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ORIGINAL PAPER

The contribution of fish to the diet of loggerhead sea turtles in the western Mediterranean revisited

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Abstract

Early juvenile loggerhead turtles (*Caretta caretta*) rely on gelatinous zooplankton, whereas individuals larger than 40 cm curved carapace length are adapted to crush hard-shelled invertebrates. Nevertheless, fish were reported to be the staple food of loggerhead turtles in the western Mediterranean 30 years ago. Here, the temporal consistency of such a fish-based diet of loggerhead turtles is assessed through gut content analysis and stable isotope analysis of samples from the Mediterranean coast of Spain spanning three decades. The gut contents of 134 juvenile loggerhead turtles (curved carapace length range: 27–71 cm) from three different periods (1991, 1999–2008 and 2010–2017) were analyzed, as well as a subsample of the same turtles ($n = 10$ in each period) for both bulk and compound-specific stable isotope ratios (CSIA-AA). Gut content analysis revealed a decline in the frequency of occurrence and numerical abundance of fish and an increasing contribution of gastropods and bivalves throughout time, although pelagic tunicates were always the most frequently observed prey. The $\delta^{15}N_{bulk}$ of turtle bone also dropped throughout the study period, but the values of the stable isotope ratio of N in phenylalanine ($\delta^{15}N_{Phe}$) indicated that 52.5% of that variability was due to a baseline shift over time. Accordingly, the trophic position estimated from CSIA-AA did not follow the decreasing pattern of $\delta^{15}N_{bulk}$, but fluctuated throughout time. The overall evidence indicates that fish consumption by loggerhead turtles in the study region declined through time, but the trophic position of loggerhead turtles did not change simultaneously. This is probably because low trophic prey such pelagic tunicates and filter-feeding bivalves and suspension-feeding gastropods were the bulk of the diet during the whole study period and fish played a minor role, even when their frequency of occurrence peaked. Past levels of high fish consumption might be due to high levels of fishery discards, currently declining because of the recent reduction of the fishing fleet.

Keywords *Caretta caretta* · CSIA-AA · Diet · Gut content analysis · Stable isotope analysis

Introduction

The accurate description of the diet of functional groups is critical when using food modelling to assess the impact of human activities on the dynamics of ecosystems (Heymans et al. 2016). However, methods to study diet composition may differ largely in prey detectability, taxonomic resolution and temporal resolution, (Davis and Pineda-Munoz 2016; Nielsen et al. 2017). Thus, combining some of them is usually needed to gain a thorough understanding of diet composition and produce reliable hypotheses on food web structure.

Loggerhead turtles (*Caretta caretta*) are carnivores widespread in the oceanic and neritic ecosystems of tropical and warm-temperate regions worldwide (Wallace et al. 2010). Historically, their populations were overexploited and are often included in food web modeling because of their relevance in marine conservation (e.g. Coll et al. 2006; Sanchez-Zulueta et al. 2023). Oceanic juvenile loggerhead turtles rely mostly on gelatinous zooplankton (Parker et al. 2005; Frick et al. 2009), but their skull morphology undergoes major changes during ontogeny (Lunardon et al.

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2020) and individuals larger than 40 cm curved carapace length (CCL hereafter) are adapted to crush hard-shelled invertebrates (Hochscheid et al. 2013; Marshall et al. 2015; Figgener et al. 2019; Chatterji et al. 2022). As a result, the diet of neritic juveniles larger than 40 cm CCL and adults is usually dominated by crabs, bivalves, and snails (Seney and Musick 2007; Casale et al. 2008; Wallace et al. 2009; Lazar et al. 2011; Hochscheid et al. 2013; Benhardouze et al. 2021; Molter et al. 2021; Palmer et al. 2021; Mariani et al. 2023; Baldi et al. 2023).

Fish has also been recorded in loggerhead turtles' diet, but their contribution is highly variable and scavenging on fishery discards or fishing bait has often been assumed to be the main source of fish for this species (Houghton et al. 2000; Tomas et al. 2001; Revelles et al. 2007; Seney and Musick 2007; Mariani et al. 2023; Baldi et al. 2023), although they may also capture healthy fish by themselves (Hirama and Witherington, 2012). In this context, the high frequency of occurrence of fishes in the diet of juvenile loggerhead turtles reported for the western Mediterranean Sea several decades ago (43–57%: Tomas et al. 2001; Revelles et al. 2007; Casale et al. 2008) was rather unusual and requires further scrutiny, particularly considering the much lower values reported in more recent studies across the Mediterranean Sea (14–35%: Benhardouze et al. 2021; Palmer et al. 2021; Baldi et al. 2023; Mariani et al. 2023). A decline in the volume of fishery discards during the past two decades (Damalas et al. 2015) may explain such pattern, but temporal analysis of loggerhead turtle diet in the same region are missing and hence no robust testing of this hypothesis is possible.

Gut content analysis is the most common approach to study the diet composition of sea turtles (Revelles et al. 2007; Casale et al. 2008; Lazar et al. 2011; Hochscheid et al. 2013; Benhardouze et al. 2021; Palmer et al. 2021; Mariani et al. 2023; Baldi et al. 2023), but result is hindered by the overestimation of prey with hard parts, such as fish, crustaceans and most mollusks, and the underestimation of soft prey, such cnidarians and pelagic tunicates (Cardona et al. 2012). Stable isotope analysis (SIA hereafter) allows tracking the fate of organic matter along food webs (Jennings et al. 1997; Vizzini et al. 2002; Cardona et al. 2007; Revelles et al. 2007) and $\delta^{15}N$ from total protein ($\delta^{15}N_{bulk}$ hereafter) is broadly used to assess the trophic position of sea turtles (Cardona et al. 2012; Moorehause et al. 2023; Velasquez-Vacca et al. 2023, 2024; Arends et al. 2024), although stable isotope analysis lacks the taxonomic resolution of gut content analysis. Moreover, stable isotope analysis offers an alternative approach to study temporal changes in the diet composition of loggerhead turtles through the study of the skeletal remains housed at museums and scientific collections (de Kock et al. 2023; Velasquez-Vacca et al. 2024), although diet reconstruction through stable isotope analysis might be hindered by the absence of appropriate diet to bone discrimination factors (Velasquez-Vacca et al. 2023).

