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$\delta^{13}C$ and $\delta^{15}N$ in ancient and recent fish bones from the Mediterranean Sea

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The naturally occurring stable isotopes of nitrogen ($^{15}N/^{14}N$) and carbon ($^{13}C/^{12}C$) in animal tissues have been used successfully to establish trophic relationships and sources of nutrients in several marine food webs. Nitrogen isotopes show a stepwise enrichment between prey and consumer tissues through assimilation processes, while carbon isotopes remain practically unchanged, reflecting the isotopic signatures of primary productivity sources. This technique has been applied to the reconstruction of past human diets, with particular emphasis on the relative importance of marine resources in prehistoric economies. In the present study, $\delta^{13}C$ and $\delta^{15}N$ values were determined for fish remains from the Early Bronze Age site of *Cova des Riuets* on the east coast of the Balearic island of Formentera and bone samples from corresponding species of modern fish were also analysed to provide a stable carbon and nitrogen baseline for palaeodietary studies in the western Mediterranean. The results show a great variability within marine ecosystems in terms of the stable isotope compositions of fish. This may be partly due to the natural complexity of marine ecosystems, but also to human influence on the environment, which can lead to a wide range of local responses to changing conditions. All this means that much caution is required when using data from modern ecosystems to infer past human diets.

Keywords: stable isotopes, Mediterranean Sea, fish bone collagen, trophic chain

Introduction

The faunal remains from archaeological sites provide important information on which species were likely to contribute to the dietary protein intake of prehistoric peoples (e.g. Grayson & Delpech 1998; Marean & Assefa 1999; Storå 2000; Rabinovich & Hovers 2004). Unfortunately, these results are often biased by the preservation of the archaeological bones, which depends on various factors surrounding their deposition and on the size and robustness of the bone itself. Small, frail bones from fish, birds and micromammals tend to be affected by taphonomic processes that can lead to their destruction at archaeological sites and thus the obliteration of any direct evidence of human consumption. The effects of post-depositional

diagenesis must be assessed on a site-by-site basis, but alterations due to human action on bones before their deposition are generally difficult to assess archaeologically. Such practices as cooking with moderate heat or the removal of fish heads at capture sites tend to weaken the bones (Andrus & Crowe 2002) or reduce their numbers at dwelling sites. Moreover, even if the bones are preserved, gentle and precise excavation techniques are needed in order to recover minute frail bones (Zohar & Belmaker 2005). For all these reasons, fish bones tend to be underrepresented in archaeological contexts.

Furthermore, in cases where a population had access to more than one protein source, it is difficult to determine the proportion derived from each source on the basis of zooarchaeological information alone.

These problems can be partially solved by means of stable isotope analysis. The stable carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) isotope ratios of animal tissues, and particularly bone collagen, can be used to quantify the human consumption of foods with different isotopic compositions (DeNiro & Epstein 1978, 1981; Schoeninger & DeNiro 1984) and to establish trophic relationships among the animal species within an ecosystem (Minagawa & Wada 1984; Fry 1988). These isotopes either behave in a predictable manner between trophic levels, thus indicating trophic position ($\delta^{15}N$), or remain virtually unchanged, reflecting the isotopic signatures of primary productivity sources ($\delta^{13}C$). Stable isotope values of fish are unpredictable, since their trophic position may change geographically and/or seasonally depending on nutrient availability (Jamieson et al. 2004). Jennings et al. (1997) suggest that many inshore reef-associated fish species have a capacity for switching their trophic position within food webs in response to local circumstances. This supports the idea that the trophic positions of species in food webs are dynamic rather than fixed, since this would allow them greater adaptive flexibility to respond to site-specific changes in food availability. In our study area, the Mediterranean, Pinnegar and Polunin (2000) have found that trophic plasticity and omnivory were widespread in the fish that they analysed. Marine food chains are more complex and less well defined isotopically than terrestrial ones (e.g. Hobson & Montevecchi 1991; Iken et al. 2001), and such factors complicate the study of marine ecosystems even more.

We are concerned here with fish remains from an archaeological site on the Balearic island of Formentera and modern counterparts caught for comparison purposes in local waters between the islands of Formentera and Ibiza. The stable isotope values obtained for the ancient and recent fish bones are then compared to see whether there are any differences and, if possible, to obtain some idea of the causes of these differences, which could stem from factors connected with seasonal/geographical variation, habitat changes due to environmental fluctuations/contamination or diagenetic processes affecting the buried bones.

