sella, 1982).

The evolutionary hypothesis presented above is in accordance with previous studies suggesting that populations of several species of marine turtles survived glacial periods in warm refugia worldwide. Reece et al. (2005) found that Mexico, South Florida and the Caribbean may have acted as Pleistocenic refugia for Western Atlantic populations of loggerhead turtles during the climate depression at the Pliocene–Pleistocene border. Green (*Chelonia mydas*) and hawksbill (*Eretmochelys imbricata*) turtles also suffered some population contractions (Reece et al., 2005) and equatorial regions such as Brazil and Guinea Bissau have been proposed as Pleistocenic refugia for Atlantic green turtles (Encalada et al., 1996). Of all sea turtle species, the leatherback turtle (*Dermochelys coriacea*) may have been the most deeply affected by climate fluctuations, since it is the only species that extensively feeds at high latitudes (James and Mrosovsky, 2004). Nonetheless, it has been suggested that leatherbacks might have survived in the Indian-Pacific during the early Pleistocene to later recolonise the Atlantic, with a subsequent genetic bottleneck (Dutton et al., 1999).

Currently, loggerhead turtles from the Atlantic rookeries abound in the Western Mediterranean (Carreras et al., 2006), where sea surface temperatures are high enough to allow them to forage year round (Revelles et al., 2007a). Some Atlantic individuals even venture into the Eastern Mediterranean, but they are scarce there (Carreras et al., 2006; Casale et al., 2008; Maffucci et al., 2006). Young loggerheads from the Atlantic rookeries reach Western Europe after drifting passively in the Gulf Stream and some may spend several years in the Mediterranean before returning to the Atlantic (Revelles et al., 2007b). This process certainly operated during the Pleistocene and allowed loggerheads to colonise the Mediterranean. However, during the cold phases of the Pleistocene, the sea surface temperature in the Western Mediterranean might have been too low (Thiede, 1978) to allow loggerheads to use it even as a foraging ground. This means that any gene flow between the Atlantic and the Mediterranean populations, mediated by dispersal of turtles from the Atlantic populations, was interrupted during the cold phases of the Pleistocene thus leading to an increased genetic differentiation between the Mediterranean and Atlantic populations. The gene flow and the colonisation events were probably restored in the following warm phase when the Western Mediterranean again became a suitable feeding ground for Atlantic loggerheads. However, contemporary gene flow rates appear to be insufficient to genetically homogenise the two areas (Carreras et al., 2011).

The presence of haplotype CC-A20.1 in Calabria could be homoplasic, as previously discussed, but may also reveal a new colonisation event from the Atlantic that occurred during the Holocene. This could explain why this Atlantic haplotype is found exclusively in the most regularly visited westernmost nesting site in the Mediterranean. If this hypothesis is true, the current genetic structure of loggerhead turtles in the Mediterranean would be the result of at least two independent colonisation events. One taking place ca. 65,000 years ago (20,000–200,000) and a recent one 15,000 years ago (5000–50,000) combined with local extinction and re-colonisation through the expansion of individuals from a few refugia following climatic fluctuations.

4.3. Conservation implications

Loggerhead turtles nesting in the Mediterranean are considered an independent regional management unit (Wallace et al., 2010) with highly reduced gene flow with other populations in the North Atlantic (Carreras et al., 2011). The rookeries within this regional management unit generally exhibit stable abundance with high genetic diversity. However, under a relatively high degree of threat due to human activities, these populations could decline in the future if threats are not abated (Wallace et al., 2011). The main human activities impacting loggerhead turtles in the region are incidental bycatch and beach loss due to tourism development. Furthermore, direct take of immatures and adults is still a problem in some countries (Casale and Margaritoulis, 2010). Although the impact of these activities should be reduced everywhere, careful planning is necessary to guarantee that the conservation actions have positive impacts on the target populations. For instance, reducing the high levels of bycatch by bottom trawlers operating in the Adriatic sea (Casale et al., 2004) or off Tunisia (Casale et al., 2008) will certainly benefit the Mediterranean management unit. However, the actual relevance of such a hypothetical reduction for each of the four major groups of rookeries in the region (Libya, Dalaman and Dalyan, Calabria and the rest of the rookeries) could only partially be anticipated with the data previously available (Casale et al., 2008; Maffucci et al., 2006). The data presented here will dramatically improve the resolution of mixed stock analysis (Carreras et al., 2006; Saied et al., 2012) for feeding grounds and hence will allow conservationists to indentify which rookeries will most likely benefit from reducing bycatch at particular feeding grounds or with a particular type of fishing gear.

The consequences of global warming are also a matter of concern, as direct impacts on marine turtles come from the flooding of nesting beaches due to the rise in sea level (Baker et al., 2006) and altered sex ratios because of the temperature-dependent sexual determination of these species (Hawkes et al., 2009). Marine turtles have adapted to previous climate fluctuations (Dutton et al., 1999; Encalada et al., 1996; Reece et al., 2005; this study), but they will have much lower chances in the context of the highly human-modified Mediterranean Sea. As temperature increases, some loggerhead populations are expected to expand northwards, colonising areas currently too cold for reproduction. However, most of the coastline in the northern shore of the Mediterranean has been intensely developed by the tourism industry and few places remain suitable for the nesting of loggerhead turtles. Furthermore, total beach surface will decrease as the sea level rises and buildings, roads and other infrastructures impede beaches moving inland. In this context, competition between the tourism industry and nesting loggerhead turtles will increase, with uncertain results for loggerhead turtles.

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Appendix A2

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ORIGINAL PAPER

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

Marcel Clusa · Carlos Carreras · Marta Pascual · Stephen J. Gaughran · Susanna Piovano · Cristina Giacoma · Gloria Fernández · Yaniv Levy · Jesús Tomás · Juan Antonio Raga · Fulvio Maffucci · Sandra Hochscheid · Alex Aguilar · Luis Cardona

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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.

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M. Clusa (✉) · C. Carreras · S. J. Gaughran · A. Aguilar · L. Cardona
Department of Animal Biology and IRBio, University of Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain
e-mail: mclusa@ub.edu

C. Carreras
Centre for Ecology and Conservation, Tremough Campus, University of Exeter, Penryn TR10 9EZ, UK

M. Pascual
Department of Genetics and IRBio, University of Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain

S. J. Gaughran
Sackler Institute for Comparative Genomics, American Museum of Natural History, Central Park West at 79th Street, New York, NY 10024, USA

S. Piovano · C. Giacoma
Department of Life Sciences and Systems Biology, University of Torino, Via Accademia Albertina 13, 10123 Turin, Italy

Present Address:
S. Piovano
School of Biological and Chemical Sciences, Laucala Campus, University of the South Pacific, Suva, Fiji Islands

G. Fernández
Fundación Aspro Natura, c/Garcilaso de la Vega 9, Costa d'en Blanes, 07181 Calvià, Spain

Y. Levy
The Israel Sea Turtle Rescue Centre, Nature Parks Authority, Mevoot Yam, Mikhmoret 40297, Israel

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Introduction

Great migrations are often found in the animal kingdom and at very different scales (Hoare 2009). By migrating, species have adapted to increase their fitness and reproductive success for millions of years, but nowadays many anthropogenic threats affect populations at their origin, destination and along migratory corridors. Only by understanding the distribution of these migratory species and the overlap with anthropogenic threats will conservation be possible.

Sea turtles are among these highly migratory species, undertaking long-distance journeys sometimes spanning entire oceans (Bolten 2003; Plotkin 2003). One of the best-known oceanic migrators is the loggerhead turtle (*Caretta caretta*), distributed in all tropical and warm-temperate areas and the most abundant sea turtle in the Mediterranean Sea (Broderick et al. 2002; Casale and Margaritoulis 2010). Loggerhead turtles of different origins coexist in this area, as juveniles from western Atlantic rookeries share foraging grounds with those clutched within the Mediterranean (Laurent et al. 1993, 1998; Bowen et al. 2003; Carreras et al. 2006, 2011). Small Atlantic juveniles enter the Mediterranean Sea through the Strait of Gibraltar during their pelagic stage and remain there until they are large enough to swim against the strong and permanent eastward current of the strait (Revelles et al. 2007a; Eckert et al. 2008). During this period, juvenile turtles of Atlantic origin use the same foraging grounds as juveniles born in Mediterranean rookeries but rarely interbreed (Carreras et al. 2011), maintaining isolation between these two genetically distinct Regional Management Units (RMU; Wallace et al. 2010).

The distribution of juvenile loggerhead turtles of Atlantic and Mediterranean origin in the Mediterranean Sea has been widely studied through the use of satellite telemetry (Cardona et al. 2005; Bentivegna et al. 2007; Revelles et al. 2007b; Cardona et al. 2009; Casale et al. 2013), mark recapture techniques (Margaritoulis et al. 2003; Casale et al. 2007; Revelles et al. 2008) and genetics (Carreras et al. 2006; Maffucci et al. 2006; Casale et al. 2008; Saied et al. 2012; Garofalo et al. 2013). In the western Mediterranean Sea, juvenile turtles of Atlantic origin mainly inhabit foraging grounds off the North African coast and juvenile turtles of Mediterranean origin forage mainly along the European coasts (Carreras et al. 2006). However, little is known about the distribution and proportion of Atlantic juveniles in other areas within the Mediterranean Sea (Laurent et al. 1998; Maffucci et al. 2006; Casale et al. 2008; Piovano et al. 2011). Furthermore, nothing is known about the distribution of young turtles from the different nesting populations existing in the Mediterranean Sea (Carreras et al. 2007; Garofalo et al. 2009; Saied et al. 2012; Clusa et al. 2013).

