Temporal variations in the feeding habits and trophic levels of three deep-sea demersal fishes from the western Mediterranean Sea, based on stomach contents and stable isotope analyses

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ABSTRACT: Temporal variations in feeding habits and trophic levels of 3 deep-sea fishes: *Hoplostethus mediterraneus*, *Hymenocephalus italicus* and *Nezumia aequalis*, were investigated off the Archipelago of Cabrera (Algerian Basin). These species are representative of the 3 main trophic guilds found at bathyal depths: plankton feeders, suprabenthos feeders and epi-endobenthos feeders. Samples were collected during 6 trawl surveys at bi-monthly intervals from August 2003 to June 2004, between 550 and 750 m depths. Stomach content and stable isotope analyses were performed to detect temporal variations in diet. Because fish were immediately fixed in 10% formalin, a parallel experiment comparing isotope composition in fixed and freshly frozen tissues was carried out. *H. mediterraneus* mainly preyed on isopods throughout the sampling period and to a lesser extent on amphipods and mysids, although mixing models revealed high consumption of mesopelagic fish in winter and spring. *H. italicus* fed on mysids and amphipods that live in the benthic boundary layer, while *N. aequali*s exhibited a more benthic diet composed of benthopelagic and benthic prey (e.g. tanaids and polychaetes). Temporal variation was observed in the diet of *H. mediterraneus*, with February being the period of change in feeding habits. Temporal changes were also evident, though weaker than in *H. mediterraneus*, in the diets of the 2 macrourids, *H. italicus* and *N. aequali*s. Patterns of temporal change in fish diets follow temporal variations in prey, which are stronger in mesopelagic than in suprabenthic or benthic fauna. Temporal variations in tissue $\delta^{15}N$ were detected in the 3 species, with the lowest values occurring in September and the highest in April to June. In general, different time lags were detected between the isotopic turnover time of consumers and that of their diets (prey) at a monthly time scale, showing that assimilation times depend on species behaviour and metabolism. Assimilation times are brief in *H. mediterraneus* but last up to 2 mo in the benthic feeder *N. aequalis*. The experiment comparing fixed versus frozen tissues showed that formalin did not affect $\delta^{15}N$ values after 6, 12 and 24 mo of preservation, while $\delta^{13}C$ signatures were depleted to different extents in the 3 species compared with controls.

KEY WORDS: *Hoplostethus mediterraneus · Hymenocephalus italicus · Nezumia aequalis* · Stomach contents · Stable isotopes · Mixing models · Temporal variations · Formalin preservation

INTRODUCTION

Knowledge of the feeding ecology of marine animals is crucial to understand how an ecological system operates, and this is particularly true for deep-sea ecosystems, which are less known and increasingly exploited. At bathyal depths most of our knowledge on deep-sea demersal communities comes principally from areas where deep-water fisheries are developed.

In terms of dietary studies many deep-water fish populations of the Mediterranean Sea have been the subject of intensive research, particularly in the Balearic Basin (e.g. Macpherson 1981, Carrassón et al. 1992, Carrassón & Matallanas 1998, 2002, Carrassón & Cartes 2002), an area for which there is also a great deal of information available on decapod crustaceans.

The deep portion of the Mediterranean Sea has high stability with respect to temperature and salinity (Mar-

galef 1985), and other conditions, such as the proportion of mud and organic matter content in sediments, also vary relatively little at mid-slope depths (Carpine 1970, Cartes et al. 2002). Therefore, this is a good ecosystem in which to study trophic relationships among the fauna, because of the relatively small influence of other environmental factors. The deep-sea environment was once thought to be quiet and calm, and lacking seasonal changes. However, several studies in recent decades have revealed that distinct seasonality exists in the marine life of the deep sea due to seasonal deposition of phytodetritus exported from the ocean surface (Gooday 1988, Thiel et al. 1989, Rice et al. 1994). Deep-sea organisms that rely on this phytodetritus as a food source have corresponding life cycles (i.e. non-continuous reproduction and/or peaks in the recruitment) and fluctuations in abundance and biomass (Billet et al. 1983, Thiel et al. 1989).

Although deep-sea fauna of the western Mediterranean basin is well known with substantial information available on specific aspects, only a few studies have addressed seasonal feeding cycles (e.g. Cartes 1994, Madurell & Cartes 2005, Fanelli & Cartes 2008), primarily because of the difficulty of collecting samples at these depths.

In addition, most deep-sea demersal fish are active predators, regarded as generalists (i.e. with highly diversified diets) and depend mainly on benthopelagic and mesopelagic prey (Mauchline & Gordon 1986). Many deep-sea fishes show diel and seasonal feeding patterns (e.g. Macpherson 1980, Mauchline & Gordon 1984, Blaber & Bulman 1987, Priede et al. 1994, Atkinson 1995, Madurell & Cartes 2005, 2006), which often have been related to the temporal vertical migrations of their mesopelagic prey (Atkinson 1995, Gartner et al. 1997). The benthic boundary layer (BBL, the zone that extends a few tens of metres into the water column immediately overlying the sea bed) macrofauna (mainly peracarid and decapod crustaceans) are a key compartment linking lower trophic levels and top predators such as fish (Cattrijsse et al. 1994, Cartes 1998, Carrassón & Cartes 2002). However, despite the accepted role of this fauna in bathyal food webs, little is known about their interactions and dynamics.