Diet is not the only source of temporal variability of $\delta^{13}C$ and $\delta^{15}N$ values in animals, as the isotopic baselines may change throughout time (Casey and Post 2011). The massive release of CO_2 resulting from fossil fuel burning has been the major driver of change in $\delta^{13}C$ values since 1850 in marine ecosystems and accounting for such a baseline shift is relatively straightforward (Eide et al. 2017; Clark et al. 2021). Other shifts, such as changes in the relative importance of phytoplankton, benthic primary producers, and terrestrial particulate matter at the base of the food web, are more difficult to address, unless the $\delta^{13}C$ values of primary consumers are known (Bas et al. 2019). Addressing changes in the $\delta^{15}N$ baseline is also challenging, because they may result from several independent processes, such as the relative intensity of nitrogen fixation and denitrification at a local scale (Somes et al. 2010), the circulation of distinct water masses at a regional scale (Sherwood et al. 2011) and anthropogenic eutrophication (Kendall et al. 2007). The latter is particularly relevant off the Mediterranean coast of Spain, where changes in waste-water management have dramatically modified the $\delta^{15}N$ baseline since the late 1990s (Roca et al. 2015).

Compound-specific isotopic analysis of amino acids (CSIA-AA) provides a mean of independently tracking shifts in the N baseline and separating baseline effects from trophic effects on $\delta^{15}N$ values and circumvents the limitations of bulk SIA to cope with shifts in the $\delta^{15}N$ baseline (Ishikawa 2018). The rationale is that the essential amino acids, such as phenylalanine and lysine, experience less trophic fractionation than trophic amino acids, such as glutamic acid, proline, leucine, isoleucine, aspartic acid and valine, as they pass from the tissues of the prey to those of the consumer (McClelland and Montoya 2002; Popp et al. 2007; Chikaraishi et al. 2009; Hannides et al. 2009; Bowes and Thorp 2015). On this ground, the $\delta^{15}N$ value of phenylalanine in the tissue of a consumer depends mostly on the local baseline, whereas the $\delta^{15}N$ value of trophic amino acids in the same tissue depends on the trophic position of each organism. This avoids the need to separately characterize the isotope baseline via field sampling of primary producers or potential prey (e.g. Hückstädt et al. 2017; Quillfeldt and Masello 2020; Durante et al. 2022). However, only a few previous studies have used CSIA-AA to analyze the trophic ecology of sea turtles (Seminoff et al. 2012; Vander Zanden et al. 2013; Arthur et al. 2014), with a recent estimate of trophic discrimination factors for individual amino acids (Lemons et al. 2020).

Here, we use gut content analysis and both bulk and compound-specific stable isotope analyses of the protein

and amino acids in the bone tissue of loggerhead sea turtles from the Mediterranean coastal waters of Spain to study the temporal changes in the composition of their diet and their trophic position from 1991 to 2017. In particular, we tested the hypothesis that fish contribution to the diet has changed through time, a process expected to cause parallel changes in the occurrence of fish in gut contents and the $\delta^{15}N$ values of the bone tissue and the trophic position of loggerhead turtles.

Materials and methods

Turtle sampling

In total, 134 loggerhead turtles were selected for gut content analysis and 30 of them also for stable isotope analysis (Table 1). Turtles in the stable isotope analysis subsample were selected according to CCL, to have a similar average in the three study periods. All the specimens were collected along the Mediterranean coast of Spain, between Cape la Nao and Cape Begur (Fig. 1).

A first subsample included 57 loggerhead turtles seized by Spanish authorities in 1991 in Barcelona (Table 1). These animals had been captured by bottom trawlers somewhere off southern Catalonia and sold illegally for human consumption. They were necropsied at Museu de Ciències Naturals de Barcelona, guts were stored in formalin for immediate dietary analysis (Tomas et al. 2001) and skeletons were preserved dry at the museum collection (https://museuciencies.cat). A second subsample of 80 dead turtles were found stranded or collected at sea, after incidental capture by bottom trawlers or trammel nets along the coast and coastal waters of the Central and North of the Valencia Region (north to Cape la Nao) from 1999 to 2017. Turtles were necropsied at the University of Valencia, guts were frozen at -20 ºC for subsequent dietary analysis, and the left humerus was preserved for both bulk stable isotope analysis and CSIA-AA. This second subsample was subdivided into two time periods: 1999–2008 and 2010–2017 (Table 1).

Gut content analysis

The mouth cavity and esophagus were examined during the necropsies. The contents of the entire digestive tract, from stomach to colon, was sieved (0.40 mm mesh size) and rinsed with water. All the food items from all the turtles with

Table 1 Number and mean curved carapace length (CCL) ± SD (with range in parentheses) of loggerhead sea turtles (*Caretta caretta*) from the western Mediterranean used for gut content analysis and bulk and compound-specific stable isotopes in three periods, i.e., 1991, 1999–2009 and 2010–2017

Period	Year	Gut content analysis		Stable isotope analysis	
		N	CCL	*N*	CCL
1	1991	57	49.6 ± 9.0 (34.0–69.0)	10	55.0 ± 9.4 (40.0–66.3)
2	1999	3		-	
	2000	3		-	
	2001	3		-	
	2002	1		-	
	2003	6		6	
	2004	1		1	
	2005	-		-	
	2006	1		1	
	2007	-		-	
	2008	2		2	
	TOTAL	20	54.6 ± 11.0 (38.5–79.0)	10	49.2 ± 7.6 (41.0–62.0)
3	2010	2		-	
	2011	7		1	
	2012	14		-	
	2013	7		1	
	2014	7		-	
	2015	7		2	
	2016	15		5	
	2017	1		1	
	TOTAL	60	41.9 ± 10.6 (27.0–71.0)	10	54.3 ± 9.0 (38.0–69.0)

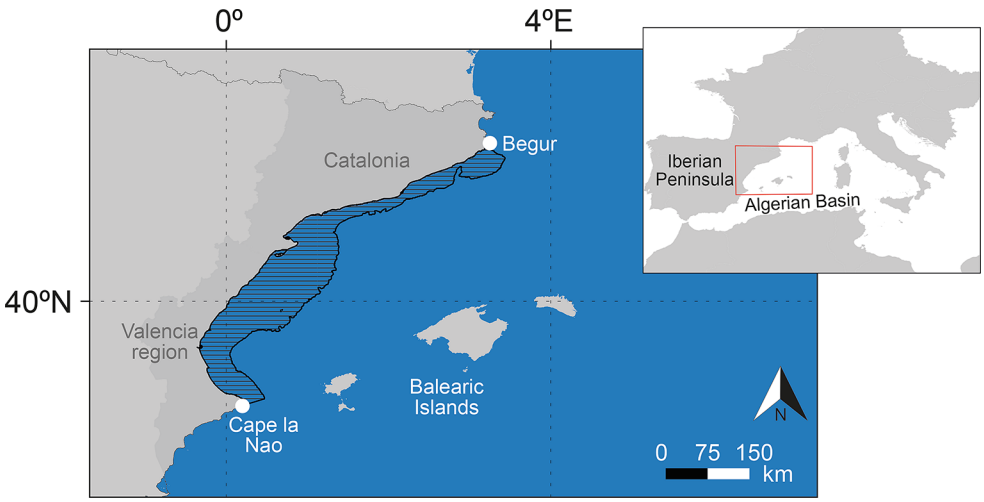

Fig. 1 Continental shelf of the study region (striped area), from Begur to Cape la Cape la Nao, Spain

food in their guts were preserved in 4% formalin (1991) or 70% ethanol (other periods), except otoliths, which were always stored dry. Food items were later identified to the lowest possible taxonomic level and counts of individual prey items (abundances) were estimated (Tomas et al. 2001). In the case of fishes, we made a reference collection of otoliths, scales and bones of western Mediterranean species for comparison. Weight data were not collected because prey were often fragmented or partially digested and identified from the remaining hard parts.