The relative contribution of each rookery to specific foraging grounds can be studied through mixed-stock analysis (MSA; Grant et al. 1980). Previous research in the Mediterranean Sea has mostly used a ~380-bp fragment of non-coding mitochondrial DNA (mtDNA) as the genetic marker for MSA (Laurent et al. 1998; Maffucci et al. 2006; Carreras et al. 2007; Casale et al. 2008; Carreras et al. 2011; Saied et al. 2012; but see Garofalo et al. 2013). However, the limited assignment power of this marker has precluded a fine-scale assessment of the contribution of Mediterranean rookeries to the Mediterranean foraging grounds. A new set of primers has been developed (Abreu-Grobois et al. 2006), which amplifies a longer segment of the mitochondrial control region (815 bp) and hence increases the resolution of genetic structuring among the different nesting areas (Monzón-Argüello et al. 2010; Shamblin et al. 2012; Clusa et al. 2013). With this increase in the genetic resolution, origin assignment power of juveniles from Mediterranean foraging grounds is expected to improve at regional and fine-scale levels, potentially unveiling previously unknown distribution patterns.

Bycatch of juvenile turtles at their foraging grounds is one of the most significant anthropogenic threats for sea turtles in the Mediterranean Sea, with over 132,000 annual captures estimated in the area (Casale and Margaritoulis 2010; Casale 2011). The impact of fisheries bycatch depends on habitat use, type of fishing gear, fishing effort, abundance of the affected populations and origin of these populations (Wallace et al. 2008). Thus, fine-scale information on the composition of bycatch in each fishing ground is essential for a proper impact assessment of turtle bycatch in the Mediterranean Sea.

This paper analyses the origin of juvenile loggerhead turtles from seven distinct foraging grounds within the Mediterranean Sea through a mixed-stock analysis with longer fragments of mtDNA with the aim to (1) describe the distribution of juveniles of Atlantic origin within the Mediterranean Sea (regional level), (2) unveil the use of Mediterranean foraging grounds by juveniles of Mediterranean origin (fine-scale level), (3) understand the mechanisms of such distributions and (4) evaluate the impact that

J. Tomás · J. A. Raga
Cavanilles Institute of Biodiversity and Evolutionary Biology, University of Valencia, Aptdo. 22085, 46071 Valencia, Spain

F. Maffucci · S. Hochscheid
Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Naples, Italy

F. Maffucci
Dipartimento di Scienze, Università Roma Tre, Viale G. Marconi 446, 00146 Rome, Italy

incidental bycatch in foraging grounds might have on nesting populations.

Materials and methods

Sample collection

Tissue samples were taken from 275 stranded or bycaught juvenile loggerhead turtles from several developmental foraging grounds in the Mediterranean Sea between 2002 and 2012 (Table 1). Only turtles smaller than 69 cm curved carapace length (CCL) were sampled, as this is the average minimum size of nesting females in the Mediterranean (Margaritoulis et al. 2003) and turtles of Atlantic origin become adults at a much larger size (Piovano et al. 2011). Sampling was designed to ensure coverage of several juvenile foraging grounds within the major sub-basins in the region (Fig. 1): the Catalano-Balearic Sea (CAB), the Algerian basin (ALG), the Tyrrhenian Sea (TYR), the northern Adriatic Sea (NADR), the southern Adriatic Sea (SADR), the northern Ionian Sea (ION) and the southern Levantine Sea (LEV). No samples could be obtained from the southern Ionian Sea or the Aegean Sea, areas also known to be used by juvenile turtles as foraging grounds (Margaritoulis et al. 2003; Casale et al. 2013).

Muscle samples were collected from dead animals and stored in 95 % ethanol. Blood samples were taken from live animals and stored frozen.

Laboratory procedures

DNA from samples was extracted with the QIAamp extraction kit (QIAGEN®), following the 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 to confirm variable sites on both strands of DNA on an ABI 3730 automated DNA analyser at the Scientific-Technical Services at the University of Barcelona or at the Molecular Biology Service of the Stazione Zoologica Anton Dohrn.

Table 1 Absolute mtDNA haplotype frequencies found in the Mediterranean foraging grounds for juvenile loggerhead turtles: CAB (the Catalano-Balearic Sea), ALG (the Algerian basin), TYR (the Tyrrhenian basin), NADR (the northern Adriatic Sea), SADR (the southern Adriatic Sea), ION (the northern Ionian Sea) and SLE (the southern Levantine Sea)

	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						1	
n	40	65	51	29	21	35	34
d	33	48	46	29	21	35	34
h	0.439 ± 0.098	0.668 ± 0.041	0.409 ± 0.084	0.197 ± 0.095	0.095 ± 0.084	0.613 ± 0.083	0.321 ± 0.101
π	0.0095 ± 0.0050	0.0248 ± 0.0123	0.0109 ± 0.0057	0.0002 ± 0.0004	0.0001 ± 0.0002	0.0010 ± 0.0008	0.0033 ± 0.0020

Total number of sampled turtles (n), number of turtles found dead (d), haplotype diversity (h) and nucleotide diversity (π) found in each foraging ground included at the bottom of the table. Mean standard deviations (±) included

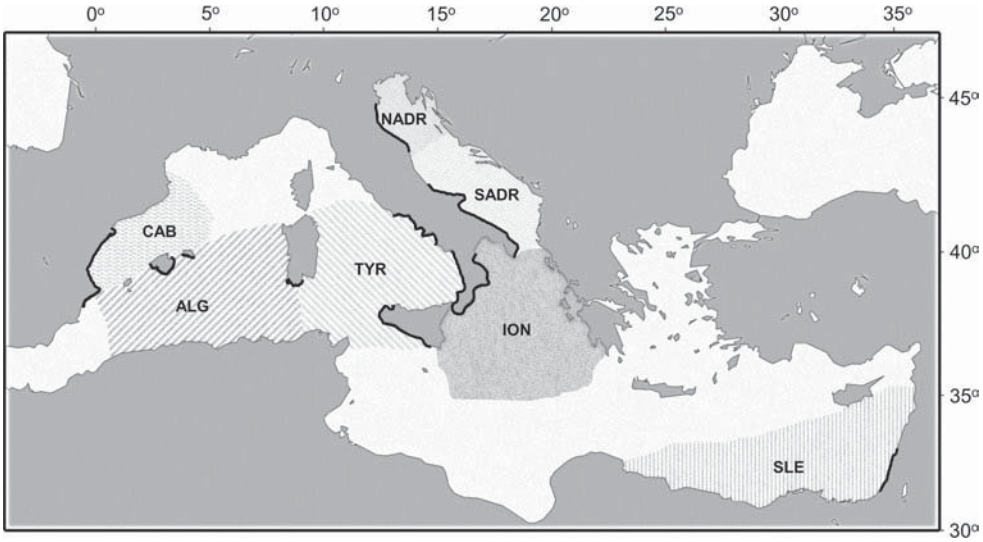

Fig. 1 Foraging grounds for juvenile loggerhead turtles sampled in this study: CAB (the Catalano-Balearic Sea), ALG (the Algerian basin), TYR (the Tyrrhenian basin), NADR (the northern Adriatic Sea), SADR (the southern Adriatic Sea), ION (the northern Ionian Sea) and SLE (the southern Levantine Sea). *Black lines* represent surveyed coastlines

Genetic structuring of foraging grounds

Sequences were aligned with BioEdit version 7.1.6 (Hall 1999) and compared to the 815-bp haplotypes previously described for this species compiled by the Archie Carr Center for Sea Turtle Research of the University of Florida (ACCSTR; http://accstr.ufl.edu). The resulting fragment also contains the 380-bp fragment, traditionally used in molecular studies on marine turtles (Norman et al. 1994).

Our results of the northern Ionian Sea were compiled with haplotype frequencies previously published from the same area (Garofalo et al. 2013) in order to increase sample size. Pseudoreplication between these two sample sets was not expected as all the individuals in this region were found dead in both studies. Compilation of haplotype frequencies for the other foraging grounds also analysed in Garofalo et al. (2013) was not done as individual carapace sizes fell off the considered range for juvenile loggerheads (Margaritoulis et al. 2003; Piovano et al. 2011).

Haplotype diversity (h; Nei 1987) and nucleotide diversity (π; Nei 1987) were estimated for each foraging ground using ARLEQUIN version 3.5 (Excoffier and Lischer 2010) to analyse the genetic diversity of the sampled areas. Pairwise genetic distances (F_{ST}) between foraging grounds were calculated with the DnaSP version 5.10 software package (Librado and Rozas 2009). The significance of genetic differentiation among these regions was assessed using Hudson's nearest neighbour statistic (S_{NN}) with 1,000 permutations. Statistical significance when analysing multiple pairwise comparisons was evaluated with a modified false discovery rate (FDR) (Narum 2006). Pairwise genetic distances between foraging grounds (F_{ST}) were plotted with a principal coordinate analysis (PCoA) inferred with GenAlEx version 6.5 (Peakall and Smouse 2012).

Stock composition

A Bayesian mixed-stock analysis (MSA) was used to assess the composition of each foraging ground as implemented in BAYES (Pella and Masuda 2001). This analysis estimates the proportion of individuals in each foraging ground coming from different rookeries. We used a baseline with a total of 23 rookeries (Supplementary Table 1) analysed in previous studies using the same primer pair (Garofalo et al. 2009; Monzón-Argüello et al. 2010; Yilmaz et al. 2011; Saied et al. 2012; Shamblin et al. 2012; Clusa et al. 2013). This baseline included haplotype frequencies from 10 Atlantic rookeries (Monzón-Argüello et al. 2010; Shamblin et al. 2012) and 13 Mediterranean rookeries (Garofalo et al. 2009; Yilmaz et al. 2011; Saied et al. 2012; Clusa et al. 2013), as loggerheads from both areas may potentially coexist in any of the Mediterranean foraging grounds considered. A 'many-to-many' MSA (Bolker et al. 2007) was not used in the present study because the genetic characterisation of Atlantic foraging grounds based on 815-bp mtDNA fragments is still unknown and this is needed for the 'many-to-many' approach.

Estimates on the size of each rookery (expressed as the mean number of nests per year; Supplementary Table 1) were included in the Bayesian approach as a weighting factor as suggested by previous studies (Bass et al. 2004). Iterated chains were only considered reliable when the Gelman–Rubin criterion was fulfilled (G-R shrink factor <1.2 for all parameters; Gelman et al. 1996). The analyses were undertaken twice: first considering two regional areas (Atlantic and Mediterranean; regional level) and second considering all rookeries as independent units (fine-scale level).