At the same time, we use the analysis of our material to compare two methods of collagen extraction from recent bone and to evaluate the use of one extra step in the standard procedure in order to eliminate lipids that might interfere with the isotopic results. The lipid content of archaeological bones is assumed to be unimportant, since it is not likely that lipids could withstand long periods of environmental exposure (Chisholm et al. 1983). Ambrose (1990), for example, found only 0.1% lipid material in a 2500-year-old antelope bone from Africa. Some results obtained for modern bones nevertheless show that lipids may interfere with stable isotope analyses and that these should consequently be removed prior to collagen extraction (Ambrose, 1990). There are not enough data to determine whether this degreasing treatment should be applied to the analysis of all kinds of recent bones, and comparison of the results is further complicated by the variety of lipid extraction protocols (DeNiro & Epstein 1978; Chisholm et al. 1983; Ambrose 1990; Lidén, Takahashi & Nelson 1995; Dufour et al. 1999). Most works applying degreasing procedures have dealt with recent mammal bones (Chisholm et al. 1983; Lidén et al. 1995) and have had little to do with fish (Dufour et al. 1999). Mammal and fish bones differ in their lipid composition, and it is important to find out when degreasing techniques should be applied.

The main goals of this study may be summarised as follows: to investigate whether there are differences in stable isotope values between ancient and modern fish of the same species, to add new stable isotope data for the understanding of ecosystems in the Mediterranean, an area where very few studies have been performed, and to compare two methods for extracting collagen from modern bone, with and without previous pre-treatment for the removal of lipids.

Material

The fish bones analysed were found at *Cova des Riuets,* Formentera, Balearic Islands. *Cova des Riuets* is a cave site situated on the hill of *La Mola*, the highest point on the island. The site lies 100 metres above sea level, but is very close to the shore (Fig. 1). The cave was

used as a midden during the Bronze Age, and it is believed that the habitation area was outside it. The fish bones, together with other animal remains, were excavated during the summer of 2002. The same archaeological level yielded pottery sherds, seeds and charcoal, and was chronoculturally ascribed to the second millennium BC (Trias & Roca 1975). This corresponds to the Early Bronze Age and constitutes one of the earliest known indications of human activity on the island. We chose to analyse all the fish bones available from the archaeological excavation, which, due to the taphonomic processes mentioned above, amounted to only five.

We also analysed recent fish bones of the same five species as were found at the site (Table 1). These fish were caught in local waters between the islands of Ibiza and Formentera (Fig. 1). Although muscle collagen composition may be regarded as just as representative as that of bone (Sholto-Douglas et al. 1991), we chose to determine the isotopic composition from bone collagen in the recent fish in order to allow a better and more direct comparison of the results with the isotopic signatures of the collagen in the archaeological fish bones. Since fish tend to show significant differences in bone morphology, size and composition, the bones analysed for each of the recent fish were anatomically the same ones as those preserved in the archaeological sample (Table 1).

All the fish bones used for this purpose were from adult specimens, as growth has a considerable influence on the composition of isotopic tissue in ectothermic