Turtles with empty guts or with unidentifiable prey only ($n=7$, all from 1991) were excluded for the dietary analysis. Values of CCL were $\log_{10}$-transfomed to homogenize variances, and potential differences of CCL between turtles of the three periods (Table 1) were examined with a one-way ANOVA, followed by a Student-Newman-Kewls *post hoc* test. The frequency of occurrence and the mean prey abundance were used to describe the diet of loggerhead turtles in each period. Bootstrapping were used to calculate the 95% confidence intervals of both the frequency of occurrence and the mean abundance per gut.

Bulk stable isotopes analysis

The left humerus of each selected turtle ($n=10$ for each of the three time periods) was drilled across a transversal section near the proximal end. The time window integrated by the bone tissue of the turtles analyzed here, ranging 38–69 cm CCL, is three to seven years, according to the number of growth layers in the cortical bone of loggerhead turtles from the western Mediterranean reported by Piovano et al. (2011).The bone powder was collected in aluminum foil and dried for 24 h in a stove at 50 ºC. A subsample of approximately 2 mg of the bone powder was mixed with 0.5 N hydrochloric acid to remove the mineral matrix and the non-collagen proteins. The pellet was dried again for 24 h at 50 ºC, rinsed with distilled water, dried again and mixed with a 2:1 chloroform: methanol solution to remove lipids (Folch et al. 1957). This solution was changed every 24 h until it was transparent, the liquid was removed and the pellet was dried for 24 h at 50 ºC. Approximately 0.3 mg of the dried pellet were weighed into tin capsules and analyzed for $\delta^{13}C_{bulk}$ and $\delta^{15}N_{bulk}$ using a continuous flow isotope ratio mass spectrometer (ThermoFinnigan Flash 1112 elemental analyzer, CE Elantech, Lakewood, NJ, USA), coupled to a Delta C isotope ratio mass spectrometer via a ConFlo III interface (both from ThermoFinnigan, Bremen, Germany) at the Centres Científics i Tecnològics de la Universitat de Barcelona (CCITUB), Barcelona, Spain (www.ccit.ub.edu).

The abundance of specific stable isotopes is expressed using the δ notation, where the relative variations in stable isotope ratios of C and N are expressed as per thousand (‰) deviations, based on predefined international standards: Vienna Pee Dee Belemnite (VPDB) calcium carbonate for $\delta^{13}C$ and atmospheric nitrogen (AIR) for $\delta^{15}N$. However, due to limited supplies of international standards (VPDB), we instead analyzed secondary isotopic reference materials, which included known isotopic compositions relative to international measurement standards (Cardona et al. 2012). For carbon, isotopic reference materials of known $^{13}C/^{12}C$ ratios, as given by the International Atomic Energy Agency (IAEA, www.iaea.org) in Vienna, Austria, were used for calibration at a precision of 0.05‰. These included polyethylene (IAEA CH_7, $\delta^{13}C=-32.1$‰), L-glutamic acid (IAEA $USGS_{40}$, $\delta^{13}C=-26.4$‰) and sucrose (IAEA CH_6, $\delta^{13}C=-10.4$‰). For nitrogen, isotopic reference materials of known $^{15}N/^{14}N$ ratios were used to a precision of 0.2‰, and these were namely: $(NH_4)_2SO_4$ (IAEA N_1, $\delta^{15}N=+0.4$‰ and IAEA N_2, $\delta^{15}N=+20.3$‰), L-glutamic acid (IAEA $USGS_{40}$, $\delta^{15}N=-4.6$‰), and KNO_3 (IAEA

NO_3, $\delta^{15}N = +4.7‰$). All these isotopic reference materials were employed to recalibrate the system once every 12 samples, and they were analyzed to compensate for any measurement drift over time. The raw data were recalculated taking into account a linear regression that had been previously calculated for isotopic reference materials (Skrzypek 2013).

Compound-specific stable isotopes analysis

For CSIA-AA, a sample of approximately 3 mg of dry bone powder from the left humerus of each of the same loggerhead turtles used for bulk stable isotope analysis ($n = 10$ for each period, see Turtle sampling section above) was hydrolyzed in 1 ml of 6 N HCl at 110 °C for 20 h. A mixture of pure amino acids dissolved in 0.1 M HCl, including alanine, serine, phenylalanine and glutamic acid (Sigma-Aldrich™) with known $\delta^{13}C_{VPDB}$ and $\delta^{15}N_{air}$ was used as external standard. Additionally, 100 µL of a 1000 ppm solution of L-norleucine (#N813, Sigma-Aldrich™) were also added to the standard mixture. The $\delta^{13}C_{VPDB}$ and $\delta^{15}N_{air}$ of each amino acid was obtained by EA-IRMS. The HCl was evaporated to dryness under a gentle stream of N_2 and infrared light. The total free amino acids from the samples and the amino acid standard mixture were derivatized by adding 100 µL of MTBSTFA (#77626, Sigma-Aldrich™), heating to 70 °C and allowing to react for 1 h. The derivatized samples and standards were previously analyzed by GC-qMS (GC6890-MS5975InertXL, Agilent Technologies), not only to ensure the complete derivatization but also to qualify the compounds by means of the elution order and their mass spectrum. Once characterized by GC-qMS, the samples were analyzed isotopically by GC-IRMS using a Trace GC Ultra coupled to a Delta V Advantage isotope ratio mass spectrometer via GC-Isolink with a combustion reactor (1030 °C) and ConfloIV interfaces (ThermoFisher ScientificTM, Germany) at CCiTUB (Figure S1). The analysis was carried out with a constant flow rate of 1.5 ml min^{-1} of He in splitless mode with a temperature of 250 °C in the injector. The amino acids (alanine, glycine, valine, leucine, isoleucine, proline, serine, threonine, phenylalanine, aspartic and glutamic acid + glutamine) were separated on a DB-X5MS column (30 m x 0.32 mm x 1.00 µm) under the following temperature program: 90 °C (1 min) @15°C/min, 140 °C (1 min) @ 6 °C/min, 220 °C (10 min) @ 12 °C/min, 300 °C (13min). The samples were analyzed in duplicate and the standard mixture was interspersed between every two injections. The daily sequence scheme used is shown in Table S1, sample chromatograms are shown in Figure S1 and the results for each turtle sample are reported in Table S2.