Table 2 Genetic distances (F_{ST}) among Mediterranean foraging grounds for juvenile loggerhead turtles (below diagonal) and S_{NN} significance p values (above diagonal)

	CAB	ALG	TYR	ADR	ION	SLE
CAB		0.032	0.660	0.037	0.100	0.492
ALG	0.194		0.006*	<0.001*	<0.001*	<0.001*
TYR	−0.019	0.164		0.022	0.005*	0.270
ADR	0.071	0.379	0.099		0.002*	0.422
ION	0.058	0.364	0.088	0.040		0.062
SLE	0.012	0.316	0.036	−0.002	0.003	

CAB (the Catalano-Balearic Sea), ALG (the Algerian basin), TYR (the Tyrrhenian basin), ADR (the Adriatic Sea), ION (the northern Ionian Sea) and SLE (the southern Levantine Sea)

* Significant S_{NN} p values after FDR correction for a threshold of $\alpha = 0.05$ ($p < 0.015$)

Results

Genetic structuring of foraging grounds

A total of 17 different haplotypes were found in the Mediterranean foraging grounds analysed (Table 1), all of them described in previous studies. Haplotype CC-A2.1 was the most dominant (70.9 %), followed by CC-A1.1 (10.2 %). Five haplotypes were exclusive to Atlantic rookeries (CC-A1.1, CC-A1.3, CC-A5.1, CC-A10.4 and CC-A14.1), six exclusive to Mediterranean rookeries (CC-A2.8, CC-A2.9, CC-A6.1, CC-A29.1, CC-A31.1 and CC-A32.1) and three shared between Atlantic and Mediterranean rookeries (CC-A2.1, CC-A3.1 and CC-A20.1). The remaining haplotypes (CC-A10.3, CC-A28.1 and CC-A55.1) have only been described in foraging grounds but have not been found in any rookery to date. However, their combined frequency was very low (1.1 %). Overall, haplotype and nucleotide diversities in foraging areas were highly variable (h range: 0.095–0.668; π range: 0.0001–0.0248), with the Algerian basin presenting the highest haplotype (0.668 ± 0.041) and nucleotide (0.0248 ± 0.0123) diversities (Table 1).

Highly significant genetic structuring was found among 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 majority of pairwise statistically significant differences occurred between the Algerian basin and the central eastern side of the Mediterranean (Table 2). PCoA ordination also reflected the deepest differentiation between the Algerian basin and the rest of foraging grounds, explaining 93.89 % of the observed variation with the first two axes (Fig. 2). This analysis also separated the Catalano-Balearic Sea and the Tyrrhenian Sea from the rest, although only by the second axis, which in turn explained only 11 % of the total variation.

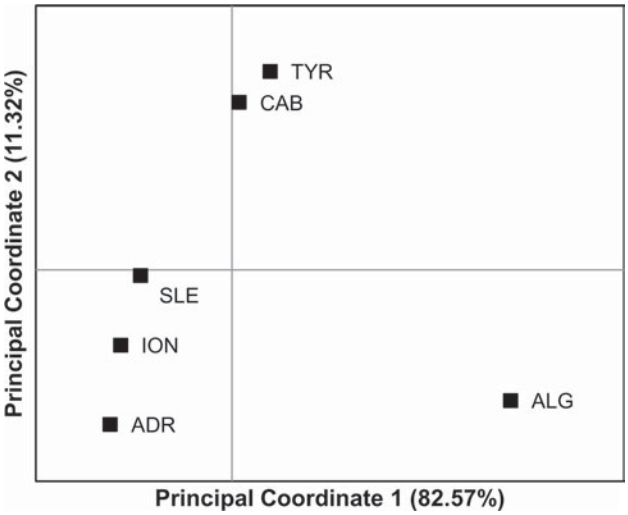

Fig. 2 Principal coordinate analysis based on genetic distances (F_{ST}) between juvenile loggerhead turtles in Mediterranean foraging grounds. Percentage of variation explained by each coordinate included in brackets. Foraging ground acronyms as shown in Table 2

Stock composition

MSA results showed that the deep differentiation between the Algerian basin and the other foraging grounds reported above was due to the overwhelming prevalence of individuals of Atlantic origin in the Algerian basin (Fig. 3). Individuals of Atlantic origin could be detected in all the foraging grounds considered but nowhere was the Atlantic contribution as strong as in the Algerian basin (58.4 ± 11.2 %). Overall, the majority of the Atlantic contribution came from central eastern Florida and south-eastern Florida (CEF and SEF; Supplementary Table 2). All the other foraging grounds studied hosted mainly Mediterranean individuals, with the strongest Mediterranean contribution (Fig. 3) found in the northern Ionian Sea (96.4 ± 3.6 %) and the Adriatic Sea (93.6 ± 16.2 %).

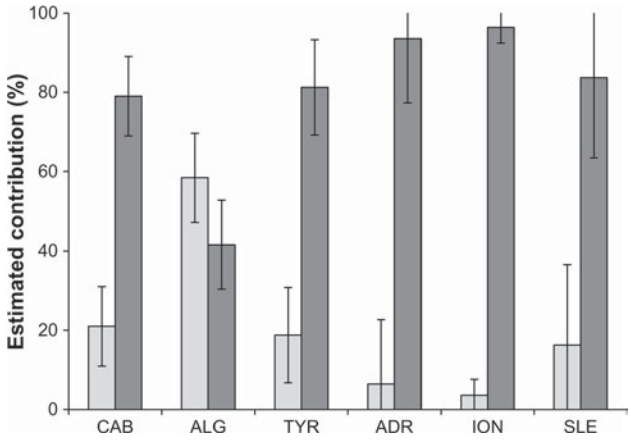

Fig. 3 Atlantic (*light grey*) and Mediterranean (*dark grey*) juvenile contributions to each Mediterranean foraging ground estimated by MSA. Standard deviation bars included. Foraging ground acronyms as shown in Table 2

Results based on unclustered rookeries (Fig. 4, Supplementary Table 2) showed that juveniles from Mediterranean rookeries were not homogenously mixed in the Mediterranean Sea, with major differences between adjoining foraging grounds. While the Adriatic Sea was inhabited by a high proportion of turtles from western Greece (57.8 ± 33.3 %), the northern Ionian Sea hosted individuals mainly from Misrata in Libya (70.4 ± 34.9 %). The Tyrrhenian Sea also hosted mainly individuals from Misrata (47.4 ± 31.3 %), but there was also relevant contribution from Calabria (14.5 ± 12.5 %). Juvenile turtles from Misrata (38.6 ± 29.1 %) and from western Greece (31.3 ± 23.7 %) had a similar abundance in the Catalano-Balearic Sea. Finally, the southern Levantine Sea showed a particularly different composition as this hosted a high proportion of individuals from the easternmost rookeries in the Mediterranean Sea: Israel, Lebanon and Turkey (Supplementary Table 2). However, their contributions were unequal and western Turkey was the source of 28.4 ± 36.6 % of its turtles in comparison with eastern Turkey or Israel and Lebanon (~10 % each).

Discussion

The contribution of different nesting beaches to any particular juvenile foraging ground will depend on the size of the population nesting at each beach and the pattern of surface currents connecting these beaches with the foraging ground (Bowen and Karl 2007; Hays et al. 2010). The largest nesting aggregation of loggerhead turtles in the North Atlantic is found along the coasts of North America (Ehrhart et al. 2003) and is connected with the European coasts by the Gulf Stream (Carr 1986; Bolten et al. 1998). Furthermore, the negative water balance of the Mediterranean Sea generates a permanent eastward flow of Atlantic water at the Strait of Gibraltar (Millot and Taupier-Letage 2004), thus connecting the Mediterranean with the Gulf Stream. The Cape Verde Archipelago hosts the second largest nesting aggregation in the North Atlantic (Marco et al. 2012), but is connected with northern South America by the North Equatorial Current rather than with the Mediterranean Sea (Mansfield and Putman 2013). In this scenario, it is hardly surprising that most of the juvenile loggerhead turtles found in the foraging grounds of the eastern Atlantic and the south-western Mediterranean had a North American origin, with only a few juveniles coming from Cape Verde (Monzón-Argüello et al. 2009, 2010; Carreras et al. 2011; this study).

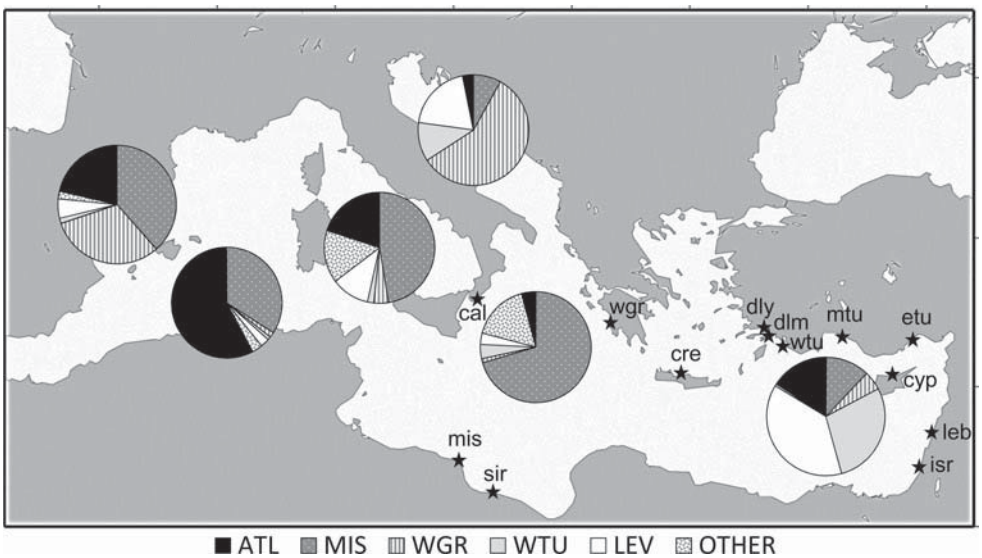

Fig. 4 Fine-scale rookery contributions (%) to Mediterranean foraging grounds estimated by MSA. Rookeries: ATL (Atlantic), MIS (Misrata, Libya), WGR (western Greece), WTU (western Turkey), LEV (Israel; Lebanon; Cyprus; eastern Turkey; middle Turkey; Dalaman and Dalyan, Turkey), OTHER (Sirte, Libya; Calabria, Italy; Crete, Greece). *Stars* show Mediterranean rookery locations