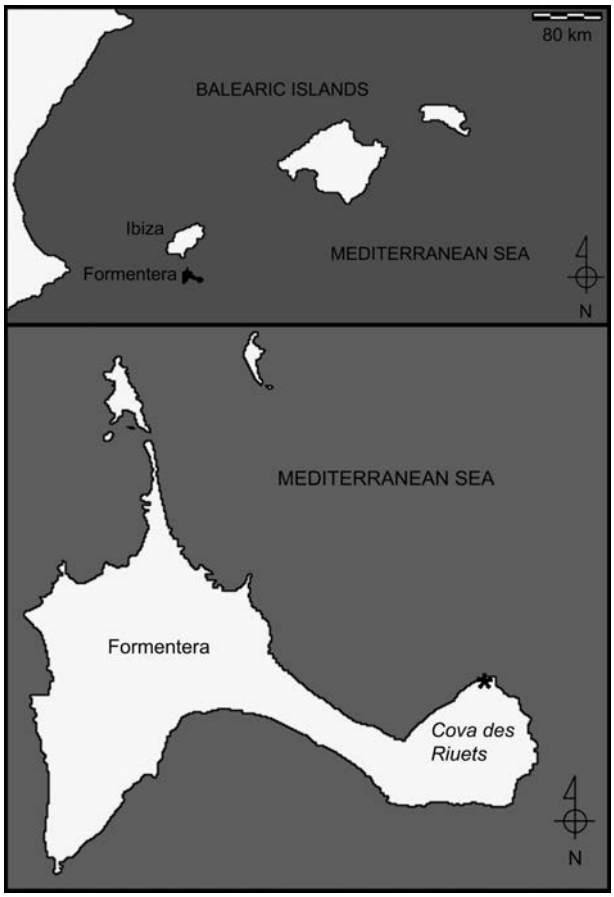

Figure 1. Above: The Balearic Islands in the western Mediterranean Sea. The recent fish studied here were caught in the waters between Ibiza and Formentera. Below: The Island of Formentera and the location of Cova des Riuets (*) on the hill of La Mola.

Table 1. Description of the fish bones analysed and the C:N ratio and stable isotopes results. The ~~marked~~ results falls outside collagen quality range (DeNiro 1985; van Klinken 1999). (d) denotes samples that underwent the degrease treatment with chloroform-methanol.

Sample	Species	Place	Bone	C:N	$\delta^{15}N$	$\delta^{13}C$
1	*Epinephelus marginatus* (Dusky grouper)	*Cova des Riuets*	Preoperculare bone	3.3	10.1	-10.5
2	*Epinephelus marginatus* (Dusky grouper)	Formentera waters	Preoperculare bone	3.2	11.4	-8.4
2d	*Epinephelus marginatus* (Dusky grouper)	Formentera waters	Preoperculare bone	3.2	10.7	-8.3
3	*Coris julis* (Rainbow wrasse)	*Cova des Riuets*	2 vertebrae	Not enough collagen		
4	*Coris julis* (Rainbow wrasse)	Formentera waters	2 vertebrae	~~4.0~~	~~7.4~~	~~-18.1~~
5	*Pagellus erythrinus* (Common pandora)	*Cova des Riuets*	1 Precaudal vertebra	3.2	8.2	-11.0
6	*Pagellus erythrinus* (Common pandora)	Formentera waters	1 Precaudal vertebra	3.3	10.1	-13.1
6d	*Pagellus erythrinus* (Common pandora)	Formentera waters	1 Precaudal vertebra	3.5	10.3	-14.1
7	*Muraena helena* (Moray eel.)	*Cova des Riuets*	1 vertebra	Not enough collagen		
8	*Muraena helena* (Moray eel.)	Formentera waters	1 vertebra	3.2	10.8	-13.7
8d	*Muraena helena* (Moray eel.)	Formentera waters	1 vertebra	3.3	10.8	-13.8
9	*Sphyraena sphyraena* (Mediterranean barracuda)	*Cova des Riuets*	1 Precaudal vertebra	3.5	9.4	-12.4
10	*Sphyraena sphyraena* (Mediterranean barracuda)	Formentera waters	1 Precaudal vertebra	3.4	7.8	-14.0
10d	*Sphyraena sphyraena* (Mediterranean barracuda)	Formentera waters	1 Precaudal vertebra	3.2	7.9	-13.7

animals (Minagawa & Wada 1984; Badalamenti et al. 2000; Gao et al. 2004; Jamieson et al. 2004) and variations in isotopic values may reflect growth processes rather than trophic position.

A detailed list of the fish bones analysed is given in Table 1 and an example of the material is shown in Figure 2.

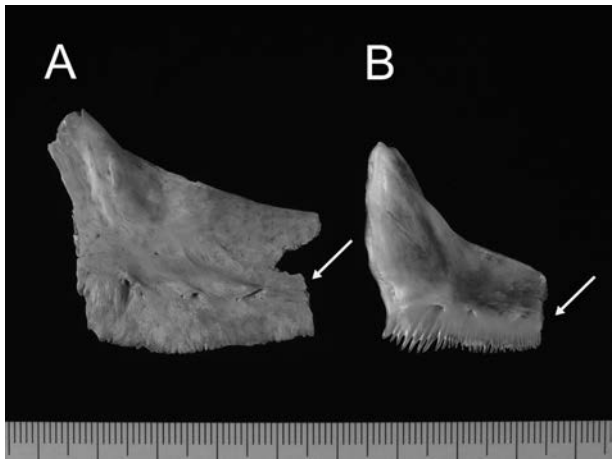

Figure 2. Ancient (A) and recent (B) preoperculare bones of *Epinephelus marginatus* as used in the comparative analysis, after sample extraction (↑). Photo by O. Clavell.