Data analysis

Permutational multivariate analysis of variance (PERMANOVA, Anderson et al. 2008) was conducted in PRIMER 6.1.1.3 with PERMANOVA + 1.0.3 (https://www.primer-e.com) to test for overall significant differences in abundance of prey types between periods, a *post hoc* pairwise comparison followed to identify the periods with significant differences (Aznar et al. 2017). Abundance data were first transformed fourth-root to reduce taxa's influential effect with numerous prey individuals, especially pelagic tunicates. After that, a Bray-Curtis similarity matrix was calculated based on the transformed data. A null distribution of pseudo-F statistics was obtained by 20,000 random permutations of elements in the matrix (see Anderson et al. 2008 for details). A test of homogeneity of dispersion based on distances to centroids of each sample (i.e. period) was also carried out (Anderson et al. 2008). When the comparison between periods was statistically significant, individual prey types' contribution to the dissimilarity between them was examined using a SIMPER decomposition of average Bray-Curtis dissimilarities (Clarke and Gorley 2006). We tested the specific hypothesis that fish consumption decreased throughout time based on two complementary tests: (i) a one-tailed Chi-square test for trends to examine changes in the frequency of turtles with fish in the gut, and (ii) a one-tailed Jonckheere-Terpstra test to examine changes in the number of fish individuals for the subsample of turtles with fish in the gut content.

The R package SuessR was used to account for the Suess and Law effects on $\delta^{13}C$ values (Clark et al. 2021). SuessR allows to choose between three regions in the North Pacific Ocean and one in the North Atlantic Ocean. We selected the last one, because the Suess effect is similar across most of the North Atlantic (Eide et al. 2017) and the Western Mediterranean is flooded with surface water from the North Atlantic (Millot and Taupier-Letage 2005). It has selected 2017 as the reference year, so the $\delta^{13}C$ values of samples from different years could be compared.

Seminal research on CSIA-AA suggested phenylalanine and glutamic acid as the reference essential and trophic amino acids respectively (Popp et al. 2007; Chikaraishi et al. 2009). Later research has confirmed the suitability of phenylalanine as the reference essential amino acid (McMahon et al. 2015) but has also revealed that both diet quality (McMahon et al. 2015; Nuche-Pascual et al. 2018) and the excretory nitrogen metabolism of the consumer (McMahon and McCarthy 2016; Ishikawa 2018) may have serious impacts on the $\delta^{15}N$ values of glutamic acid. As a result, McMahon et al. (2015) recommended using proline as the choice of trophic amino acid. Here, the trophic position of turtles was calculated independently using glutamic

acid + glutamine (Glx hereafter) and proline (Pro) as the trophic amino acid, as follows:

$$TP_{CSIA-AA} = 1 + [(\delta^{15}N_{tro} - \delta^{15}N_{Phe} - \beta)/TDF]$$

where $TP_{CSIA-AA}$ is the trophic position of the turtle, $\delta^{15}N_{tro}$ and $\delta^{15}N_{Phe}$ are the $\delta^{15}N$ values in the trophic amino acid considered and phenylalanine of turtles respectively, β is the average difference between the $\delta^{15}N$ value in the trophic amino acid considered and phenylalanine in primary producers and TDF is the average trophic discrimination factor between the trophic amino acid considered and phenylalanine. It should be noted that β differs largely between non-vascular and vascular primary producers, due to the transamination of phenylalanine during the synthesis of lignin in vascular plants (Ishikawa 2018). Furthermore, β differs between Glx and Pro (Chikaraishi et al. 2009, 2010; Ramirez et al. 2021). Here, we calculated the trophic position of loggerhead turtles using Glx or Pro as the trophic amino acid and assumed that phytoplankton is the only source of organic matter for the food web. Accordingly, we used $\beta_{Glx-Phe} = 2.95$ (Ramirez et al. 2021) and $\beta_{Pro-Phe} = 3.10$ (Chikaraishi et al. 2009, 2010). Lemons et al. (2020) reported experimental TDF values for individual amino acids in the skin and blood of green turtles *Chelonia mydas*. Here we used TDF_{skin} (3.21 for Glx and 2.53 for Pro), as collagen is the most abundant protein both on skin and the organic matrix of bone.

General linear models (GLM), followed by a Student-Newman-Kewls *post hoc* test, were used to assess differences in the average values of stable isotope ratios between the three periods (Table 1). The Levene test was used to check for homoscedasticity. All these analyses were done using IBM SPSS Statistics 27 for Windows (https://www.ibm.com).

Results

Gut content analysis

Significant differences were found in the mean CCL of loggerhead turtles sampled for gut content analysis from different periods ($F_{(1, 124)} = 18.24$, $P < 0.001$) and the *post hoc* test revealed that turtles from period 3 were smaller compared to those from periods 1 and 2 ($P < 0.001$) (Table 1). We aimed to reduce this size disparity for SIA and CSIA-AA by subsampling turtles of a similar size (Table 1).

The prey categories most frequently observed in the gut contents of turtles from period 1 were fish, crustaceans and pelagic tunicates, in decreasing order of Fo, and pelagic tunicates, fish and crustaceans in decreasing order of mean abundance (Table 2). In contrast, pelagic tunicates were the prey category most frequently observed in the gut contents of turtles from period 2, both in Fo and numerically, followed by gastropods and crustaceans, with a smaller contribution of fish or cephalopods (Table 2). Finally, bivalves, gastropods and pelagic tunicates, in decreasing order of Fo, or gastropods, bivalves, annelids and pelagic tunicates, in decreased order of abundance (Table 2), were the most frequently observed prey categories in the gut contents of turtles from period 3. While annelids had a similar abundance to pelagic tunicates in period 3, they had a much lower Fo.