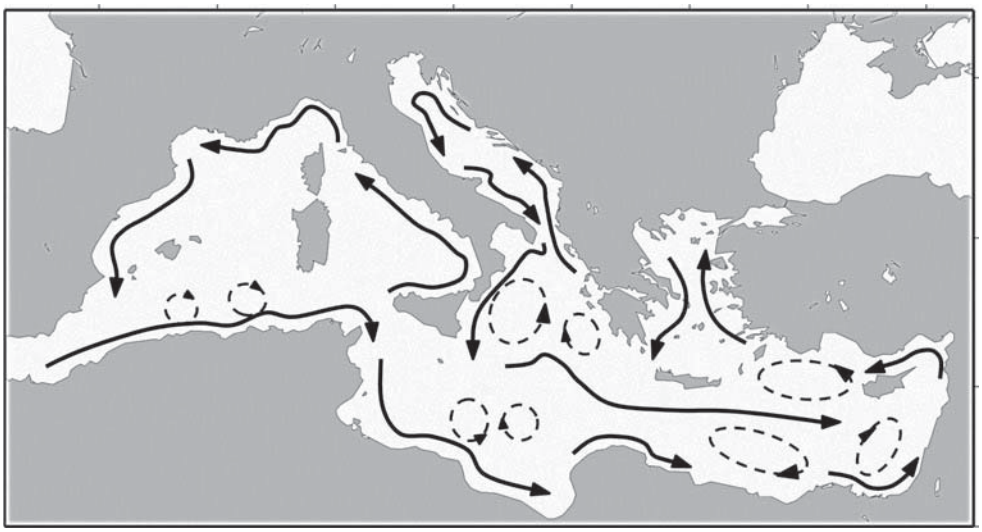

Fig. 5 Main surface circulation patterns of the Mediterranean Sea. Thin dashed lines show transient gyres and eddies. Adapted and modified after Robinson et al. 2001 and Millot and Taupier-Letage 2004

Once into the Mediterranean Sea, Atlantic water flows initially eastwards along the slope of Northern Africa (Fig. 5) and then splits in two major currents, one flowing northwards into the Tyrrhenian Sea and the other flowing eastwards along the coast of Libya to the southern Levantine Sea (Millot and Taupier-Letage 2004). Accordingly, the relative abundance of juvenile loggerhead turtles of Atlantic origin decreases downstream, from the Algerian basin to the Adriatic Sea (Carreras et al. 2006; Maffucci et al. 2006; this study). However, the contribution of Atlantic rookeries to the Algerian basin reported here is lower than that detected in previous studies (Carreras et al. 2006; Carreras et al. 2011). This is because the longer mtDNA fragment allowed the differentiation of the Libyan CC-A2.9 haplotype from the widespread CC-A2.1 haplotype, something impossible with the short fragment. Thus, some of the turtles occurring in the Algerian basin and previously considered of Atlantic origin come actually from Libya.

Conversely, the occurrence of turtles of Atlantic origin in the eastern Mediterranean is higher than previously reported. This is likely to be a consequence of analysing only turtles shorter than 69 cm CCL, as turtles of Atlantic origin migrate back to the Atlantic at an average length of 58.8 cm CCL (Revelles et al. 2007a), and hence, the proportion of turtles of Atlantic origin in any foraging ground will decline when larger turtles are considered. Casale et al. (2008), on the basis of data from Laurent et al. (1998), estimated that only 11 % of the turtles in the southern Levantine Sea had an Atlantic origin, whereas our MSA results based on long fragments indicate a much higher proportion (20 %). It should be noted that the turtles sampled by Laurent et al. (1998) ranged in size from 49.4 to 86.3 cm CCL, whereas here only turtles shorter than 69 cm have been considered. This might also explain why the proportion of turtles of Atlantic origin present in the Adriatic Sea is slightly larger than that previously estimated on the basis of a wider size range (Giovannotti et al. 2010; Yilmaz et al. 2012).

Another methodological difference is the use of population size as a weighting factor for the MSA (Bass et al. 2004), while other studies in the region did not use it (Maffucci et al. 2006). Thus, an underestimation of the contribution of juveniles from Atlantic rookeries could have also occurred in these previous studies as they did not consider the much larger number of nests per year recorded in Atlantic beaches (ca. 100,000 nests per year; SWOT 2007) compared to the Mediterranean (ca. 7,200 nests per year; Casale and Margaritoulis 2010).

The surface circulation pattern might also explain the distribution patterns of turtles from Mediterranean nesting beaches to the different sub-basins. The prevalence in the Adriatic Sea of turtles from western Greece might be explained by the pattern of water entering the Adriatic Sea having previously flowed past the coast of western Greece (Fig. 5; Millot and Taupier-Letage 2004). Likewise, the prevalence of turtles from Libyan beaches in the Ionian Sea may be linked to the mesoscale eddies present in the Ionian Sea (Robinson et al. 2001; Hamad et al. 2006; Hays et al. 2010), which might trap the hatchlings and juveniles swimming off Libya in the sub-basin and prevent dispersal across the eastern Mediterranean (Fig. 5). A proportion of juveniles from Libya might also be trapped in coastal systems and pushed by a westward current to the Algerian basin, the Catalano-Balearic Sea and the Tyrrhenian Sea, where its contribution is also relevant. This westwards dispersal perfectly fits the one suggested by Hays et al. (2010) for hatchlings drifting in the Mediterranean Sea.

Nevertheless, if the hypothesis that currents determine the observed distribution patterns of juveniles is true, a higher proportion of juvenile turtles from western Greece

would be expected to occur in the northern Ionian Sea, as hatchlings swimming off western Greece encounter a water current bifurcation, with one current flowing northwards into the Adriatic Sea and another one flowing south-eastwards (Fig. 5; Hays et al. 2010). Accordingly, half of the adult turtles departing from western Greece migrate to the Ionian Sea after nesting and the other half to the Adriatic Sea (Zbinden et al. 2011; Schofield et al. 2013). In this scenario, the low estimated contribution of western Greece to the foraging grounds in the northern Ionian Sea might be caused by two non-excluding processes. On the one hand, currents flowing off western Greece fluctuate seasonally (Hays et al. 2010) and most hatchlings might emerge when northward flowing prevails, thus drifting to the Adriatic Sea. This hypothesis could be tested combining particle-tracking modelling with detailed data about the seasonality of hatchling emergence at rookeries in western Greece. Expanding this kind of studies to the remaining rookeries in the Mediterranean would improve our understanding of hatchling dispersal within the whole basin. On the other hand, a very large nesting population might exist in Libya (Laurent et al. 1999), which might result in the dilution of contributions from western Greece. Although recently published figures do not support that claim (Casale and Margaritoulis 2010), nest numbers in Libya are poorly known due to political unrest and further research in the region is urgently needed.

The turtles considered in this study ranged from 30 to 69 cm CCL and hence were capable of dispersing independently of prevailing currents within the Mediterranean, except in the Strait of Gibraltar, the Alboran Sea and the Algerian Stream (Revelles et al. 2007a). However, the results reported here revealed genetic structuring consistent with the distribution of water masses and the pattern of surface currents. There is increasing evidence that young turtles become imprinted by the habitats they visit during their developmental migration, which in turn determines the habitats where they will settle and forage as adults (Hatase et al. 2002; Hays et al. 2010; Fossette et al. 2010; Eder et al. 2012). Turtles of Mediterranean origin begin settlement at approximately 40 cm CCL (Casale et al. 2008), which suggests that the genetic structuring here reported might emerge from such a process as imprinting. This, however, might not apply to turtles of Atlantic origin, as their natal rookeries are more than 6,000 km away from the Mediterranean foraging grounds they used as juveniles. This results in a remarkable trade-off between philopatry and habitat knowledge that finally leads them to leave the Mediterranean once they are large enough to overcome the currents in the Alboran Sea and the Strait of Gibraltar and settle in the western Atlantic (Bowen et al. 2005). Accordingly, adult turtles of Atlantic origin are highly scarce in the Mediterranean Sea.

The contributions from specific rookeries to Mediterranean foraging grounds described here are important not only for a better understanding of the biology of this species but also for its conservation. Fisheries bycatch stands as one of the major anthropogenic factors threatening sea turtle populations worldwide (Lewison et al. 2004; Lewison and Crowder 2007, Wallace et al. 2008), and available evidence indicates that tens of thousands of turtles are bycaught incidentally every year around the Mediterranean Sea (Carreras et al. 2004, Lewison et al. 2004; Alessandro and Antonello 2010; Casale 2011; Álvarez de Quevedo et al. 2010, 2013). However, the impact of these high levels of bycatch is unevenly distributed among nesting areas, according to the heterogeneous admixture revealed by genetic markers in this study. For example, bycatch in the western Mediterranean might be a threat for populations nesting in North America and in Libya, but less of a threat for those nesting elsewhere. Likewise, the Tyrrhenian Sea is an important foraging area for turtles not only from Libya but also from Calabria. Thus, bycatch in the Tyrrhenian Sea may directly impact the small nesting population of Calabria. Bycatch in the Adriatic Sea might primarily affect the population nesting in western Greece, whereas bycatch in the Levantine Sea might affect primarily the populations nesting in Turkey, Lebanon and Israel. This shows that knowing the degree of overlap between fishing and foraging grounds is a key factor to protect specific populations nesting in the Mediterranean Sea.

Overall, the present study has revealed previously unknown distributions of Atlantic and Mediterranean juvenile turtles within the Mediterranean Sea at a regional and fine-scale level through the use of population genetics. We highlighted the importance of large studies comprising vast sampling areas (particularly in the case of migratory species) and the use of long fragments of mtDNA as these highly enhance genetic resolution. We have underlined MSA as a useful tool in conservation biology, and with it, we suggest that future management plans include updated genetic assessments of wild populations as a conservation method to unveil population structuring and life-stage-specific distributions.