Method

Sample preparation

The archaeological fish bones were cleaned manually with a brush and scalpel to remove all visible contaminants. The bones were further cleaned by air abrasion.

The recent fish specimens were boiled and dissected. After removing the flesh by scraping, the bones were rinsed several times by sonication in distilled water and then oven-dried at 50°C.

Bone collagen extraction

Two collagen extraction methods were employed.

All the samples, archaeological and recent, were subjected to the standard collagen extraction procedure used at the Department of Archaeological Science, University of Bradford (United Kingdom), as outlined by Richards and Hedges (1999). Depending on the amount available, 10 to 700 mg of bone was demineralized in a 0.5 M HCl solution at 4°C for 2–3 days and then rinsed 3 times with deionized water until the pH became neutral. Collagen was extracted by solubilization in a hot weak HCl solution for 48 hours (70°C, pH 3). The product was then filtered, ultrafiltered (30 kDa), frozen and freeze-dried for 48 hours.

Collagen was extracted from a second set of the recent samples (excluding sample 4 due to its small size) with an additional chloroform-methanol treatment in order to remove lipids that might interfere with the results (Chisholm et al. 1983; Dufour et al. 1999). The bones were soaked for 24 hours in a 2:1 methanol:chloroform solution prior to the demineralization in HCl, following the method suggested by Kates (1986). The extracting organic solvent was removed by soaking the bone in distilled water.

Analytical measurements

Elemental (C and N) and isotopic ($\delta^{13}C$ and $\delta^{15}N$) measurements of collagen extracts were performed with a CN elemental analyser (Roboprep) coupled to a Europa Scientific Geo 20/20 isotope ratio mass spectrometer. Elemental carbon and nitrogen were measured, allowing calculation of the C:N ratio, given as a percentage (%), which denotes the biochemical quality of samples (DeNiro 1985; van Klinken 1999). The isotopic results are reported in terms of $\delta^{13}C$ and $\delta^{15}N$ relative to the vPDB standard and atmospheric N_2 (AIR), respectively, in parts per thousand (‰). Each sample was run in duplicate, except samples 1 and 4, which were run once due to the small amount of collagen recovered. Internal standards were routinely analysed in the same way as the samples in order to control the analytical accuracy (one standard with each set of 10 samples). The analytical error (1σ) for both $\delta^{13}C$ and $\delta^{15}N$ was ±0.2‰ or less.

Results and Discussion

Reliability of the results

The carbon and nitrogen isotopic values and atomic C:N ratio for the fish bone collagen samples are presented in Table 1. For various reasons it was not possible to include all the samples in the interpretation. Samples 3 and 7 did not yield enough collagen for isotopic measurement; sample 4d lacked the initial bone weight required for the degreasing treatment, and sample 4 was excluded as it had a C:N ratio above 3.5. All the other samples had atomic carbon to nitrogen ratios in the range 3.2–3.5 and therefore retain their *in vivo* isotopic signatures according to the criterion proposed by DeNiro (1985).

Table 2. Comparison of the two collagen extraction methods used on modern fish bones. Values marked with (d) were obtained after the degreasing treatment. Δ indicates the value for the untreated sample minus that for the treated sample.

Sample	C:N	C:N d	Δ C:N	$\delta^{15}N$	$\delta^{15}N$ d	Δ $\delta^{15}N$	$\delta^{13}C$	$\delta^{13}C$ d	Δ $\delta^{13}C$
Dusky grouper	3.2	3.2	0	11.4	10.7	0.7	-8.4	-8.3	-0.1
Common pandora	3.3	3.5	-0.2	10.1	10.3	-0.2	-13.1	-14.1	1.0
Moray eel.	3.2	3.3	-0.1	10.8	10.8	0	-13.7	-13.8	0.1
Med. barracuda	3.4	3.2	0.2	7.8	7.9	-0.1	-14.0	-13.7	-0.3

Table 3. Comparison of C:N ratios and isotopic values between recent (R) and ancient (A) fish bones, and the differences (Δ) between them.