In general, studies on feeding relationships of deep-sea organisms have concentrated on gut contents. These data, however, have some limitations. For example, gut contents reflect diets at particular points in time and space, and neglect certain types of dietary materials such as gelatinous plankton and detritus that may nevertheless be important in sustaining marine food webs. Other limitations include the voiding of gut contents upon capture and the crushing or grinding of food that makes identification of prey difficult (Fanelli & Cartes 2008). These limitations are especially

intense in the study of food webs in the deep ocean, because of the constraints associated with sampling at great depths.

The use of stable isotopes of nitrogen (N) and carbon (C) for the study of trophic interactions is now common in aquatic ecosystems (Pinnegar & Polunin 2000). Analysis of stable isotope composition provides an indication of the origin and transformations of organic matter. Stable isotopes integrate short-term variations in diet and, thus, are less subject to temporal bias. In fish, tissue turnover rates may be as slow as 0.1 to 0.2% d^{-1} (Hesslein et al. 1993). The $\delta^{15}N$ in tissues of consumers is typically greater by 2 to 3‰ relative to their prey, so that stable N-isotope data have been used to estimate the trophic levels of organisms (Owens 1988). In contrast, tissues tend to be rather weakly enriched in ^{13}C at progressively higher trophic levels (<1‰); thus, ^{13}C may act as a useful indicator of the primary organic carbon sources of an organism's diet. Stable isotopes have rarely been applied to detect seasonal changes in fish diet (Polunin et al. 2001, MacNeil et al. 2006), and applications of stable isotope analysis (SIA) in deep-sea ecosystems to elucidate temporal changes in trophic levels are scarce (Fanelli & Cartes 2008, Madurell et al. 2008, Fanelli et al. 2009). Despite their advantages, results of SIA often need the knowledge of dietary composition from gut content analyses to be interpreted correctly, because isotope composition gives only an average indication of the trophic level of food that has been assimilated into tissues (see Johnston & Kennedy, 1998 and Owens & Watts 1998 for reviews). Hence, we chose to study stomach contents and stable isotopic composition simultaneously.

In this study we combined information on stomach contents of 3 deep-sea fishes: *Hoplostethus mediterraneus*, *Hymenocephalus italicus* and *Nezumia aequalis*, with stable isotope composition of predators and prey to test for (1) temporal variations in feeding habits and trophic levels of predators and (2) the lag between the isotopic turnover time of consumers and that of their diets at a seasonal scale (i.e. the assimilation rate of species). These 3 species were chosen as representative of 3 different trophic guilds based on literature data: *H. mediterraneus* actively forages on natatory benthopelagic prey (Pais 2002, Madurell & Cartes 2005), *H. italicus* consumes mainly suprabenthic resources and *N. aequalis* preys on infauna (i.e. polychaetes) (Macpherson 1979, Mauchline & Gordon 1984, Madurell & Cartes 2006). Temporal changes in food resources available for fish and the changes in trophic levels elucidated by SIA are important aspects of deep-sea trophic ecology scarcely considered in previous approaches. We gave particular emphasis to the possible influence of different environmental factors (e.g. primary production, temperature and/or

salinity that represent water mass dynamics) and biological features (e.g. size, weight and density of species present) on the seasonal trends observed in fish diets. This aspect is particularly relevant in the context of the deep-sea environment, which is not completely stable (see above), and studies on the influence of environmental variables on different biological aspects of deep-sea species are almost non-existent.

Another important aspect of this study was to test for fixative-dependent shifts of $\delta^{13}C$ and $\delta^{15}N$ in preserved material. Fish samples used in this study were fixed in formalin on board ship, so that subsequent exchange and leaching needed evaluation. Thus, a comparison was performed between frozen samples and samples preserved in formalin for different intervals (6, 12 and 24 mo). This was also necessary because, among the increasing number of published studies of SIA on preserved materials, contradictions and variability are evident in results and interpretations (Bosley & Wainright 1999, Kaehler & Pakhomov 2001, Arrington & Winemiller 2002, Edwards et al. 2002, Sarakinos et al. 2002, Feuchtmayr & Grey 2003, Sweeting et al. 2004, Kelly et al. 2006). Reported differences in isotope ratios between preserved and control samples have also been highly variable (from negligible differences up to 2.5‰ difference). Moreover, effects of preservatives (especially formalin) on fish tissues have been studied to some extent in fresh and brackish water fishes (Arrington & Winemiller 2002, Vander Zanden et al. 2003, Kelly et al. 2006, Syväranta et al. 2008) and cultured seabass *Dicentrarchus labrax* (Sweeting et al. 2004), but this aspect never has been considered before for deep-sea species with lower tissue turnover (slower growth) than shelf-living species.

MATERIALS AND METHODS

Study area and sampling. Samples of *Hoplostethus mediterraneus*, *Hymenocephalus italicus* and *Nezumia aequalis* were collected (Table 1) over a period of 10 mo, on 6 experimental survey cruises from August 2003 to June 2004, at approximately bimonthly time intervals, within the framework of project IDEA (Influence of oceanographic structure and dynamics on demersal populations in waters of the Balearic Islands, see Massutí et al. 2008 for details). Sampling was carried out in the Algerian Basin off the Archipelago of Cabrera (38.8° to 39.0° N, 2.8° to 3.0° E) at depths ranging from 576 to 752 m. Each survey comprised a single haul at each of 3 depths (ca. 580, ca. 660 and ca. 750 m; see also Fanelli & Cartes 2008). All hauls were carried out with a commercial otter trawling vessel. The horizontal opening of the gear, on average, was ca. 25.5 m (SD = 1.76 m) and the vertical opening was ca. 2.2 m. Cod-end mesh size was 40 mm. Haul duration was about 1 h. Abundance (ind. km^{-2}) and biomass (g km^{-2}) were calculated after standardising catches to the swept area. Distance and trawl mouth width were recorded using a SCANMAR system attached to the trawl for each haul.