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Appendix A3

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ORIGINAL PAPER

Different growth rates between loggerhead sea turtles (*Caretta caretta*) of Mediterranean and Atlantic origin in the Mediterranean Sea

Susanna Piovano · Marcel Clusa · Carlos Carreras · Cristina Giacoma · Marta Pascual · Luis Cardona

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Abstract We estimated for the first time the growth rates of loggerhead sea turtles of Mediterranean and of Atlantic origin found in the Mediterranean Sea, combining both skeletochronological and genetic analyses. Our growth models suggested that the growth rate of loggerhead sea turtles of Mediterranean origin was faster than that of their conspecifics with an Atlantic origin exploiting the feeding grounds in the Mediterranean Sea. The age at maturity for Mediterranean origin loggerhead sea turtles, estimated using our best fitting model, was 24 years, which suggests that loggerhead sea turtles nesting in the Mediterranean are not only smaller than those nesting in the western North Atlantic but also younger.

Introduction

Large marine vertebrates have some traits in common, such as late age at maturity and low reproductive rates, that make them highly vulnerable to negative effects of human activities (Lewison et al. 2004). Many threats, such as incidental catch in fishing gear, hunting and habitat degradation, operate on local populations, resulting in a global population decline. As a consequence, many of those large vertebrates, including all seven sea turtle species, are now listed in the IUCN Red List of Threatened Species (IUCN 2010).

In the present study, we focused on the loggerhead sea turtle *Caretta caretta*, a species circumglobally distributed from tropical to temperate waters and currently categorized as "Endangered" (IUCN 2010). The loggerhead sea turtle is the most common sea turtle species in the Mediterranean Sea (Margaritoulis et al. 2003), and it is also a highly migratory species, with individuals capable of migrations spanning thousands of kilometres (Carr 1987; Bolten et al. 1998). Atlantic loggerhead sea turtles enter the Mediterranean Sea through the Strait of Gibraltar (Revelles et al. 2007a; Eckert et al. 2008), so individuals with Atlantic and Mediterranean origin are both present in Mediterranean waters (Laurent et al. 1998; Carreras et al. 2006). However, there is increasing evidence that the proportional contribution of turtles carrying an Atlantic genotype is higher in the western basin and is lower in the eastern basin of the Mediterranean Sea (Carreras et al. 2006).

Analyses using mitochondrial DNA (mtDNA) markers have demonstrated that Atlantic females do not nest regularly in the eastern Mediterranean, as some haplotypes that are frequent in Atlantic nesting beaches (Encalada et al. 1998; Monzón-Argüello et al. 2010) are not detected in the Mediterranean ones (Carreras et al. 2007; Garofalo et al. 2009). Furthermore, Mediterranean loggerhead sea turtles are significantly smaller at maturity than loggerhead sea turtles from other populations (Tiwari and Bjorndal 2000; Margaritoulis et al. 2003) and are thought to settle earlier on neritic habitats than their Atlantic conspecifics (Revelles

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S. Piovano (✉) · C. Giacoma
Dipartimento di Biologia Animale e dell'Uomo, University of Torino, Via Accademia Albertina 13, 10123 Turin, Italy
e-mail: susanna.piovano@gmail.com

M. Clusa · C. Carreras · L. Cardona
Department of Animal Biology and IrBio, Faculty of Biology, University of Barcelona, Avenida Diagonal 645, 08028 Barcelona, Spain

M. Pascual
Department of Genetics and IrBio, Faculty of Biology, University of Barcelona, Avenida Diagonal 645, 08028 Barcelona, Spain

et al. 2007b; Casale et al. 2008a; Cardona et al. 2009). Whether these traits are adaptive or just the result of phenotypic plasticity remains unknown, although biparentally inherited genetic markers indicate a limited gene flow between Atlantic and Mediterranean populations reflected in the high genetic differentiation between them (Carreras et al. in press).

Growth rates in sea turtles have been traditionally estimated from capture-tagging-recapture data (Frazer and Ehrhart 1985; Shaver 1994). This approach suffers from two main problems: variability in recapture interval and researchers' tendency to exclude negative growth rates from data analyses (Snover et al. 2007a). The first leads to an overestimation of annual growth rate if based on summer recaptures and to an underestimation if based on winter-early spring recaptures. The second problem affects the distribution of error in measurements, biasing it towards errors that overestimate growth. Additionally, capture-tagging-recapture methods require long-term labour-intensive efforts (Bjorndal et al. 2001).

Skeletochronology, which relies on the count of growth marks deposited in bone tissue to estimate age, was applied for the first time by Zug et al. (1986) on loggerhead sea turtles. Since then, the technique has been used for ageing green turtles *Chelonia mydas* (Bjorndal et al. 1998; Zug and Glor 1998; Zug et al. 2002; Goshe et al. 2010), Kemp's ridley turtles *Lepidochelys kempii* (Zug et al. 1997; Avens and Goshe 2007; Snover et al. 2007a), olive ridley turtles *Lepidochelys olivacea* (Zug et al. 2006), leatherback turtles *Dermochelys coriacea* (Zug and Parham 1996; Avens et al. 2009) and loggerhead sea turtles from the Pacific (Zug et al. 1995) and Atlantic Oceans (Klinger and Musick 1992, 1995; Parham and Zug 1997; Bjorndal et al. 2003; Snover et al. 2007b, 2010; Snover and Hohn 2004). Previous attempts at using skeletochronology on loggerhead sea turtles found in the Mediterranean Sea (Guarino et al. 2004; Casale et al. 2011a) did not include genotype analyses, and hence, these findings could not be used to separately characterize age and growth for loggerhead sea turtles from the Mediterranean and Atlantic populations.

The primary aim of this study was to estimate growth rates of loggerhead sea turtles with Mediterranean and Atlantic origin, combining skeletochronological and genetic methods.

Materials and methods

Study area

Italy extends into the middle of the Mediterranean Sea, and with its peninsula and main island, Sicily, it geographically divides the eastern from the western part of the Sea. The two parts remain connected through the Strait of Sicily and the Strait of Messina. Data from loggerhead sea turtles stranded along Italian coasts, incidentally captured by Italian fishing vessels or recovered by Italian rescue centres, showed that the size of individuals ranged from small juvenile to adult (bancadati.tartanet.it). Moreover, nesting beaches are known to occur along the south Italian coasts (Mingozzi et al. 2007). These characteristics make Italy an ideal candidate area for the investigation of the growth rates of loggerhead sea turtles carrying a Mediterranean or an Atlantic genotype across sizes spanning small juvenile to adult life stages.

Sample collection

Front flippers from a total of 95 individuals were sampled from 2007 to 2009 for genetic analysis and skeletochronology. Samples were collected from dead loggerhead sea turtles coming from the Adriatic Sea, the Ionian, the Tyrrhenian and the Sardinian Seas, as well as from the Strait of Sicily and the Strait of Messina. The individuals had either been stranded dead (75%) or died at the local Tartanet network of rescue centres during rehabilitation (25%). In addition, two dead-in-nest hatchlings of Atlantic origin found during nest excavation were provided by Brancaleone CTS rescue centre (Calabria, nesting season 2007), and two additionally dead-in-nest hatchlings, previously identified as having a Mediterranean origin, were provided by Riserva Naturale Orientata "Isola di Lampedusa" (Pelagie Islands, nesting season 2006). For all individuals, only curved carapace length (CCL) measured notch-to-tip (Bolten 1999) was available.

The right front flipper was removed during post-mortem examination; muscle or skin samples were collected and stored in 95% ethanol, and the humerus bone was dissected, flensed of tissue, boiled and then allowed to dry in the air for 4 weeks.

Molecular methods

DNA was extracted from the muscle or skin samples using the QIAamp extraction kit (QIAGEN) following the manufacturer's instructions (http://www.qiagen.com).

We amplified a fragment of 815 bp of the control region of the mitochondrial DNA of all the samples using primers LCM15382 (5′-GCTTAACCCTAAAGCATTGG-3′) and H950 (5′-GTCTCGGATTTAGGGGTTT-3′) (Abreu-Grobois et al. 2006), which included the 380-bp region historically surveyed for this species in previous studies within the same area (Carreras et al. 2006, 2007; Casale et al. 2008b; Encalada et al. 1998; Laurent et al. 1998). Sequences were aligned by eye using the program BioEdit version 5.0.9 (Hall 1999) and compared with the short

(~380 bp) and long (~815 bp) haplotypes described for the species in the Archie Carr Centre for Sea Turtle Research Database (accstr.ufl.edu). Furthermore, samples bearing mtDNA haplotypes common to Atlantic and Mediterranean nesting beaches or samples that failed to amplify were genotyped for seven nuclear DNA (nDNA) microsatellites previously used in the species: Cm84, Cc117, Cm72 and Ei8 (Fitzsimmons et al. 1995); Cc141 and Cc7 (Fitzsimmons et al. 1996); and Ccar176 (Moore and Ball 2002), the last one modified as described in Carreras et al. (2007).

Origin assessment of individuals

Individual assignments, including that of hatchlings, were done for all individuals using a combination of microsatellites and mtDNA as described in Revelles et al. (2007a) and Carreras et al. (in press). When a mtDNA exclusive haplotype, from either the Atlantic or Mediterranean nesting area, was present in an individual, this individual was assumed to have originated from the corresponding nesting area. All individuals with mtDNA common haplotypes or haplotypes not assigned to any nesting area were assigned using the seven microsatellites and the STRUCTURE version 2.1 software (Pritchard et al. 2000), considering the baseline developed in Carreras et al. (in press). This baseline included microsatellite data from individuals born in Mediterranean nesting beaches sampled in Carreras et al. (2007) and microsatellite data from Atlantic migrants find in western Mediterranean feeding grounds (Carreras et al. in press) and identified by means of mtDNA Atlantic exclusive haplotypes (Carreras et al. 2007). The probability of each individual to be from either the Atlantic or Mediterranean populations was obtained. Assignation of each individual to either group was accepted when probability was higher than 0.7 for that group.