Sample	C:N A	C:N R	Δ C:N	$\delta^{15}N$ A	$\delta^{15}N$ R	Δ $\delta^{15}N$	$\delta^{13}C$ A	$\delta^{13}C$ R	Δ $\delta^{13}C$
Dusky grouper	3.3	3.2	-0.1	10.1	11.4	1.3	-10.5	-8.4	2.1
Common pandora	3.2	3.3	0.1	8.2	10.1	1.9	-11.0	-13.1	-2.1
Med. barracuda	3.5	3.4	-0.1	9.4	7.8	-1.6	-12.4	-14.0	-1.6

Comparison between the two collagen extraction methods used with modern fish bones

The comparison between isotopic values and C:N ratios obtained using the two extraction methods is presented in Table 2, with the differences expressed as Δ (value for the untreated sample minus value for the treated sample)**.** The Δ C:N values range from -0.2 to 0.2‰, with a mean of -0.02 (S.D. = 0.2, n = 4), the Δ $\delta^{15}N$ values from -0.2 to 0.7‰, with a mean of 0.1‰ (S.D. = 0.4, n = 4), and the Δ $\delta^{13}C$ values from -0.3 to 1‰, with a mean of 0.17‰ (S.D. = 0.57, n = 4). Thus the chloroform-methanol treatment seems to have had little influence on the bone collagen isotopic values and their C:N ratios, the variations in which are close to the value for the analytical precision (±0.2‰ or less).

We did not compare the yield values (mg of final lyophilized collagen/mg of initial dry bone weight) obtained by the two collagen extraction methods since yield values are of relatively little significance because of their dependence on various parameters (Dufour et al. 1999). Due to the small amount of bone available for the experiment, the initial weight was very low, in some cases only 10 mg. This could lead to a low collagen yield, since proportionately more material is lost at each extraction step than with larger samples. The use of ultrafilters, as described under Methods, also lowers the collagen yield by about 50% or more (Müldner & Richards 2005). Nicholson (1998) found in her investigations on bone diagenesis that, in contrast to mammal and bird bones, most fish bones exhibit a substantially reduced collagen yield.

Lipids are more depleted in ^{13}C than other biochemical fractions (DeNiro & Epstein 1977), and lipid contamination will lead to a decrease in $\delta^{13}C$ values by as much as -7‰ as compared with bone protein (DeNiro & Epstein 1978). Moreover, since lipids lack nitrogen atoms, lipid-contaminated bones would have higher C:N ratios with $\delta^{15}N$ remaining unchanged. This is not the tendency observed in our results.

For this reason, we believe that it may not be necessary to follow the lipid extraction procedure when analysing modern fish bones, though this assumption should be treated with caution due to the small number of samples involved. Dufour et al. (1999), who extracted collagen from two recent marine specimens by the same two procedures, found only a minor isotopic difference between the methods (Δ C:N = -0.1‰ and Δ $\delta^{13}C$ = -0.3‰ for both samples). Elsewhere, Ambrose (1990) has reported stable isotope values for one modern fish bone (Nile perch) in which lipids represented 0.11% of the bone and 0.62% of the bone collagen. It is possible that the small amount of initial bone used in our experiment made the influence of the lipid content negligible, whereas this may not be the case when analysing recent mammal bones, as shown by other authors (Chisholm et al. 1983; Lidén et al. 1995).

On the other hand, fish lipid content is known to vary with the reproductive stage or nutritional state of the fish and also seasonally (Focken & Becker 1998), which suggests caution and a need for further work on this subject.