Immediately after capture, specimens were fixed in 10% buffered formalin. Once in the laboratory, fish were measured to the nearest 1 mm (total length [TL] for *Hoplostethus mediterraneus* and pre-anal length [PAL] for the two macrourids, corresponding to 26.4% of TL in *Hymenocephalus italicus* and to 26% of TL in *Nezumia aequalis*, according to the information available in fishbase, www.fishbase.org), weighed to the nearest 0.1 g and dissected for stomach content analysis. A sample of dorsal white muscle was taken from some specimens for stable isotope analysis.

Stomach contents analysis and data processing. Prey items were identified to the lowest possible taxonomic level, counted under a binocular microscope and weighed (precision, 0.0001 g) after excess moisture had been removed by blotting with tissue paper. The contribution of each prey item in the fishes' diet was estimated using the percentage frequency of occurrence (%F), the percentage of numerical abundance (%N), the percentage of gravimetric composition (%W) and index of relative importance (IRI) = (%N + %W) × %F, expressed as percent IRI (%IRI) = (IRI/ΣIRI) × 100. Prey items were sorted in decreasing order according to their %IRI contribution, and then cumulative %IRI was calculated (see Hyslop 1980 for a review). Stomach fullness (stomach weight/fish weight × 100) and trophic diversity (Shannon-Wiener diversity index *H'*) indices were also calculated. Diet was characterised for each species by month.

Table 1. *Hoplostethus mediterraneus*, *Hymenocephalus italicus* and *Nezumia aequalis.* Number of specimens dissected (number of fishes with food in stomachs in brackets) by month and year. Length ranges (total length for *H. mediterraneus* and PAL for *H. italicus* and *N. aequalis*) are also included

Species	2003			2004			Length range (mm)
	August	September	November	February	April	June	
H. mediterraneus	59 (37)	53 (33)	44 (29)	34 (22)	15 (8)	39 (22)	67–214
H. italicus	41 (30)	31 (22)	60 (41)	36 (24)	27 (23)	32 (25)	25–43
N. aequalis	45 (34)	21 (18)	56 (34)	69 (46)	28 (22)	39 (31)	23–52

To measure sample size sufficiency (i.e. the number of stomachs to be examined), the cumulative H' was plotted against the cumulative number of randomly pooled analysed stomachs (Ferry & Cailliet 1996) for each species. We repeated this procedure 5 times using a progressively smaller number of stomachs (100, 95, 85, 75 and 50% of sample size). We calculated logistic and linear regressions each time and compared the goodness of fit coefficient R^2 calculated for a logistic curve with the one calculated for the linear relation. The sample size was considered sufficient when R^2 from the logistic curve was higher than R^2 from the linear regression. This was the case for all three species during the 6 mo (for macrourids a mean sample number of 15 stomachs per month was considered adequate, while for *Hoplostethus mediterraneus* it was 20) except for *H. mediterraneus* from February to June for which few individuals (only 15 individuals in April, 8 with full stomachs) were collected in this period due to the abundance pattern of this species in the area (Moranta et al. 2008). With 15 stomachs the R^2 value for the linear relation in *Hymenocephalus italicus* was 0.98, while for the logistic curve it was 0.99; for *Nezumia aequalis* the R^2 from the logistic curve (R^2_{log}) was higher than that from the linear regression (R^2_{lin}) using 12 stomachs ($R^2_{log} = 0.99$ versus $R^2_{lin} = 0.97$), while for *H. mediterraneus* at least 20 stomachs were necessary (with 20 stomachs, $R^2_{log} = 0.98$ versus $R^2_{lin} = 0.93$).

Dietary analyses were performed with non-parametric univariate and multivariate techniques. First, we tested whether the rationale for choosing the 3 species, based on literature data, was consistent with our own results. Thus, separations among the diets of the species were visualised by principal coordinates analysis (PCA), based on the Bray-Curtis resemblance matrix of 4th-root transformed, aggregated weight data. The significance of separation among the 3 species' diets was tested by a 1-way analysis of similarities (ANOSIM) (Clarke & Warwick 1995). The most typifying food species for each fish was determined by SIMPER procedure (Clarke 1993) and appears as a vector overlay on the PCA plot.

Second, we analysed temporal changes in the diet of each species. Permutational multivariate analysis of variance (PERMANOVA) (Anderson et al. 2008) was used to analyse the multivariate data set for the complete experimental design. This method allows multivariate data to be analysed on the basis of any distance or dissimilarity measure of choice, with p-values obtained using permutations. In the case of *Hoplostethus mediterraneus* the experimental design had one factor, month (fixed with 6 levels, corresponding to the 6 mo when sampling was carried out). For the 2 macrourids the experimental design had 2 factors (month with 6 levels and depth, nested in month, with 3 levels). PERMANOVA was based on modified Gower distance after a 4th-root transformation of the multivariate set of data. This measure of resemblance was deemed the most suitable for stomach content data, which are generally skewed and contain many zeros (Anderson et al. 2008, Fanelli & Cartes 2008). The 4th-root transformation has been considered the most appropriate for weight data (Clarke & Warwick 1995, Anderson et al. 2008); it reduces the contribution of highly abundant species in relation to less abundant ones. Significance was set at p = 0.05 and p-values were obtained using 9999 permutations of residuals under restricted permutations of the raw data. That is the recommended method when there is only a single factor (e.g. for *H. mediterraneus*) and under the reduced model when there are 2 factors (Anderson et al. 2008), as in the case of the macrourids. A SIMPER analysis was applied to each species to determine the most typifying prey species for month and depth, when those variables were found to be significant.