Skeletochronology: LAG interpretation and age estimation

Humeri were selected because of their capability of retaining more periosteal growth marks than other bones (Zug et al. 1986). Sections were cut at diaphyseal level just distal to the deltopectoral crest (Zug et al. 1986). The medial width was measured with digital callipers to the nearest 0.01 mm, prior to cross-sectioning. A preliminary section 8-10 mm thick was prepared using a diamond saw petrographic cutter (Remet Hergon MT60). Preliminary sections of bone were decalcified in 5% nitric acid (range of decalcification time: 2–71 h) and then washed in tap water to remove any trace of acid. Thin cross-sections 25 μm thick were obtained using a freezing-stage microtome (Reichert-Jung cryocut 1800) and then stained with Mayer's haematoxylin and successively mounted with an aqueous medium (Aquovitrex, Erba).

Digital images of stained cross-sections were acquired at a suitable magnification (ranging from 8× to 12.5×, depending on the size of the section) with Leica Application System LAS EZ v.2.3.0 combined with Leica EZ4 D dissecting microscope. When a section was too large for the camera, partial images were acquired and stitched together using Adobe Photoshop (Adobe System Inc.). Lines of arrested growth (LAGs) were counted by two independent readers (SP and RC) using the microscope. Each section was read three times at a minimum of 7-day intervals by each reader. Each LAG was marked on digital images. A consensus on LAGs count and position was reached for each humerus. To compare pairwise LAGs counts between readers, the Wilcoxon signed rank test was used (Ramsey and Schafer 2002). High-resolution digital images of each cross-section enabled LAGs measurements with the image analysis software ImageJ version 1.43u (http://rsb.info.nih.gov/ij). Humerus diameter, LAG diameter and resorption core diameter were measured along an axis parallel to the dorsal edge of the bone (Goshe et al. 2010). Resorption core diameter included the medullary cavity and any secondary (endosteal) bone deposited in the area of resorption (Curtin et al. 2009), where LAGs were removed (Castanet and Smirina 1990).

Cyclic annual growth mark deposition has been described for loggerhead sea turtles in the Atlantic Ocean (Klinger and Musick 1992; Coles et al. 2001). Injuries, illness or reduction in food supply may have an influence on growth and may cause the development of accessory lines in the bone (Zug et al. 1986). These lines are usually incomplete or less chromophilic than LAGs. To limit the possibility of overestimating the age, we counted only chromophilic complete lines.

A small number of our samples was from turtles which had experienced, on average, 1 month in captivity (mean = 31 days, SD = 35, $N = 24$) in a rescue centre after being found injured. A diffuse mark was detected in the outermost edge of the cross-section of five of those individuals. The outermost edge of the periosteal bone is where the most recent bone is deposited (Enlow 1969); the outermost LAGs were fully visible and could be discriminated from the border of the cross-sections by June in captive-reared European pond turtles *Emys orbicularis* (Castanet 1985) and in Kemp's ridley sea turtles in the Atlantic Ocean (Snover and Hohn 2004). Under the assumption that the same would happen to loggerhead sea turtles in the Mediterranean Sea, the date of recovery for rehabilitation and the date of death of the five individuals were checked, and the diffuse outermost mark was counted as an annual growth mark in the three individuals that died in spring, while it was interpreted as a non-annual accessory mark in the two individuals that died in autumn.

A common feature in sea turtles is resorption and remodelling of the innermost part of the humerus (Zug et al. 1986), which destroys the growth marks deposited earliest in life (Castanet and Smirina 1990). In our study, age estimation was obtained by summing the number of measurable LAGs and the estimation of resorbed LAGs through application of a correction factor protocol (Parham and Zug 1997). Strictly speaking, the number and the diameter of LAGs from humeri that retained the first growth mark were used to estimate the number of resorbed LAGs of remodelled humeri. Based on validation for Kemp's ridley sea turtles (Snover and Hohn 2004, Snover et al. 2007a), a diffuse annulus representing the first year mark was assumed in this study for loggerhead sea turtles. The protocol can be applied only to humeri with a resorption core smaller than the maximum LAG diameter of humeri already aged (Zug et al. 2002). Following Goshe et al. (2010), additional correction factors were developed to extend the protocol and allow age estimation of the whole sample of humeri. Each time, several regression models were assessed to identify the relationship between LAG diameter and LAG number. The best fitting model was chosen on examination of the residuals and R^2 values. The analysis was performed on turtles of Atlantic and Mediterranean origin separately.

Back-calculation and growth rates

To model the relationship between humerus diameter and individual carapace length, we used the equation proposed by Snover et al. (2007b) after validation on Atlantic loggerhead sea turtles:

$$L = L_{\mathrm{op}} + b(D - D_{\mathrm{op}})^{c} \quad (1)$$

where L is the estimated carapace length, L_{op} is the minimum carapace length of a hatchling, D is the medial width of the humerus, D_{op} is the minimum width of a hatchling humerus, b is the slope and c is the coefficient of proportionality.

The back-calculation technique relies on the body proportional hypothesis (Francis 1990) and uses the relationship between marks in hard parts of the body and body length to estimate the length of an individual's body at the time of the formation of the mark.

Growth rates were calculated by subtracting the back-calculated CCL of the inner LAG from that of the outer LAG for each pair of neighbouring LAGs. Growth rates were then assigned to size classes based on the CCL at the beginning of the growth interval (Parham and Zug 1997). Mean growth rate and standard deviation were calculated for each 10 cm size class. Analyses were performed separately for turtles of Atlantic and Mediterranean genotype assignation.

Growth models

Two different approaches were used to model growth. The main approach, based on ageing, was carried out as follows: first, the estimate of age, obtained by skeletochronology (Zug et al. 1986) and application of the correction factor protocol (Parham and Zug 1997; Goshe et al. 2010); then the estimate of the length at time since the last LAG deposition, obtained from back-calculation (Snover et al. 2007b); finally, the fitting of logistic, Gompertz and von Bertalanffy growth curves to length-at-age data, separately for turtles of Atlantic and Mediterranean origin. The asymptotes were fixed using biological data from the literature on Atlantic and Mediterranean populations ($CCL_{max} = 124$ cm in Ehrhart and Yoder 1978; $CCL_{max} = 99$ cm in Margaritoulis et al. 2003, respectively). Akaike's information criterion corrected for small sample sizes (AIC_c) was calculated for each model. The best fitting model was selected on AIC_c scores, ΔAIC_c and Akaike's weights. Additional analyses on nested models were done using the F test (Ramsey and Schafer 2002).

To support these results, we used a secondary approach that was not based on ageing but on the mark-recapture concept. We used back-calculated lengths (Snover et al. 2007b) and time lapse to fit a Fabens' von Bertalanffy growth interval model (as first applied on sea turtles by Frazer and Ehrhart 1985).

The Fabens' (1965) modified von Bertalanffy equation for mark and recapture data:

$$L_r = A - (A - L_c)\mathrm{e}^{-kd} \quad (2)$$

where L_r is the carapace length at recapture, A is the asymptotic carapace length, L_c is the carapace length at first capture, k is the intrinsic growth rate and d is the time between capture and recapture expressed in years. In our case, L_r was the carapace length at the outermost LAG and L_c was the carapace length at the innermost LAG, both estimated using back-calculation, d was the number of measured LAGs and A was fixed using biological data as described above.

Statistical analyses were performed using R version 2.12.1 (R-Development Core Team 2010).

Results

The mtDNA or nDNA markers were amplified successfully from 99 samples, but amplification success was much higher for nDNA markers (77 successfully amplified samples for mtDNA and 99 for nDNA). This differential amplification success was probably because shorter nDNA markers were better preserved in partially degraded samples from dead stranded individuals than the much longer

mtDNA marker used in this study. Furthermore, 26 individuals were impossible to allocate due to the presence of the commonly shared haplotype CC-A2.1 and the lack of conclusive microsatellite results (assigning probability lower than 0.7). As a consequence, only 73 samples yielded reliable origin assessments.

Seven different mtDNA haplotypes were found within the study area: CC-A1.1 (2.6% of the samples), CC-A2.1 (79.2%), CC-A2.9 (6.5%), CC-A3.1 (6.5%), CC-A6.1 (1.3%), CC-A10.3 (1.3%) and CC-A20.1 (2.6%). While unique haplotypes from both the Mediterranean (CC-A6.1 and CC-A2.9) and the Atlantic (CC-A1.1 and CC-A10.3) allowed immediate assignment, samples with shared haplotypes were genotyped by microsatellites to increase assignment capability. A high polymorphism degree was found for all loci, these presenting different alleles ranging from 14 (cc7) to eight (cm72, ccar176, cc117, Ei8) alleles per locus. Overall, eight individuals could be assigned from mtDNA analyses and 65 from microsatellite genotyping, yielding 33 individuals assigned to nesting beaches in the Mediterranean and 40 individuals assigned to nesting beaches in the Atlantic. Skeletochronology was focused on a subset of 65 individuals (30 Mediterranean and 35 Atlantic).