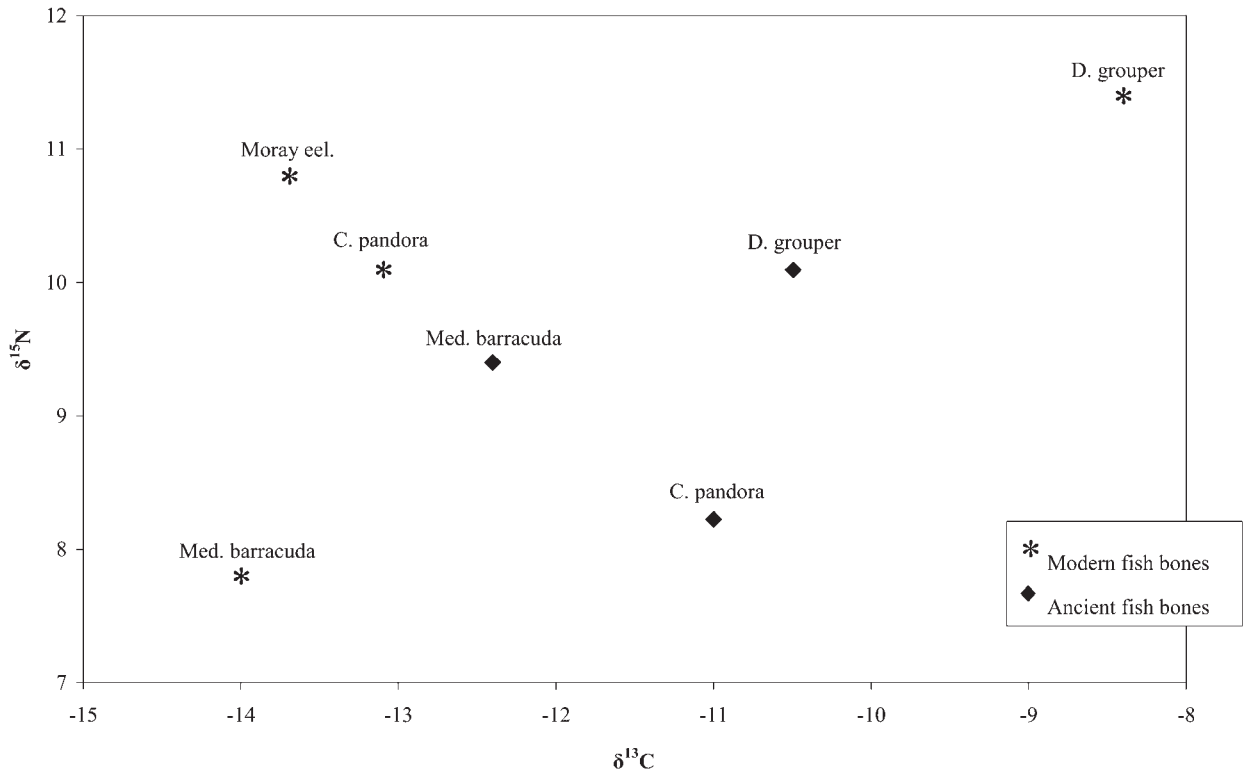

Figure 3. Plot of the $\delta^{15}N$ and $\delta^{13}C$ values for fish bone collagen (‰).

Comparison of collagen isotopic values and C:N ratios between recent and ancient fish bones

The isotopic values and C:N ratios for recent and ancient fish are shown in Table 3 and Figure 3. The difference (Δ) is expressed in terms of the modern sample value minus the ancient sample value. The C:N ratios did not change significantly between recent and ancient specimens. $\delta^{15}N$ shows a relatively large variation, with a range from -1.6 to 1.3‰ (Mean = 0.5‰, n = 3), but the greatest variation is observed in the $\delta^{13}C$ values, which range from -2.1 to 2.1‰ (Mean = -0.5‰, n = 3). These results may reflect a change in the trophic structure of the rocky littoral fish communities of the area throughout time, possibly related to habitat change. This agrees with findings by other authors who have studied recent fish from protected areas *versus* non-protected ones (Macpherson 2000). Conditions in protected areas unaffected by intensive fishing can be presumed to approach those which prevailed thousands of years ago, long before the human overexploitation of marine ecosystems.

Human impact on marine environments could play an important role in the food web structure, since contaminants and other products derived from human activity (nitrates from agriculture, synthetic fertilizers etc.) modify the isotopic signatures of primary producers and this change is transmitted throughout the food chain (Hansson et al. 1997). As pointed out by France (1995), no particular $\delta^{15}N$ value in a fish is an inviolate marker of food-web position alone, but rather a reflection of a combination of both trophic and source fractionation. If there is a change in the isotopic signatures of the sources, this may be reflected in the organisms that feed of these resources. Although it is difficult to determine whether it has had any impact on western Mediterranean environments, we must bear in mind the fact that the Suez connection with the Red Sea has been active for the last 140 years.

The diagenetic processes that affect buried bones could also be responsible for the differences between the isotopic signatures of the ancient and recent fish. The C:N ratio is nevertheless a good test for assessing the quality of the collagen in archaeological samples, and the use of ultrafilters in the collagen extraction method also guarantees the integrity of the collagen.

Isotopic values for ancient marine fish

The isotopic values and C:N ratios from the ancient Mediterranean fish are given in Table 1 (samples 1, 5 and 9) and shown graphically in Figure 3. The dusky

Table 4. Some general characteristics of the fish species studied.