Stable isotope analysis. *Sample preparation:* From 3 specimens of each species from each month, a portion of white muscle close to the dorsal fin was dissected and prepared for stable isotope analysis. For each species we chose 3 specimens of the same size throughout the sampling period (*Hoplostethus mediterraneus*: mean TL = 164.9 ± 12.1 mm, ANOVA $F_{1,12} = 1.81$, p > 0.05; *Hymenocephalus italicus*: mean PAL = 35.4 ± 2.5 mm, $F_{1,12} = 1.48$, p > 0.05; *Nezumia aequalis*: mean PAL = 44.0 ± 1.0 mm, $F_{1,12} = 0.33$, p > 0.05) to ensure that observed changes in stable isotope signatures were due to a temporal effect (month/season) and not to size differences. Three replicates (with no significant differences in the size of analyzed specimens) are widely accepted and used in isotope-based trophic web studies. However, to add power to our analysis and reduce Type II error, we randomly analyzed supplementary samples for *N. aequalis* (IDEA0803; n = 3) and *H. italicus* (IDEA0204; n = 3). We performed a power analysis to calculate the minimum sample size required to accept the outcome of the statistical two-tailed *t*-test with a particular level of confidence (power). The power of a statistical test is the probability that the test will reject a false null hypothesis (i.e. that it will not make a Type II error). As power increases, the chances of a Type II error decrease. The probability of a Type II error is referred to as the false negative rate (β). Therefore, power is equal to 1–β. Power analysis was performed by using the software Statistica 6.0. The results confirmed that 3 replicates are enough in our case (critical *t* = 1.98, actual power = 0.81, α = 0.05, β = 0.8).

Samples were dried to constant weight at 60°C, then ground to a fine powder. Using continuous flow, the samples were introduced into a Finnigan Delta XP plus

isotope-ratio mass spectrometer for isotopic analysis (CNR-IAMC, Naples). Three capsules of an international standard (urea) were analysed at the beginning of each sequence, plus 1 capsule every 6 samples to compensate for machine drift and for quality control. Experimental precision (based on the SD of replicates of the internal standard) was <0.2‰ for $\delta^{15}N$ and <0.1‰ for $\delta^{13}C$. The $\delta^{13}C$ and $\delta^{15}N$ values were obtained in parts per thousand (‰) relative to Vienna Pee Dee Belemnite (VPDB) and atmospheric N_2 standards, respectively, according to the following formula: $\delta^{13}C$ or $\delta^{15}N = [(R_{sample}/R_{standard}) - 1] \times 10^3$, where $R = {}^{13}C/{}^{12}C$ or ${}^{15}N/{}^{14}N$.

Organic carbon and total nitrogen elemental composition were determined from the residues of CO_2 and N_2 gases. C:N ratios were measured simultaneously during stable isotope analysis from the percent element data.

Fixative dependent shifts of $\delta^{13}C$ and $\delta^{15}N$ in preserved fish samples: Since all samples during IDEA cruises were fixed in formalin, some specimens were collected in the Algerian Basin (i.e. in the area where IDEA cruises were carried out) in May 2005 within the BALAR–MEDITS survey (Massutí & Reñones 2005) to assess for the fixative dependent shifts of $\delta^{13}C$ and $\delta^{15}N$ in preserved fish samples compared with frozen samples. Portions of white muscle were dissected from the fish. From the same specimens (3 of each species – *Hoplostethus mediterraneus*: mean TL = 155.1 ± 7.2 mm; *Hymenocephalus italicus*: mean PAL = 39.8 ± 6.3 mm; *Nezumia aequalis*: mean PAL = 48.8 ± 5.1 mm; ANOVA p > 0.05 for the 3 species) several tissue cubes were prepared, oven-dried and used as controls, while other tissue cubes were fixed in formalin and removed from the treatment after 6, 12 and 24 mo. They were then oven dried for SIA. Differences between the size of specimens used for the experiment and those used to assess for temporal differences were negligible and in any case not significant.

Data analysis: To assess the fixative-dependant shift of $\delta^{13}C$ and $\delta^{15}N$ in preserved material, differences in the isotopic composition of unfixed samples (C) and samples for each period of formalin preservation (F1, F2 and F3, respectively, after 6, 12 and 24 mo) were tested by 1-way ANOVA, with time of preservation as a fixed factor. When results showed an effect of fixation on tissue samples, they were investigated by *a posteriori* comparisons using Student-Neuman-Keuls (SNK) test (Underwood 1997).

If the results of the experiment were negative (no effect of fixation on tissue samples after different time intervals), temporal variations in trophic levels ($\delta^{15}N$) and sources of carbon ($\delta^{13}C$) of samples collected during IDEA cruises were tested by one-way ANOVA for each species, with month as a fixed factor. The $\delta^{13}C$

values of samples collected during IDEA were also normalised for lipid concentration according to Post et al. (2007); thus, $\delta^{13}C$ values of our untreated samples (not defatted) were converted to normalised $\delta^{13}C$ values according to $\delta^{13}C_{normalised} = \delta^{13}C_{untreated} - 3.32 + 0.99 \times C:N_{sample}$. $C:N_{sample}$ was the C:N ratio of samples collected during IDEA. Afterwards we analysed temporal trends for normalised samples and compared results with values before accounting for lipid content.