Age estimation

LAGs counts were not statistically different between the two readers (Wilcoxon signed rank test: $p = 0.773$); in addition, consensus on LAGs count and position was reached for each humerus. The age was equal to the number of LAGs in six of the Mediterranean origin turtles, which retained all LAGs (range: 2–4 year). The function that best fit the relationship between LAG diameter (dLAG), in mm, and LAG number (nLAG) in this group of turtles was a power function ($R^2 = 0.69$, $N_{\mathrm{LAGs}} = 14$):

$$\mathrm{dLAG} = 63873 \times (\mathrm{nLAG})^{0.3256} \quad (3)$$

Equation 3 was used to estimate the number of resorbed LAGs for 15 humeri with resorption core diameters smaller than 10.08 mm (corresponding to the largest LAG diameter measured in the previous group of turtles). Visible LAGs were renumbered according to the estimated number of resorbed LAGs, and then data from the two previous groups were joined together to estimate the number of lost LAGs in humeri with a resorption core diameter smaller than 18.15 mm, which included all the remaining samples. dLAG and nLAG in this group followed a linear relationship ($R^2 = 0.89$, $N_{\mathrm{LAGs}} = 101$):

$$\mathrm{dLAG} = 5.7177 + 1.2842 \times (\mathrm{nLAG}) \quad (4)$$

The same correction protocol was applied to turtles of Atlantic origin. The age was equal to the number of LAGs counted in six humeri (range: 1–5 year). In this case, the function that best fit the relationship between dLAG, in mm, and nLAG was a power function ($R^2 = 0.76$, $N_{\mathrm{LAGs}} = 19$):

$$\mathrm{dLAG} = 57307 \times (\mathrm{nLAG})^{0.3704} \quad (5)$$

Equation 5 was used to estimate the number of resorbed LAGs of 10 humeri with resorption core diameters smaller than 10.61 mm. Visible LAGs were renumbered according to the estimated number of resorbed LAGs, and then data from the two previous groups were pooled together to estimate the number of lost LAGs in humeri with a resorption core diameter smaller than 16.65 mm. dLAG and nLAG in this group followed a linear relationship ($R^2 = 0.88$, $N_{\mathrm{LAGs}} = 58$):

$$\mathrm{dLAG} = 5.2598 + 1.1476 \times (\mathrm{nLAG}) \quad (6)$$

Once again, measurable LAGs were renumbered according to the estimated number of resorbed LAGs, and then data from the two previous groups were joined together to estimate the number of lost LAGs in humeri with a resorption core diameter smaller than 25.59 mm, which included all the remaining samples. Also, in this group, dLAG and nLAG followed a linear relationship ($R^2 = 0.91$, $N_{\mathrm{LAGs}} = 130$):

$$\mathrm{dLAG} = 6.3655 + 0.7712 \times (\mathrm{nLAG}) \quad (7)$$

Growth rates

Measured CCL of turtles assigned to the Mediterranean origin ranged in size from 4.2 to 76 cm (mean = 38.1 cm, SD = 15.7; Fig. 1), while measured CCL of turtles with an Atlantic origin ranged from 4.5 cm to 80 cm in size (mean = 45.9 cm, SD = 19.6; Fig. 1). Despite the small sample of hatchlings available, the length of the two hatchlings from Pelagie Islands previously assigned to a Mediterranean origin (mean = 4.2 cm, SD = 0.4) was consistent with the size of hatchlings from Mediterranean nesting beaches (Dodd 1988; Margaritoulis et al. 2003), and the length of the two hatchlings from Calabria assigned to an Atlantic origin (mean = 4.5 cm, SD = 0.1) was consistent with the size of hatchlings originating from western Atlantic nesting beaches (Dodd 1988).

Parameter estimates of Eq. 1 were $b = 37.5524$ and $c = 0.8887$ with $L_{\mathrm{op}} = 4.21$ and $D_{\mathrm{op}} = 1.782$ for turtles assigned to the Mediterranean origin; $b = 30.1919$ and $c = 0.9678$ with $L_{\mathrm{op}} = 4.54$ and $D_{\mathrm{op}} = 1.794$ for turtles with an Atlantic origin. Back-calculated CCLs ranged from 16.5 cm at the innermost LAG to 76.7 cm at the outermost LAG (mean = 35.8 cm, SD = 11.0) for turtles with a Mediterranean origin and from 13.0 cm at innermost LAG to 78.9 cm at outermost LAG (mean = 44.0 cm, SD = 16.3) for turtles assigned to the Atlantic origin.

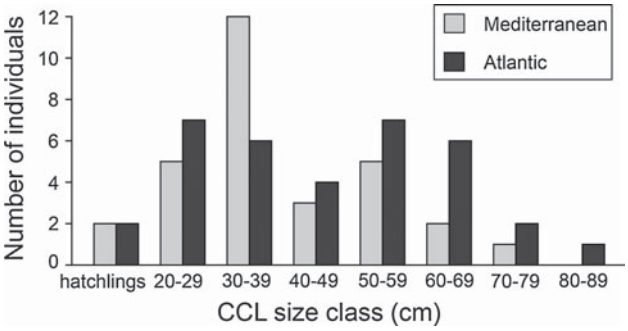

Fig. 1 Frequency distribution of measured CCL class for the subset of loggerhead sea turtles assigned to Mediterranean and Atlantic origin and used for skeletochronological analysis

Size-specific growth rates and standard deviations were calculated on turtles of Mediterranean and Atlantic origin separately (Table 1).

Growth models

Length-at-age data for turtles of Mediterranean and Atlantic assignation (Table 2) were best fitted by the von Bertalanffy growth model (Table 3; Fig. 2). For the Mediterranean group, the von Bertalanffy and the Gompertz growth models were more or less equivalent ($\Delta AIC_c < 2$), while the logistic model was distinguishable; for the Atlantic group, the three growth models were clearly distinguishable based on ΔAIC_c values (Table 3).

The von Bertalanffy models were then the object of further analyses. A full model with two sets of parameters for the Mediterranean and the Atlantic origin data was compared with a reduced model with a common set of parameters for all data. The two models were significantly different (F test: $p = 0.007$), providing evidence that the two populations display different growth rates over the size range of turtles examined in this study. This result was obtained using two different asymptotic values, 99 cm for the Mediterranean group and 124 cm for the Atlantic group. The same statistical difference (F test: $p = 0.008$) was obtained by using a unique value of 99 cm for both groups, which excluded the possibility that the use of two asymptotic values was the cause of the difference in growth. Both the Mediterranean and the Atlantic growth models, which were characterized by a fixed value (asymptote) and two parameters, were then compared with the respective three parameters full models. In this case, we could not discard the null hypothesis (F test: $p = 0.935$ for the Mediterranean and $p = 0.114$ for the Atlantic) so, based on the criterion of parsimony, we chose the reduced models with a fixed value and two parameters.

Using the best fitting model, age at maturation was estimated at 24 years for turtles with a Mediterranean assignation based on the average minimum size of nesting females in the Mediterranean basin (69 cm, in Margaritoulis et al. 2003). A similar result of 23 years was obtained from the Fabens' von Bertalanffy growth interval model. Average size of first-time nesting females from Atlantic populations (98 cm, in Turtle Expert Working Group 2009) was beyond the size range of our sample; thus, estimate of age at maturity could not be extrapolated (Bjorndal and Zug 1995).

The Brody growth coefficient resulting from the model fits was different between the two origin groups, with a higher value for the Mediterranean ($k = 0.042$ year^{-1}, bootstrapped 95% CI: 0.036–0.049; Table 2) and lower value for the Atlantic origin group ($k = 0.023$ year^{-1}, bootstrapped 95% CI: 0.020–0.025; Table 2). Differences in intrinsic growth rates were consistent from both the best fitting model (Table 2) and the Fabens' von Bertalanffy growth interval model, with the estimated Mediterranean rate being higher than the estimated Atlantic rate (Fabens' $k = 0.051$ year^{-1} for turtles with a Mediterranean assignation and 0.036 year^{-1} for turtles with Atlantic assignation).

Table 1 Size-specific growth rates (cm year^{-1}) from estimated CCL at all measurable LAG diameters (total pair of neighbouring LAGs = 254), for individuals assigned to Atlantic and Mediterranean origin

CCL size class (cm)	Atlantic assignation					Mediterranean assignation				
	Mean growth rate (cm year^{-1})	SD	Min	Max	n	Mean growth rate (cm year^{-1})	SD	Min	Max	n
13.0–19.9	4.6	1.8	2.3	8.3	12	5.1	0.6	4.4	6.1	6
20.0–29.9	3.2	1.0	2.3	6.1	17	3.5	1.7	1.5	8.6	31
30.0–39.9	3.0	1.3	0.8	5.6	26	2.9	1.5	0.4	8.6	46
40.0–49.9	3.0	1.2	0.9	5.3	32	2.9	1.0	1.3	5.1	29
50.0–59.9	2.1	1.5	0.2	5.6	25	4.1	1.4	2.5	5.0	3
60.0–69.9	2.7	1.1	0.5	4.0	12	4.4	0.4	4.2	4.7	2
70.0–78.9	1.5	0.4	0.5	2.2	11	3.0	0.6	2.6	3.4	2

Table 2 Growth function parameter estimates for Mediterranean and Atlantic origin loggerhead sea turtles length-at-age data

	Logistic $y = a/(1 + e^{((b-x)/c)})$		Gompertz $y = ae(-b \times c^x)$		von Bertalanffy $y = a(1 - e^{(-b(x-c))})$	
Parameter	b	c	b	c	b	c
Atlantic	24.075	18.278	1.649	0.964	0.023	−7.722
Mediterranean	12.077	10.472	1.513	0.936	0.042	−4.848

Parameter "a" was fixed at 124 cm for the Atlantic and at 99 cm for the Mediterranean populations (maximum length from Ehrhart and Yoder 1978 and from Margaritoulis et al. 2003, respectively)

Table 3 Growth function fitting criteria for loggerhead sea turtles length-at-age data

Model	AIC_c	ΔAIC_c	Akaike's weight
Atlantic			
von Bertalanffy	360.080	0	0.9880
Gompertz	368.943	8.863	0.0117
Logistic	376.424	16.345	0.0003
Mediterranean			
von Bertalanffy	305.677	0	0.6230
Gompertz	307.345	1.668	0.2706
Logistic	309.210	3.533	0.1064

The lowest AIC_c and greatest Akaike's weight indicate the best fitting model

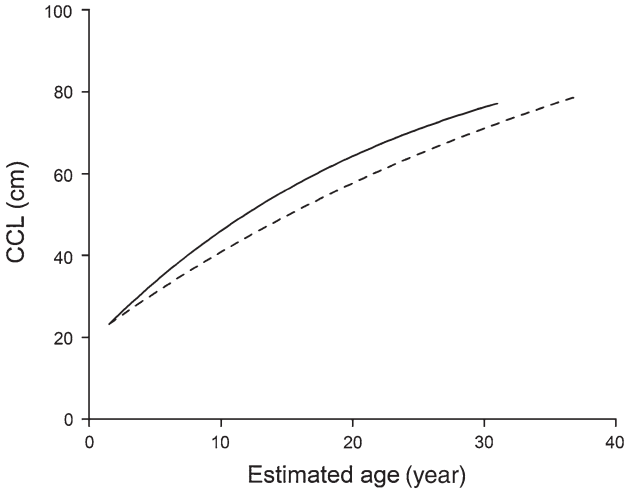

Fig. 2 Length-at-age relationship for loggerhead sea turtles of Mediterranean (*solid line*) and Atlantic (*dashed line*) origin in the Mediterranean Sea as described by the best fitting model, the von Bertalanffy growth model. Curves are limited to the size range of turtles examined in this study. The model predicts that 24 years are required for loggerheads of Mediterranean origin to reach maturation at a size of 69 cm CCL and that 38 years are required for loggerheads of Atlantic origin to grow to 80 cm CCL

Discussion

Despite the fact that at least 25 years have passed since the first application of the skeletochronological method to investigate the age of a sea turtle (Zug et al. 1986), and different histological and LAG measurement techniques have been more or less successfully applied over the years (reviewed in Snover et al. 2007b; Goshe et al. 2009), consensus on the use of the same protocol has not been reached yet.