Name	Size (adults)	Habitat	Diet	Trophic level
Epinephelus marginatus (Dusky grouper)	0.7–0.9m Max.1.5m	4–400m Benthic	Cephalopods, crustaceans, small fish	Macro-carnivore
Coris julis (Rainbow wrasse)	0.15–0.2m Max.0.25m	1–60m Benthic	Small crustaceans, echinoderms	Meso-carnivore 1
Pagellus erythrinus (Common pandora)	0.2–0.5m Max.0.6m	1–50m Benthic	Small fish, crustaceans, molluscs, algae	Meso-carnivore 2
Muraena helena (Moray eel.)	1–1.5m Max.2m	2–100m Benthic	Fish, cephalopods	Macro-carnivore
Sphyraena sphyraena (Med. barracuda)	0.5–0.7m Max.1.2m	1–20m Pelagic	Cephalopods, crustaceans, small fish	Macro-carnivore

grouper specimen from *Cova des Riuets* has the highest $\delta^{15}N$ and $\delta^{13}C$ values, 10.1 and -10.5‰ respectively, whereas the pandora specimen exhibits the lowest $\delta^{15}N$ value (8.2‰) and the barracuda shows the lowest $\delta^{13}C$ value (-12.4‰). The maximum difference between fish from the same site is 1.9‰, for both $\delta^{15}N$ and $\delta^{13}C$ values. There are greater differences between ancient and recent individuals of the same species than between ancient individuals of different species.

As far as we know, there is only one published study of stable isotopes in ancient fish from the Mediterranean, in which Francalacci (1988) analysed three fish bones from the Mesolithic Uzzo Cave, Italy, dated to 9000 BP. The fish were not identified to species, but the $\delta^{13}C$ and $\delta^{15}N$ values obtained are close to ours ($\delta^{15}N$: 9.8, 10.6 and 11.4‰, and $\delta^{13}C$: -10.2 and -10.6‰). These results may suggest that Mediterranean marine ecosystems did not change significantly between 9000 and 4000 years BP.

Isotopic values for modern marine fish: reconstructing the trophic chain

The isotopic values and C:N ratios for the modern Mediterranean fish are given in Table 1 (samples 2, 6, 8 and 10) and shown graphically in Figure 3. The results show that marine fish as a dietary resource exhibit variable isotopic compositions, the total range being 3.6‰ for $\delta^{15}N$ and 5.6‰ for $\delta^{13}C$ (n = 4). Although the $\delta^{13}C$ values show the greatest range of variability, $\delta^{15}N$ analysis is a more reliable tool for food web reconstruction (Hobson & Welch 1992). All the fish species analysed here are carnivores that feed basically on herbivorous fish, crustaceans and molluscs (Table 4), but the observed range of $\delta^{15}N$ values indicates a certain degree of trophic niche separation within this feeding type. The barracuda had the lowest $\delta^{13}C$ and $\delta^{15}N$ values, the pandora and moray eel showed intermediate values, and the dusky grouper exhibited the highest values for both isotopes. The values indicate that these fish species are operating at different trophic levels, the dusky grouper being at the highest (each successive step in the food chain corresponds to an increase of about 3‰ in $\delta^{15}N$ values, DeNiro & Epstein 1981). Raw $\delta^{13}C$ and $\delta^{15}N$ data for fish tend to be quite variable, however, because they may be more or less omnivorous or feed at different trophic levels, and the same fish species may exhibit different $\delta^{15}N$ values in different ecosystems according to the food chain length in each system (Cabana & Rasmussen 1994), because the increase in ^{15}N content during assimilation from one trophic level to another depends on the number of steps in the food web.

Hobson and Montevecchi (1991), summarizing $\delta^{15}N$ and $\delta^{13}C$ values for a variety of marine organisms off the north-eastern coast of Newfoundland from several trophic levels, observed that planktivorous fish showed a mean $\delta^{15}N$ value of 10.6‰ whereas those at a higher trophic level, piscivorous and benthic fish, including the Atlantic cod (a predator at the highest trophic level), averaged 14.5‰. Similar values were obtained by Richards and Hedges (1999), showing an enrichment of 2–3‰ in $\delta^{15}N$ between successive trophic levels: planktivores ($\delta^{15}N$=11.5‰), opportunistic generalists or fish with an unknown diet ($\delta^{15}N$=13‰) and piscivores ($\delta^{15}N$=14‰). France (1995) summarized $\delta^{15}N$ values for marine fish given in the literature as ranging from 8 to 20‰, with a mean of 14±3‰. Our results show less $\delta^{15}N$ enrichment, which may be due to the small sample size. When comparing food-web structures from different habitats we must nevertheless pay attention to the variability in source isotopic signatures. For this reason comparisons should be made with data from the same