Since data on the isotopic signatures of dominant mesopelagic and suprabenthic fauna are available from the same area where fish were collected (Fanelli et al. 2009), and because diet-switch experiments confirm that the isotopic turnover of consumers frequently lags the isotopic turnover of their diets (Hesslein et al. 1993, Herzka & Holt 2000, Harvey et al. 2002), we calculated the dietary $\delta^{15}N$ values of the 3 species expected from the isotopic values of their prey. Thus for each species, the nitrogen isotope signature predicted from its diet was calculated using a weighted-average equation of the following form (Clarke et al. 2005):

$$\sum_{i=1}^{K} \delta^{15}N \frac{b_i}{b_t} + f, \quad \text{where } b_t = \sum_{i=1}^{k} b_i \quad (1)$$

and where $\delta^{15}N$ is the nitrogen isotope signature of the ith prey item, b_i/b_t is the proportion of diet biomass observed for that prey item, and f is the trophic fractionation coefficient, which was taken as 2.54‰ for $\delta^{15}N$ (Vanderklift & Ponsard 2003). We then compared the dietary $\delta^{15}N$ values with the values resulting from the direct analysis of fish tissues. Finally, we estimated the goodness of fit between the dietary and fish tissue values, delayed by 1, 2 or more months, by calculating the R^2 of the correlations between dietary and observed values.

It was only possible to collect a limited number of *Hoplostethus mediterraneus* from February to June, due to the behavioral dynamics of this species (Moranta et al. 2008), which probably includes a period of aggregation and dispersion, as earlier observed for *H. atlanticus* (Rosecchi et al. 1988). For this reason we calculated the range of all possible food sources of *H. mediterraneus* by a mixing models computation to integrate the results obtained by stomach contents analysis. For this estimation we used Iso-Source, a Visual Basic program created to perform these procedures and described in Philips & Gregg (2003). This model was chosen among many others (e.g. MixSIR, STEP and SOURCE, SIAR) because it is quite easy to use, available free of charge and complete. Many of the assumptions of IsoSource are implicit in other highly spread mixing models such as STEP and SOURCE (Lubetkin & Simenstad 2004). IsoSource employs a mass balance approach to esti-

mate the range of all possible diet proportions from 0 to 100% for as many as 10 contributing prey sources. The model outputs all isotopic combinations that sum to the isotopic signature of the consumer as viable solutions (Phillips & Gregg 2003). Briefly, the user supplies the isotopic signatures of the sources (the prey) and the mixture (in our case the predator, *H. mediterraneus*), along with the desired source increment (1%) and the mass balance tolerance (0.1‰). Output files include all the feasible source combinations, with histograms and descriptive statistics on the distributions for each source. Isotopic signatures of the sources (prey) include the isotopic values of the dominant species in the diet throughout the whole sampling period (data of prey species used in mixing models are presented in Table 2). Mixing polygons created around the adjusted consumer on a $\delta^{13}C$ and $\delta^{15}N$ scatter plot indicate possible source contributions. IsoSource uses the location and distance of sources around the adjusted consumer on a $\delta^{13}C$ and $\delta^{15}N$ scatterplot to estimate possible diet contributions (Phillips & Gregg 2003). A 'constrained solution' occurs when the consumer isotopic signature is nearest the line connecting 2 sources. Those 2 sources contribute the greatest to that consumer's diet, which is indicated by a more complete bell-shaped curve of possible solutions. A consumer signature located more toward the middle of the polygon is considered 'diffuse,' where multiple sources contribute comparably to the organism's diet, often indicated by incomplete curves illustrating a more intermittent diet contribution. We expressed the range of possible solutions in histogram form with bell-shaped curves representative of sources contributing the most to the diet of a consumer.

We also considered a 'per-step' trophic fractionation between fish and their prey of 2.54‰ according to Vanderklift & Ponsard (2003). Output files include all the feasible source combinations, with histograms and descriptive statistics on the distributions for each source.

Table 2. *Hoplostethus mediterraneus*. Mean ± SD $\delta^{15}N$ and $\delta^{13}C$ values of the prey used for the computation of mixing models for *H. mediterraneus*, number of replicates (n) per species/taxon is also given. Amp: Amphipoda; Iso: Isopoda; Mys: Mysidacea; Dec: Decapoda

Taxon	Prey	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)	n
Amp	*Tryphosites longipes*	5.76 ± 0.84	−20.04 ± 0.9	20
Iso	*Natatolana borealis*	8.10 ± 1.18	−19.8 ± 2.1	16
Mys	*Boreomysis arctica*	6.33 ± 0.27	−19.81 ± 0.7	16
Dec	*Pasiphaea* spp.	7.45 ± 0.23	−19.97 ± 0.13	4
Dec	Sergestidae	5.56 ± 0.75	−20.36 ± 0.07	16
Dec	*Plesionika martia*	7.35 ± 0.67	−19.28 ± 0.87	20
Fish	Myctophidae	7.80 ± 0.27	−19.03 ± 0.68	27

Trophic level estimates: Trophic levels were calculated with $\delta^{15}N$ data from fish using different species as references: the hyperiid amphipod *Vibilia armata* (mean (±SD) $\delta^{15}N$ = 3.36 ± 0.14‰, Fanelli et al. 2009) for *Hoplostethus mediterraneus* and 2 selective surface deposit feeders, the cumaceans *Cyclaspis longicaudata* and *Plathysymphus typicus* (mean (±SD) $\delta^{15}N$ = 2.26 ± 0.89‰, Fanelli et al. 2009), for the 2 macrourid fishes. The rationale behind these choices was that *H. mediterraneus* has strongly pelagic habits and is more linked to the pelagic food web, while macrourids are connected to the detritus-based food web. Thus, deposit feeders are better suited as reference material for macrourids than filter feeders.