The numerical composition of the diet of loggerhead turtles differed significantly among periods (PERMANOVA, $F_{(2, 124)} = 14.59$, $P < 0.001$), as did all the pairwise *post hoc*

Table 2 Frequency of occurrence (fo) and mean abundance per turtle (with 95% CI in brackets) of gut contents of neritic loggerhead turtles off the Mediterranean coast of Spain north to Cape La Nao (see Fig. 1) in three periods (see Table 1). The dominant prey in each period are highlighted in bold type

Prey categories	Period					
	1991 ($n = 47$)		1999–2008 ($n = 20$)		2010–2017 ($n = 60$)	
	Fo (%)	Abundance	Fo (%)	Abundance	Fo (%)	Abundance
Annelids	7.4 (5.6–9.3)	< 0.1 (< 0.1-01)	0.0	0.0	37.5 (27.5–45.0)	2.3 (1.0-3.5)
Bivalves	1.8 (0–2.0)	< 0.1 (< 0.1-1)	25.0 (10–40)	0.6 (0.4-1)	**90.0 (87.5–92.5)**	**4.7 (4-5.3)**
Bryozoans	0.0	0.0	0.0	0.0	2.5 (0.0–5.0)	< 0.1 (0.0-0.1)
Cephalopods	18.5 (13.2–20.1)	0.3 (0.2–0.6)	40.0 (10.0–40.0)	0.6 (0.2–0.6)	30 (20.0–40.0)	0.5 (0.3–0.7)
Cnidarians	5.6 (3.7–7.4)	0.2 (< 0.1–0.3)	15.0 (10.0-250)	0.35 (0.1–0.9)	25.0 (20.0–30.0)	0.4 (0.3–0.5
Crustaceans	**51.9 (48.1–55.6)**	**1.2 (1-1.3)**	**40.0 (15.0–45.0)**	**2 (0.5–2.4)**	27.5 (20.0-35.5)	1.2 (0.7–2.1)
Echinoderms	5.6 (3.7–7.4)	< 0.1 (< 0.1-01)	0.0	0.0	17.5 (15.0–20.0)	0.2 (0.2–0.3)
Gastropods	31.5 (28.7–35.6)	0.4 (0.2–0.6)	**50.0 (25–65)**	**3.7 (1.6–6.2)**	**72.5 (67.5–75.0)**	**4.5 (3.7–5.7)**
Insects	3.7 (< 0.1-5.0)	< 0.1 (0.0-0.02)	5.0 (0.0–5.0)	0.2 (1–02)	22.5 (17.5–25.0)	0.3 (0.3–0.4)
Poriferans	11.1 (9.3–12.9)	0.11 (< 0.1–0.13)	5.0 (0–10)	0.1 (0.0-0.2)	22.5 (20.0-27.5)	0.4 (0.3–0.5)
Scaphopods	0.0	0.0	0.0	0.0	17.5 (12.5–27.5)	0.4 (0.2–0.6)
Fish	**57.4 (53.7–59.3)**	**3.8 (3.1–4.8)**	35 (35.0–45.0)	0.85 (0.6–1.2)	32.5 (27.5–37.5)	0.5 (0.4–0.7)
Benthic tunicates	12.8 (4.3–25.5)	0.5 (< 0.1–0.6)	5 (0.0–15.0)	0.1 (0.0-0.3)	15.0 (5.0-23.3)	0.5(0.3–0.6)
Pelagic tunicates	**42.6 (37.0-47.6)**	**49.7 (43-74.7)**	**75.0 (65.0–95.0)**	**53.0 (31.5–70.5)**	**73.3 (67.7–80.0)**	**1.8 (1.5-2.0)**

comparisons between periods ($P < 0.001$ in all comparisons). The SIMPER analysis indicated that for the comparison between periods 1 and 2, pelagic tunicates and fish (more abundant in period 1), and crustaceans and gastropods (more abundant in period 2) were the main contributors (> 10%) to dissimilarity (Table 3). Between periods 1 and 3, the main contributors were fish (period 1) vs. bivalves, pelagic tunicates and gastropods (period 2), and between periods 2 and 3, the groups were pelagic tunicates (period 2) vs. bivalves and gastropods (Table 3).

Both the frequency of occurrence of fish in the gut contents of loggerhead turtles (Chi-square test from trends, $\chi^2 = 16.19$, 1 df, one-tailed $P < 0.001$) and the number of fish individuals in the subsample of turtles that consumed fish (Jonckheere-Terpstra test, Z= -4.21, $n = 54$, one-tailed $P < 0.001$) decreased in the two most recent periods (Fig. 2).

Table 3 SIMPER pairwise comparisons between three periods: 1 (1991), 2 (1999–2008) and 3 (2010–2017) of the abundance of prey groups that account for ~90% of dissimilarity in the diet of western Mediterranean loggerhead turtles (*Caretta caretta*). Abundance values are fourth-root transformed, and the highest abundance of each pair appears in bold. The percent contribution of each prey group to overall dissimilarity is also shown

	Abundance		Contribution (%)
	Period 1	Period 2	
Pelagic tunicates	0.77	**1.73**	28.4
Fish	**0.95**	0.41	16.7
Crustaceans	**0.63**	0.54	13.2
Gastropods	0.33	**0.71**	12.9
Cephalopods	0.27	**0.43**	8.5
Bivalves	0.02	**0.31**	4.7
Cnidarians	0.08	**0.17**	3.7
Benthic tunicates	**0.19**	0.06	3.1
	Period 1	Period 3	
Bivalves	0.02	**1.21**	17.2
Pelagic tunicates	0.77	**0.87**	13.3
Gastropods	0.33	**1.07**	12.7
Fish	**0.95**	0.27	12.5
Crustaceans	**0.63**	0.45	9.5
Cephalopods	0.27	**0.49**	7.5
Annellids	0.13	**0.38**	5.2
Benthic tunicates	0.19	**0.20**	4.4
Cnidarians	0.08	**0.31**	4.2
Sponges	0.13	**0.26**	3.8
	Period 2	Period 3	
Pelagic tunicates	**1.73**	0.87	19.2
Bivalves	0.31	**1.21**	16.1
Gastropods	0.71	**1.07**	13.1
Crustaceans	**0.54**	0.45	9.5
Cephalopods	0.43	**0.49**	8.2
Fish	**0.41**	0.27	6.7
Cnidarians	0.17	**0.31**	5.5
Annellids	0.00	**0.38**	4.6
Insects	0.11	**0.26**	4.2
Sponges	0.06	**0.26**	3.6

Conversely, the frequency of occurrence of pelagic tunicates ($\chi^2 = 10.11$, 1 df, one-tailed $P = 0.001$) increased in the two most recent periods, but the number of tunicates in the subsample of turtles that consumed them (Jonckheere-Terpstra test, Z = 2.21, $n = 79$, one-tailed $P = 0.011$) decreased in the third period.

Stable isotope analysis

The loggerhead turtles from the three study periods selected for stable isotope analysis ($n = 10$ per period for both bulk and CSIA-AA stable isotope analysis) did not differ in average CCL (Fig. 3A, Levene statistics = 0.312, df = 2,27, $P = 0.735$, GLM, $F_{2,29} = 1.312$, $P = 0.286$). The average $\delta^{15}N_{bulk}$ of loggerhead turtles varied throughout the study period (Levene statistics = 0.270, df = 2,27, $P = 0.765$, GLM, $F_{2,29} = 5.088$, $P = 0.013$) and statistically significant differences were observed between 1991 and the other two periods, as revealed by a *post hoc* Student-Newman-Kewls test (Fig. 3B). The average $\delta^{15}N_{Phe}$ of the loggerhead turtles also varied throughout the study period (Fig. 3C, Levene statistics = 0.092, df = 2,27, $p = 0.912$, GLM, $F_{2,29} = 5.088$, $P = 0.013$) and statistically significant differences existed also only between 1991 and the other two periods, according to a post hoc Student-Newman-Kewls test. It should be noted that $\delta^{15}N_{bulk}$ and $\delta^{15}N_{Phe}$ were positively and significantly correlated ($r = 0.743$, $p < 0.001$), but changes in $\delta^{15}N_{Phe}$ explained only 52.5% of the variance in the $\delta^{15}N_{bulk}$ values. This might suggest that most of the drop in the $\delta^{15}N_{bulk}$ values observed from 1991 to the other two study periods resulted from changes in the baseline.