In this study, we chose to stain thin cross-sections prior to LAG counting, which proved to make LAGs more readable when compared to unstained cross-sections (Goshe et al. 2009). The staining technique was preferred in a large portion of skeletochronological literature on other reptiles (Ehret 2007; Curtin et al. 2008; Kolarov et al. 2010) as well as on amphibians (Leclair and Castanet 1987; Guarino et al. 1995; Seglie et al. 2010).

A serious problem with skeletochronology studies in sea turtles is extensive bone remodelling (Zug et al. 1997), and our study was not an exception. The phenomenon results in erosion of the inner periosteal bone of the humeri, deleting a number of innermost LAGs and leaving fragments of not completely resorbed LAGs that were not measurable. Parham and Zug (1997), who first applied correction protocols, stated that among the three protocols they used, the correction factor protocol "matches best the observed pattern of bone growth in *Caretta caretta*". However, their samples lacked small juveniles and wild, aged individuals. Bjorndal et al. (2003) stated that the correction protocol for age estimation was problematic and avoided its use, but the size of the medullary cavity in their sample of humeri allowed them to estimate that a maximum of two LAGs was lost. On the contrary, our sample was mostly composed of humeri with a medullary cavity larger than the average diameter measured for the first and second LAGs, indicating that in many humeri, more than two LAGs had been resorbed. The correction factor protocol was later applied by Zug et al. (2006), Goshe et al. (2010) and Casale et al. (2011a) on sea turtles, but also by Curtin et al. (2008) on tortoises.

We chose to measure LAG diameters, as previously done by Zug et al. (1995, 1997, 2002, 2006), Zug and Glor (1998) and Snover et al. (2007a, b, 2010). If we had chosen to measure the ventral radii instead, as done by Bjorndal

et al. (2003), probably we would have been able to measure a few more LAGs, but such a method would have been difficult to perform on our sample and would have left us unconfident in the results. We agree with Snover et al. (2007b), who reported that the position of the medullary cavity was generally asymmetrical and that the position of the focus differed among individuals. Thus, the measurements of the radii would have been highly subjective. Even though fragmented LAGs were not used for age estimation, their count proved to be useful in this study as an additional tool to evaluate the goodness of the estimation of the number of missing LAGs produced by each regression model based on the correction factor protocol.

The model that best fit our length-at-age data was the von Bertalanffy, as in previous studies (Klinger and Musick 1995; Zug et al. 1995, 1997; Parham and Zug 1997; Bjorndal et al. 2000, 2001; Snover 2002; Wallace et al. 2008; Casale et al. 2009a, b), but all models suggested a faster growth rate for loggerhead sea turtles of Mediterranean origin than for those of Atlantic origin. The difference in the growth rate of both groups was remarkable, as turtles came from the same feeding grounds, and might be related to differences in physiology or in the habitat use. For example, previous evidence indicates that juvenile loggerhead sea turtles are primarily oceanic in Mediterranean regions where turtles of Atlantic origin prevail (Cardona et al. 2005; Revelles et al. 2007b), whereas juvenile loggerhead sea turtles within the same size range are primarily neritic in areas where turtles of Mediterranean origin prevail (Casale et al. 2008a; Cardona et al. 2009). Differences in the primary productivity of coastal and oceanic regions in the Mediterranean are very large (Bosc et al. 2004), and hence, turtles recruiting earlier to more productive, neritic habitats are expected to grow faster. The reason why turtles of Atlantic origin remain in oceanic environments for an extended time is unknown, but might be related to the necessity of undertaking the long return, migration across the Atlantic (Bolten and Balazs 1995).

The carapace length of female loggerhead sea turtles nesting on Mediterranean beaches is smaller than that of females nesting on the Pacific and the Atlantic coasts (Margaritoulis et al. 2003). The lowest mean CCL, 66.5 cm (range: 60.0–90.0 cm), is from Cyprus, while the highest mean CCL, 84.7 cm (range: 71.9–93.0 cm, in Margaritoulis et al. 2003), is from Kefalonia, Greece. Loggerhead sea turtles in this range of sizes are usually assigned to the subadult stage in the Atlantic and to the adult stage in the Mediterranean. Predictions based on the von Bertalanffy growth model fitted on our Mediterranean origin turtles suggested that loggerhead sea turtles from the Mediterranean population require an estimated average 24 years (bootstrapped 95% CI: 21–27 year) to reach the average minimum CCL for nesting in the Mediterranean, that is 69 cm (Margaritoulis et al. 2003).

Wallace et al. (2008) estimated that loggerhead sea turtles in the Mediterranean Sea would take 14 years in a fast growth scenario and 25 years in a low growth scenario to reach a size of 70 cm CCL. Genetic origin was not ascertained in their study. Our estimation at the same size of 70 cm was of 25 years (bootstrapped 95% CI: 22–28 year) for the Mediterranean origin group, which is in accordance with the low growth scenario, and of 29 years (bootstrapped 95% CI: 27–32 year) for the Atlantic origin group.

Three previous studies based on different methodologies reported the estimation of age at maturity of loggerhead sea turtles from Mediterranean waters. Estimates of years required to reach the lowest mean CCL size of 66.5 cm of females nesting in the Mediterranean ranged 15–16 years based on skeletochronology (Casale et al. 2011a), 16 years based on capture-mark-recapture (Casale et al. 2009a) and 19–23 years based on length frequency analysis (Casale et al. 2011b). Genetics were not considered, so turtles of Atlantic and Mediterranean nesting grounds were likely to be mixed. However, differences in estimations in those studies could be primarily due to the methodologies applied to obtain the growth curve (see Snover et al. 2007b). Our estimation at the same size of 66.5 cm was of 22 years (bootstrapped 95% CI: 19–24 year) for the Mediterranean origin group and 26 years (bootstrapped 95% CI: 24–29 year) for the Atlantic origin group.

Most of the loggerhead sea turtles of Atlantic origin found in the Mediterranean come from the western North Atlantic (Carreras et al. 2006), although a small number from the Cape Verde Islands occur in the western Mediterranean (Monzón-Argüello et al. 2010). Some of the turtles considered for the present study had haplotypes exclusive to the western North Atlantic, and none of them had any of the exclusive haplotypes reported by Monzón-Argüello et al. (2010) for the Cape Verde Islands. As a consequence, most of the turtles assigned to the Atlantic populations are likely to come from the western North Atlantic. The average size of first-time nester female loggerhead sea turtles nesting along the western North Atlantic coasts is 98 cm CCL (range: 87–104, in Turtle Expert Working Group 2009), which is larger than those recorded in our Atlantic group (<80 cm CCL). We did not use our data to estimate the age at maturity for this group because the inference would have required extrapolation of the model beyond the size range of our sample. Despite the different methods used for age estimation, there is increasing evidence that loggerhead sea turtles from the North Atlantic reach sexual maturity around 30 years of age (Frazer and Ehrhart 1985; Crouse et al. 1987; Parham and Zug 1997; Snover 2002), or even later (Bjorndal et al. 2000, 2001; Heppell et al. 2003), and lower projections (Mendonca 1981; Crowder et al. 1994) appear to be

underestimates (Braun-McNeill et al. 2008). If this is true, mature loggerhead sea turtles nesting in the Mediterranean are not only smaller than those nesting in the western North Atlantic, but they are also younger.

According to the above reported size at maturity for Atlantic loggerhead sea turtles, the individuals with Atlantic origin analysed in this study should have been assigned to the juvenile or subadult life stages. However, humeri of the five larger individuals with Atlantic origin, with a CCL ranging from 68 to 80 cm, showed a typical sign of ageing, an ectepicondylar foramen, formed due to the gradual closure of the ectepicondylar groove as age increased (Zug et al. 1986). In the western Atlantic, the minimum size of the CCL of an adult is 87 cm (Turtle Expert Working Group 2009), and complete closure of the groove is reached in a turtle of around 90 cm CCL (Zug et al. 1986) and an age close to 30 years (Bjorndal et al. 2000). Predictions based on the von Bertalanffy growth model suggested that, in the Mediterranean, turtles with Atlantic origin and a CCL ranging in size 68–80 cm should be assigned to an age of around 27 years or older (68 cm CCL: mean 27 year, bootstrapped 95% CI: 25–30 year), which agrees with the closure of the ectepicondylar groove and potential adulthood. Accordingly, these findings suggested that loggerhead sea turtles with Atlantic origin living in Italian waters had a lower rate of growth than loggerhead sea turtles with the same origin but living in the Atlantic Ocean. This might be explained by the much lower productivity of the Mediterranean when compared with the shelf waters along North America (Longhurst 1998). Furthermore, these potentially adult turtles of Atlantic origin have a size similar to that of adult loggerhead sea turtles of Mediterranean origin, which indicates that the turtles of both populations could reach adulthood at the same size if they remain in the Mediterranean long enough. As a consequence, the differences in size at first maturity reported for the Atlantic and the Mediterranean are probably the result of phenotypic plasticity, but should be considered when using demographic models to understand human impacts on these populations.

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