The $\delta^{15}N$ values were converted to trophic levels based on the assumption that there was a fractionation of 2.54‰ per trophic level (Vanderklift & Ponsard 2003) and that primary consumers have a trophic level of 2:

$$\text{Trophic Level} = \left(\frac{\delta^{15}N_{\text{Fish}} - \delta^{15}N_{\text{PC}}}{2.54}\right) \qquad (2)$$

where $\delta^{15}N_{\text{Fish}}$ is the mean $\delta^{15}N$ of that species and $\delta^{15}N_{\text{PC}}$ is the mean $\delta^{15}N$ of primary consumers (filter feeders and deposit feeders). Since different investigators have dissimilar opinions about the 2.54‰ fractionation point, we decided to also calculate the trophic level by using the value of per-step trophic fractionation of 3.25‰, reported by Vanderklift & Ponsard (2003) for carnivorous vertebrates, and compare the trophic levels obtained with the 2 values. We chose the values reported in Vanderklift & Ponsard (2003) because they were generated by a meta-analysis of 134 estimates of fractionation (Δ) from 32 peer-reviewed papers including 14 estimates for fish (9 publications).

Correlation with abiotic and biotic factors. To check for possible correlations between fullness and the sampling characteristics (depth and month), the environmental variables (salinity and temperature on the bottom and 5 m above the bottom, grain size [mean and mode], chlorophyll *a* [chl *a*] recorded at different time intervals before and simultaneously with sampling) and the biological features of the fish species (TL or PAL, weight and density of the species in the environment), a Spearman's rank correlation was performed for each species separately (see also Fanelli & Cartes 2008). All of the above variables were tested as possible factors that influence feeding (fullness). Spearman's rank correlation is a non-parametric measure that can test the direction and strength of the relationship between 2 variables; thus, when correlations are significant, the sign of the correlation (positive or negative) can give a useful indication of the ecological relationship.

All the statistical analyses were performed with PRIMER 6 and PERMANOVA+ (Clarke & Warwick

1995, Anderson et al. 2008) and STATISTICA 6.0. Mixing models were computed with IsoSource (available at www.epa.gov/wed/pages/models.htm).

RESULTS

Preserved versus frozen samples

No significant differences (ANOVA, p > 0.05 for all 3 species) were observed in $\delta^{15}N$ values between controls (frozen) and preserved samples after 6, 12 or 24 mo (Table 3a). The duration of preservation (from 6 to 24 mo) did affect carbon isotopic ratios of muscle in all the analyzed species, but in different ways (Table 3b). In all 3 species, $\delta^{13}C$ values were depleted with respect to frozen samples after 24 mo. For the macrourids there was a slight enrichment after 6 mo of preservation, while in *Hoplostethus mediterraneus* depletion increased with time of preservation, reaching ca. 2‰ after 24 mo. Depletion after 24 mo was ca. 2‰ in *Hymenocephalus italicus* and 0.8‰ in *Nezumia aequalis*. Thus, temporal variations in $\delta^{13}C$ must be interpreted with caution because there are fixative-dependent shifts. There are previously published studies, when $\delta^{13}C$ shifts are ca. 2‰ or lower, in which $\delta^{13}C$ values from formaldehyde-preserved specimens have been used to elucidate food web structure. No significant differences were detected between the C:N ratios of frozen and preserved samples.

Overall species analysis

PCA analysis showed a well-defined segregation among diets of the 3 species (Fig. 1), as expected. The first axis, which explains 34.5% of the total variation, separates the macrourids, *Nezumia aequalis* and *Hymenocephalus italicus*, from *Hoplostethus mediterraneus*. The second axis, explaining 20.3% of the total variation, is probably linked to depth, separating *H.*
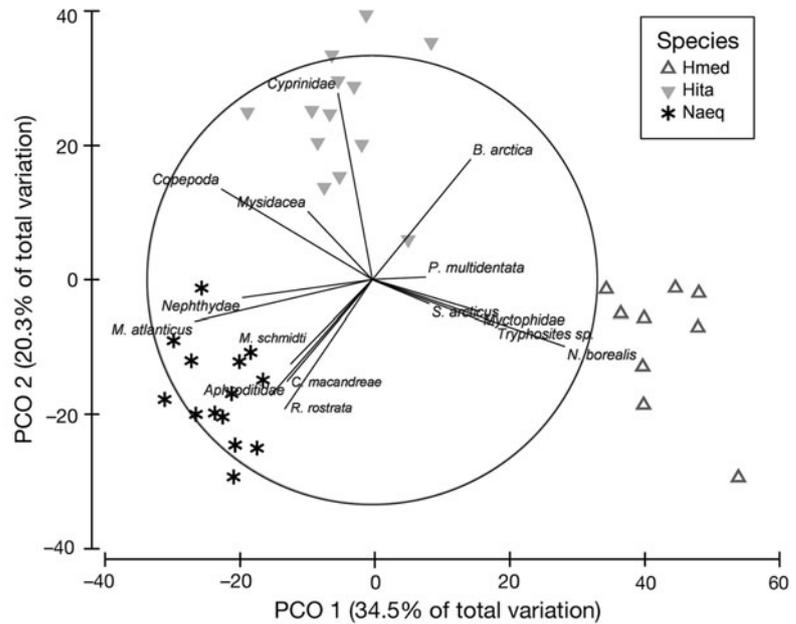
Fig. 1. *Hoplostethus mediterraneus*, *Hymenocephalus italicus* and *Nezumia aequalis*. Principal coordinates analysis of stomach contents samples of *H. mediterraneus* (Hmed), *H. italicus* (Hita) and *N. aequalis* (Naeq), based on the Bray-Curtis resemblance matrix of 4th-root transformed weight data; vectors indicate the species that contributed most to similarity with each species and/or to dissimilarity among species, based on SIMPER results. PCO: principal coordinate