The trophic position ($TP_{CSIA-AA}$) of loggerhead turtles estimated using Glx as the trophic amino acid were statistically lower than those based on Pro ($TP_{Glx-Phe} = 2.1 \pm 0.9$, $TP_{Pro-Phe} = 3.6 \pm 0.9$) and they evolved differently through the study period (Leven statistics = 1.672, df = 5, 54, $P = 0.157$; GLM: model: $F_{5,59} = 56.506$, $P < 0.01$; trophic amino acid: $F_{1,59} = 117.924$, $P < 0.01$; period: $F_{2,59} = 57.290$, $P < 0.001$; interaction: $F_{2,59} = 25.012$ $P < 0.001$).

The average $\delta^{13}C_{bulk}$ changed significantly through the study period (Fig. 2E, Levene statistics = 0.042, df = 2,27, $p = 0.959$, GLM, $F_{2,29} = 4.364$, $P = 0.019$), as dropped from the first to the second study period and increased again in the third one, according to a *post hoc* Sudent-Newman-Kewls test. Correcting for the Suess effect ($\delta^{13}C_{Suess}$) did not change this pattern (Fig. 2F, Levene statistics = 0.142, df = 2,27, $P = 0.864$, GLM, $F_{2,29} = 3.672$, $P = 0.039$).

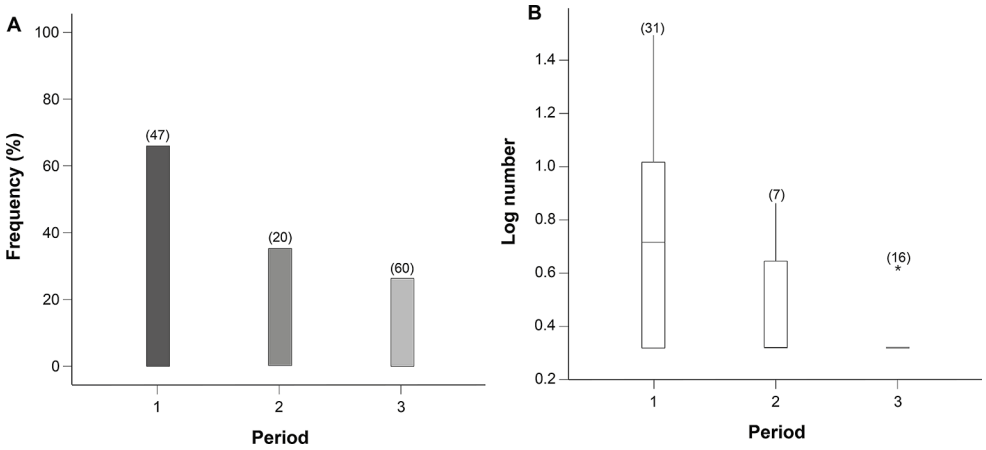

Fig. 2 Changes in fish consumption by western Mediterranean loggerhead sea turtles, *Caretta caretta*, in three periods: period 1 corresponds to 1991, period 2 to 1999–2008 and period 3 to 2010–2017. (**A**) Percent frequency of occurrence of fish during each time period is shown by the gray bars and (**B**) $\log_{10}$-transformed number of fish individuals in loggerhead turtle gut content samples. Lines represent medians and the asterisk marks an outlier value. Numbers above bars indicate the number of turtles in each category

Discussion

The results of the gut content analysis reported here revealed a diet shift for neritic loggerhead turtles off the Mediterranean coast of Spain throughout the study period, as fishes and crustaceans were replaced by gastropods and bivalves as major prey from 1991 to 2017. However, CSIA-AA did not detect any consistent drop in the trophic position of loggerhead turtles. These contrasting results might be explained by the high contribution of low trophic position prey to the diet of loggerhead sea turtle throughout the whole study period (pelagic tunicates in the two first periods and gastropods and bivalves in the third one) and also because of the existence of several confounding factors discussed below.

Both pelagic and benthic tunicates are primarily herbivores (Dadon-Pilosof et al. 2019), Mediterranean bivalves are suspension feeders (Antit et al. 2016) and Mediterranean gastropods exhibit a diversity of feeding modes (Antit et al. 2016), although *Turritella* spp., the gastropod genus most often consumed by the loggerhead turtles studied here, are deposit feeders (Graham et al. 1938). Conversely, most Mediterranean benthic crustaceans are omnivores or carnivores (Cartes et al. 2002) and most Mediterranean coastal fishes are carnivores, with trophic positions higher than 3 and usually higher than 4 (Karachle and Stergiou 2017). This is also true for the fish species most often recovered from the gut contents analyzed, such as European sardine (*Sardina pilchardus*), European hake (*Merluccius merluccius*) and horse mackerels (*Trachurus* spp.). On this ground, a drop in the consumption of fish and crustaceans and an increase in the consumption of bivalves and gastropods from 1991 to 2017 was expected to result in a drop in the trophic position of loggerhead turtles during the study period, as far as these groups make a relevant contribution to the nutrients assimilated by loggerhead turtles.

The $\delta^{15}N_{bulk}$ of turtle bone certainly dropped from 1991 to 1999–2008 and 2010–2017, but $\delta^{15}N_{Phe}$ values also dropped in parallel, thus indicating a shift in the isotope baseline. Actually, the variation in the $\delta^{15}N_{Phe}$ values explained 52.5% of the variability in the $\delta^{15}N_{bulk}$ values of the loggerhead turtles analyzed here and hence most of the temporal change observed was due to a baseline shift. This conclusion is consistent with the baseline shift already observed in seagrasses and deep-sea fishes from the study area (Roca et al. 2015; Fanelli et al. 2016) and attributed to a dramatic increase in the number of functional waste-water treatment plants from 1990 to 2010 (Roca et al. 2015). This is because waste-water is highly enriched in ^{15}N compared to natural sources and waste-water treatment plants reduce both the total amount of N compounds in water discharges and its $\delta^{15}N$ values (Kendall et al. 2007).