area. Jennings et al. (1997), in their study of trophic pathways around the Mediterranean island of Mallorca, reported that $\delta^{13}C$ ranged from -19.2 to -16.1‰ and $\delta^{15}N$ from 8.4 to 13.8‰ in fish with different feeding strategies (benthic invertebrate, pelagic plankton and/or fish feeders). Pinnegar and Polunin (2000), studying food webs in Calvi Bay (Corsica), found that $\delta^{13}C$ and $\delta^{15}N$ for fish ranged from -19.6 to -16.3‰ and from 4.6 to 9.8‰, respectively. Flatfish in the Gulf of Lions (NW Mediterranean) exhibited quite similar values to the above for both $\delta^{13}C$ (from -19.9 to -17.7‰) and $\delta^{15}N$ (from 9.4 to 10.9‰) (Darnaude et al. 2004). Our results fall within the expected range for $\delta^{15}N$, but the $\delta^{13}C$ values are less negative than those reported elsewhere.

The results for modern marine ecosystems may be influenced by anthropogenic factors such as fishing (e.g. Harmelin-Vivien 2000; Morales-Nin et al. 2000; Jennings et al. 2002), aquaculture (e.g. Dosdat 2001) or contamination of the sea water with pollutants (e.g. Hansson et al. 1997). Fishing has an important effect on marine ecosystems by altering the structure and heterogeneity of benthic habitats, changing the species composition and diversity, reducing stocks, affecting predation and competition rates and changing trophic structures, energy flows and trophic cascades, etc. Moreover, at the species level, fishing reduces mean body size and biomass, lowers the mean size/age at maturity, alters sex ratios and detracts from reproductive potential (Macpherson 2000; Stergiou & Koulouris 2000). Overfishing in coastal environments can lead to the local disappearance of some species or to changes in their ecological habits. The dusky grouper (*Epinephelus marginatus*), for example, as studied here, is becoming rare along our coasts due to traditional exploitation techniques, diving and environmental factors connected with human activity (Gracia & Castello-Orvay 1995). Consequently, the effects of fishing on the trophic structure of fish communities may be much more complex than is generally assumed. All these factors can be blamed for the wide range of isotopic results obtained, which are simply a reflection of changes in trophic structures and feeding strategies.

Conclusions

The differences in stable isotope values observed between ancient and recent fish specimens and between our results and those of other authors suggest a great diversity in the trophic structure of marine ecosystems, and particularly in the feeding strategies adopted by various fish species. These differences may reflect responses to natural changes in local environmental conditions, or else they could be due to modern human activities such as overfishing, aquaculture or other sources of sea water contamination such as agriculture, all of which provoke changes in primary producers that are transmitted throughout the food chain.

Marine fish show extremely variable isotopic compositions that are related to local environments and to the trophic positions of given species, and it is difficult to use such data as a basis for predicting isotopic values in consumers of marine fish such as humans without testing fish remains from archaeological sites and/or recent fish from a marine system close to the site. We have to be very cautious when investigating past human diets by means of stable isotope analysis using recent animals as a reflection of past ecosystems. Whenever possible one should use animals from the same site and epoch as the human bones to allow back-calculation of the diet.

There seems to be little difference between stable isotope results obtained with or without a degreasing treatment when analysing recent fish bones. This may be due to the low lipid content of fish bones, which becomes practically negligible when the small quantity of sample processed is taken into account. More data are needed to support this, however.

Variations between seasons, sites, species, individuals and tissues and in the metabolic and nutritional status of the animals concerned present methodological and interpretative challenges for stable isotope research. Further work is needed in order to provide a better understanding of the processes responsible for the differences in isotopic composition observed between living organisms. Stable isotope analysis is becoming a valuable tool for interpreting the past diets of animals and humans, but biological systems, especially marine ones, are characterized by a marked variability and unpredictability which calls for a deepening of studies on trophic web structures and feeding relationships between organisms and reminds us that much caution is required in the proposed interpretations.

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