Table 3. *Hoplostethus mediterraneus*, *Hymenocephalus italicus* and *Nezumia aequalis*. (a) Mean ±SD $\delta^{15}N$ and $\delta^{13}C$ values of control (C) and preserved samples removed from fixative after 6 (F1), 12 (F2) and 24 (F3) mo. (b) ANOVA results for $\delta^{13}C$ values between C samples and F1, F2 and F3. Results of post hoc Student-Neuman-Keuls tests (SNK) are also reported for each species. **p < 0.01, ***p < 0.001

(a)	*H. mediterraneus*		*H. italicus*		*N. aequalis*	
Sample	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$
C	11.29 ± 0.09	−17.15 ± 0.05	9.20 ± 0.07	−17.62 ± 0.31	11.09 ± 0.58	−16.98 ± 0.21
F1	11.45 ± 0.05	−17.21 ± 0.18	9.15 ± 0.14	−16.62 ± 0.26	11.18 ± 0.21	−16.20 ± 0.08
F2	10.75 ± 0.26	−17.86 ± 0.22	8.98 ± 0.43	−17.52 ± 0.11	11.03 ± 0.98	−17.13 ± 0.14
F3	11.45 ± 0.09	−19.23 ± 0.04	9.55 ± 0.26	−19.54 ± 0.14	11.40 ± 0.33	−17.86 ± 0.08

(b)		*H. mediterraneus*		*H. italicus*		*N. aequalis*	
Source	df	MS	*F*	MS	*F*	MS	*F*
$\delta^{13}C$	3	4.18	17.35***	3.52	8.29**	2.08	107.0***
Error	8	0.24		0.42		0.02	
SNK		F3 < F2 < F1 = C		F3 < F2 = F1 = C		F3 < F2 < C < F1	

mediterraneus and *N. aequalis* from *H. italicus*. The former species have their distributional centres of gravity (COG) deeper (normally at 700 m) than *H. italicus*. The ANOSIM routine provided evidence of a significant separation (global R = 0.80, p < 0.001) among the diets of the 3 species. Differences were greater between *H. mediterraneus* and *N. aequalis* (pairwise comparison, R = 0.95, p < 0.001), than between the 2 macrourids (R = 0.69, p < 0.001). As shown by SIMPER analysis, *Natatolana borealis* was the most typifying species in the *H. mediterraneus* diet (mean percentage contribution = 51.9%), while the diet of *H. italicus* was dominated by mysids (40.9%), copepods (10.4%) and gammarids (8.8%) (See also Fig. 1). Amphipods (17.9%), isopods (8.7%) and mysids (20.3%) typified the diet of *N. aequalis* (see also Fig. 1). Benthic species such as polychaetes (including Nephthydae, Aphroditidae and some unidentified species) were also important, contributing more than 13% to the diet of *N. aequalis*. There was a gradient in the average dissimilarity among the species, in accordance with their feeding behaviours; dissimilarity was high between *H. mediterraneus* and *N. aequalis* (80.1%), but less between *H. mediterraneus* and *H. italicus* (73.8%) and still less between the 2 macrourids (64.2%).

Temporal variations in food sources

Hoplosthetus mediterraneus

A total of 244 individuals of *Hoplostethus mediterraneus* were examined; among these 24 (9.8%) were everted and 68 (27.9%) were empty. A total of 152 stomachs were analysed for diet composition and contained 32 identified prey items (Table S1 in the supplement at www.int-res.com/articles/suppl/m402p213_app.pdf). The diet consisted mainly of crustaceans and fish. Isopods (*Natatolana borealis*) were the dominant prey throughout the whole period studied, and ranged in size from 6.3 to 21.1 mm TL. They occurred in large numbers, up to 32 specimens in a single stomach. However, significant variations in their proportions (in term of %IRI) were observed among seasons (Table S1): *N. borealis* was the most abundant prey from August to November (%IRI values ranged from 97.5 to 98.8) and in June (%IRI = 85.5). In February and April, *N. borealis* was replaced by *Tryphosites* spp. (%IRI = 51.2), *Boreomysis arctica* (%IRI = 24) and mesopelagic fish (%IRI = 11 in April).