$TP_{CSIA-AA}$ was expected to account for such a shift in the $\delta^{15}N$ baseline and still detect the replacement of fish and crustaceans by bivalves and gastropods as major prey of loggerhead turtles, but this was not true. $TP_{CSIA-AA}$ based on Pro revealed only small, fluctuating and biologically non-significant differences in the trophic position of loggerhead turtles throughout the study period, whereas $TP_{CSIA-AA}$ based on Glx increased from study period 1 to study period 2 and remained high during study period 3. It should be noted, however, that Glx resulted in $TP_{CSIA-AA}$ values well below those expected for a consumer relying heavily on suspension feeders. Conversely, Pro resulted in $TP_{CSIA-AA}$ values highly consistent with those expected for a consumer with such a diet. The reason for such differences is beyond the scope of the present study, but supports the evidence indicating that Glx can be more sensitive than Pro

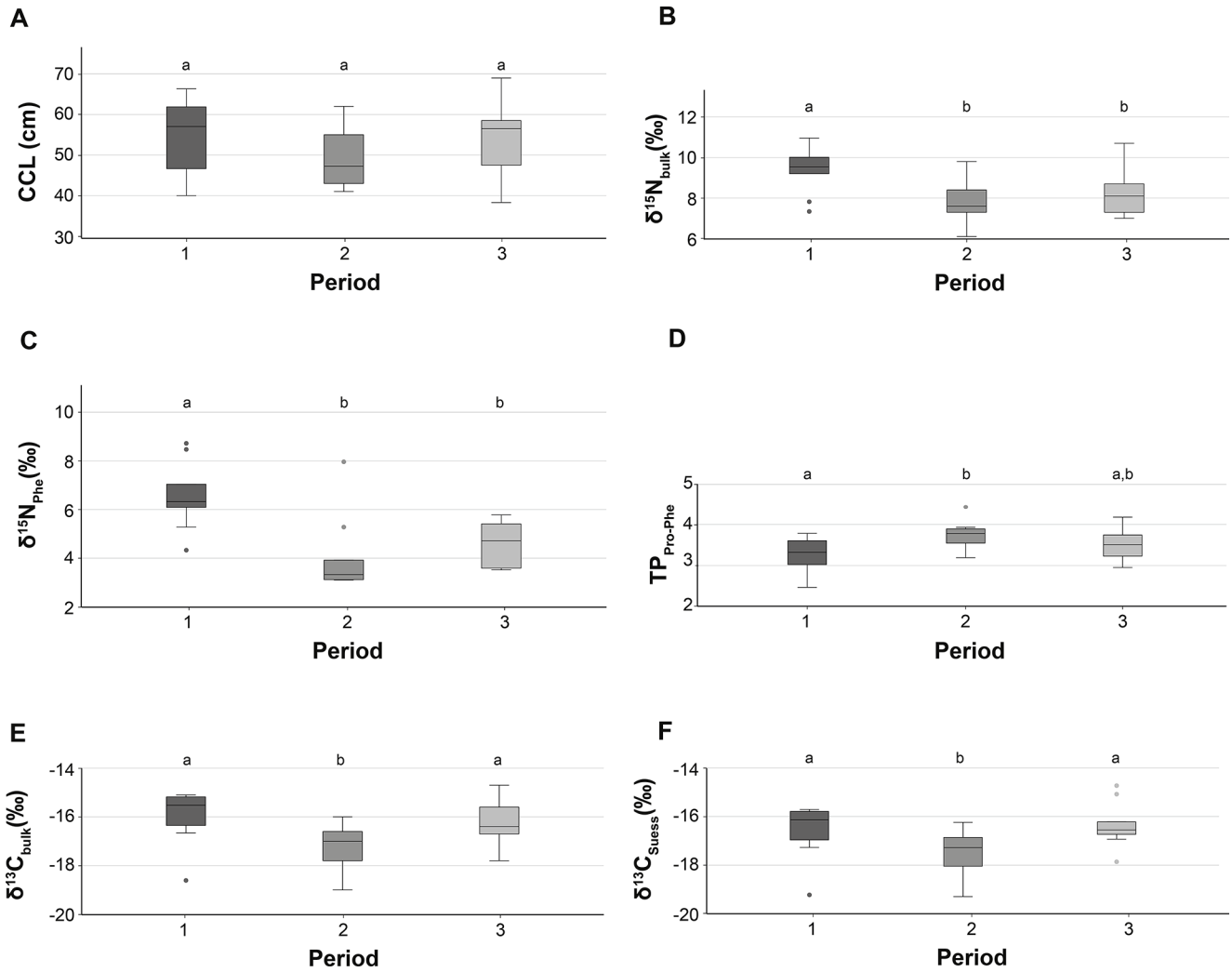

Fig. 3 Boxplots for curved carapace length (CCL; **A**), $\delta^{15}N$ in bone collagen ($\delta^{15}N_{bulk}$;**B**), $\delta^{15}N$ in bone phenylalanine ($\delta^{15}N_{Phe}$;**C**), trophic position according to CSIA-AA ($TP_{CSIA-AA}$;**D**), $\delta^{13}C$ in bone collagen ($\delta^{13}C_{bulk}$,**E**) and $\delta^{13}C$ after correcting for the Suess and Laws effects ($\delta^{13}C_{Suess}$,**F**). Period 1 corresponds to 1991, period 2 to 2007–2009 and period 3 to 2015–2017. The box (50% of the data) represents first (25% of the data) and third quartiles (75% of the data), the line the median, and the whiskers the 95% confidence interval of the median. Different superscripts indicate statistically significant differences between periods. Sample size is 10 for each period

to diet quality (McMahon et al. 2015 and 2016). Furthermore, the results reported here are influenced by four additional confounding factors.

First, the carapace length of the loggerhead turtles whose gut contents were analyzed varied largely within and among the three studied periods, whereas individuals in the smallest size classes were avoided for CSIA-AA and hence the average CCL was slightly higher. This might have reduced the variability in the trophic position estimated through CSIA-AA and resulted in a more consistent trophic position throughout the study period. Additionally, some of the changes through time in the frequency of occurrence and abundance of prey may result from changes in the size structure of the sea turtle samples and the smaller average CCL of the loggerhead turtles whose gut contents were analyzed in the third study period might explain a smaller consumption of fish. This hypothesis could be tested easily by removing the smallest turtles from the sample, but sample size was too small to produce meaningful results.

Second, gut content analysis informs only about the prey ingested in the last days before death, whereas bone integrates dietary information over several years (Velasquez-Vacca et al. 2023). The time window integrated by the bone tissue of the turtles analyzed here, ranging 38–69 cm CCL, is three to seven years, according to the number of growth layers in the cortical bone of loggerhead turtles from the western Mediterranean reported by Piovano et al. (2011). As a result, there is no temporal overlap between the dietary information captured by the stable isotope ratios of the turtles from the two first study periods, whereas only one of the

turtles from the third study period might overlapped temporally with two turtles from the study period 2.

Third, geographic scale is another major difference between the dietary information captured by gut content analysis and stable isotope ratios in the bone tissue. This is because immature loggerhead turtles roam broadly over the continental shelf of the study area throughout the year (Cardona et al. 2009) and hence the stable isotope ratios in their bones integrate dietary information also from a broad area. Conversely, the stomach content comes from a discrete feeding patch, exploited only a few days or hours before capture. If fish is consumed only sporadically by loggerhead turtles, and scavenging on discarded fish increases significantly the risk of bycatch, the contribution of fish to the diet of loggerhead turtles will be overestimated by stomach content analysis, whereas little evidence of fish consumption will be incorporated to the stable isotope ratios of their tissue. This temporal and geographic mismatch explains, for instance, the dissimilarity between the stomach contents of bottlenose dolphins (*Tursiops truncatus*) entangled in trammel nets and the diet revealed by the stable isotope analysis of biopsies from live dolphins (Giménez et al. 2017). It should also be noted that the smaller size of the sample used for stable isotope analysis is not a concern here, as the longer time window of the bone tissue integrated reduces variability compared to the snapshot revealed by gut content analysis (Davis and Pineda-Munoz 2016).

Fourth, gelatinous zooplankton is often overlooked in gut content analysis, particularly fragile jellyfish, whereas the contribution of prey species with hard skeletal elements is overestimated (Cardona et al. 2012). This suggests that the actual contribution of fish to the diet of loggerhead turtles might be much lower than suggested by the results of the gut content analysis reported here. On the contrary, pelagic tunicates are often overlooked, but they occurred in most of the gut content analyzed in the three periods, although abundance decreased in the most recent study period. Pelagic tunicates may form dense blooms, which explain why hundreds of tunicate individuals occurred in the gut of a few turtles. The decrease in the average number of tunicates may suggest less frequent and intense tunicate blooming during the third study period, but there is no additional evidence confirming such hypothesis. In any case, the overall evidence suggests that low trophic position prey, such pelagic tunicates, deposit-feeding gastropods and suspension-feeding bivalves, may represent the bulk of the diet of loggerhead turtles and hence determine largely their trophic position, although weight data would be needed to better assess their relevance. If this group was the bulk of the diet of loggerhead turtles, changes in the frequency of occurrence of other prey items would be irrelevant for the trophic position of loggerhead turtles, although their contribution would be magnified by the high detectability of otoliths. This hypothesis is consistent with the average trophic position of loggerhead turtles estimated by CSIA-AA, which ranged from 3.2 to 3.7 through the study period and hence was close to the value expected for a predator relying massively on primary consumers such as pelagic tunicates, suspension-feeding bivalves and deposit-feeding gastropods (somewhere between 3 and 4). Mixing models would offer an independent test for this hypothesis, but prey from the two first study periods are not available for analysis and published TDF values are unsuitable for samples including both cortical and medullar bone (Velasquez-Vacca et al. 2023).

Fifth, $TP_{CSIA-AA}$ is highly sensitive to the relative contribution of vascular and non-vascular plants at the base of food webs. This is because vascular and non-vascular primary producers differ dramatically in the average difference between $\delta^{15}N_{Pro}$-$\delta^{15}N_{Phe}$, due to the deamination of phenylalanine to synthesize lignin increased largely $\delta^{15}N_{Phe}$ in vascular plants (Chikaraishi et al. 2009, 2010; Ishikawa 2018). Although phytoplankton is often assumed to be the main source of baseline organic matter in marine food webs, this is not necessarily true in coastal areas, where seagrasses and terrestrial particulate organic matter can also be relevant. The relative contribution of detritus from terrestrial C3 plants and phytoplankton to the organic matter in the sediments off the Mediterranean coast of Spain depends largely on the distance to river mouths and depth (Rumolo et al. 2015; Quirós-Collazos et al. 2017; Ausín et al. 2023), but the actual contribution to the pool of organic matter fueling the food web is poorly known. Assuming phytoplankton as the only relevant source of organic matter and using Pro as the trophic amino acid results into a $TP_{CSIA-AA}$ estimate for loggerhead sea turtles highly consistent with the results of gut content analysis (3.6 ± 0.9 throughout the study period). However, changes in the actual contribution of phytoplankton and vascular plants to the diet of the prey consumed by loggerhead sea turtles may explain some of the temporal variability observed in the $TP_{CSIA-AA}$ of loggerhead sea turtles and have obscured the expected drop resulting from a lower availability of discarded fish. This hypothesis is supported by the slight drop in the $\delta^{13}C_{Suess}$ values of loggerhead sea turtles from 1991 to 2007–2009, consistent with a higher contribution of ^{13}C-depleted terrestrial detritus to the food web. Additional studies using essential amino acid fingerprinting (Arthur et al. 2014) would help disentangling this question.

Despite all these drawbacks, CSIA-AA has been useful here to reveal that the decline in the $\delta^{15}N_{bulk}$ of loggerhead sea turtles from the Mediterranean coast of Spain from 1991 to 1999–2008 was mostly the result of a baseline shift. As the latter explained 52.5% of the observed variability

in the $\delta^{15}N_{bulk}$ of loggerhead sea turtles, the reduction in the consumption of fish and crustaceans decreased the average $\delta^{15}N_{bulk}$ of loggerhead sea turtles by only 0.75‰. If the diet to consumer TDF of the cortical bone of loggerhead sea turtles is similar to that of green sea turtles (5.1 ± 1.1‰, Turner Tomaszewicz et al. 2017), the trophic position of loggerhead sea turtles decreased only by 0.15 from 1991 to 1999–2008. Hence, the overall evidence reported here indicates that fish consumption by loggerhead sea turtles off the Mediterranean coast of Spain has declined dramatically during the past three decades, although such reduction had no relevance for their trophic position. Past levels of high fish consumption might be linked to high levels of fishery discards (Tsagarakis et al. 2014; Damalas et al. 2015) and the recent reduction of the fishing fleet in the study area (Martín 1991; Anuario Digital de Estadística https://www.mapa.gob.es/es/estadistica/temas/estadistica-digital/powerbi-pesca.aspx) might have resulted in a lower availably of discarded fish for loggerhead sea turtles. In any case, fish currently play a minor role in the diet of loggerhead sea turtles off the Mediterranean coast of Spain, where gastropods, bivalves and pelagic tunicates prevail. This conclusion indicates that the diet of juvenile loggerhead sea turtles in the western Mediterranean has been rather constant throughout the past three decades and hence their ecological niche has not changed. Furthermore, the results reported here stress the need to combine several methods (gut contents, bulk SIA, and CSIA-AA) to better understand the diet of consumers.

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Data availability Data will be made available at the repository of Universitat of Barcelona upon acceptance.

Declarations

Conflict of interest The authors disclose no conflict of interest.

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