The lowest values of diversity (*H′*) were found in September and November due to the nearly complete dominance of *Natatolana borealis* in the diet (Table S1). A significant decrease in the mean number of prey ingested (ANOVA, $F_{5,151}$ = 3.09, p = 0.01) was

observed in spring, whereas higher values were found in late summer (August/September). No significant differences were found in mean prey weight or stomach fullness (ANOVA, p > 0.05) by month.

PERMANOVA (1 factor: month, with 6 levels) provided evidence for statistical differences in diet as function of month ($F_{5,151}$ = 2.19, p < 0.01), and the pairwise comparison located the source of variation between November and February (Table 4a). SIMPER analysis showed that the most typifying species was *Natatolana borealis* throughout the sampling period, its contribution to the similarity between pairs of samples per month ranged between 91.6% in August to 98.9% in November. The species that most contributed to similarity in February and April were *Tryphosites* spp. (43.4% in February and 90.3% in April) and *Boreomysis arctica* (18.9% in February and 9.7% in April). In June, *N. borealis* contributed 84.2% to the diet, with *Tryphosites* spp. and gammarids as secondary typifying species (5.3% and 4.7%, respectively). The highest values of similarity between paired samples were observed from August to November (more than 50%).

Hymenocephalus italicus

A total of 227 *Hymenocephalus italicus* individuals were analysed, among which 14 (6%) had the stomachs everted and 48 (21%) were empty. From 165 stomachs containing food, 52 categories of prey items were identified (Table S2 in the supplement at www.int-res.com/articles/suppl/m402p213_app.pdf). Mysids dominated the diet of *H. italicus* all through the year, being most abundant in August (%IRI = 84.7), November and June (both %IRI = 77.7) but still the most ingested prey during the other months (%IRI ranged between 57 and 69). Other important prey items were copepods (%IRI = 14.2), isopods (%IRI = 11.1), ostra-

Table 4. *Hoplostethus mediterraneus*, *Hymenocephalus italicus* and *Nezumia aequalis.* PERMANOVA pairwise comparisons based on a Modified Gower resemblance matrix of 4th-root transformed biomass data of stomach contents under 9999 permutations of *H. mediterraneus*, *H. italicus* and *N. aequalis* (75 items). Values are *t*-values. *p < 0.05; **p < 0.01; ns: not significant

Groups	*H. mediterraneus*	*H. italicans*	*H. aequalis*
Aug, Sep	1.03 ns	1.09 ns	0.77 ns
Sep, Nov	0.56 ns	1.06 ns	0.86 ns
Nov, Feb	2.45**	1.57*	0.69 ns
Feb, Apr	1.13 ns	0.93 ns	1.76**
Apr, Jun	1.34 ns	0.94 ns	1.02 ns
Jun, Aug	0.91 ns	1.37*	1.18 ns
n	32	52	75

cods (%IRI = 9.9) and amphipods (%IRI = 4.9), whereas polychaetes, fish and other crustaceans (e.g. cumaceans and decapods) were ingested only occasionally. At a species level, *Boreomysis arctica*, *Natatolana borealis* and *Munnopsurus atlanticus* were the main prey items in terms of %IRI (Table S2). Trophic diversity values (*H'*, 2.42 ± 0.18; see also Table S2) were approximately the same in all sampling periods. Mean prey weight varied significantly among months (Kruskal-Wallis test, $H = 13.72$, $p = 0.02$), being higher in August and February, while number of prey and stomach fullness did not. Two-factor PERMANOVA provided evidence for significant differences among months ($F_{5,164} = 1.53$, $p < 0.05$) and depths ($F_{9,164} = 1.49$, $p < 0.01$). At a temporal scale, major shifts occurred between November and February and between June and August (Table 4b). SIMPER analysis showed that a different contribution of benthopelagic prey to the diet was responsible for these separations (Table 5). In November, cyprinids contributed more than 57% to the diet of *H. italicus*, while in February *B. arctica* (65%), *Andaniexis mimonectes* (10%) and *N. borealis* (8%) were the most typifying species. Mysids and cyprinids were the most important prey in June (accounting for more than 60%), while in August *H. italicus* mostly preyed on *B. arctica* (70%).

Depth was also an important factor associated with separation between samples: the contribution of *Boreomysis arctica* to the diet increased from 30.4% at 580 m to 63.1% at 750 m, with a corresponding opposite trend in the consumption of other mysids (18.6% at 580 m, 15.7% at 660 m, and not a typifying taxon at 750 m) and copepods (14.3% at 580 m, 9.8% at 660 m, and not typifying taxon at 750 m).

Nezumia aequalis

A total of 258 *Nezumia aequalis* individuals were examined; however, 50 (19.8%) stomachs were everted and 23 (9.1%) were empty. A total of 185 stomachs were analyszed for diet composition and 75 prey items were identified (Table S3 in the supplement at www.int-res.com/articles/suppl/m402p213_app.pdf). Gammarids were the most abundant prey in the diet of *N. aequalis* in August and September (%IRI = 44.5 and 36.5, respectively), polychaetes were dominant in the diet from September to February (average %IRI = 31.7) and isopods were mainly ingested in April (%IRI = 40.7). Other crustaceans (e.g. cumaceans, decapods, tanaids and ostracods) were ingested less consistently (Table S3). At the species level, *Munnupsurus atlanticus*, *Boreomysis arctica* and *Maera schmidti* were the main prey items ingested in terms of %IRI (Table S3). Ostracods occurred frequently in stomach contents, but their contribution in terms of weight was low (mean %W = 2); they are probably ingested accidentally. Trophic diversity was higher than in the other species examined (*H'*, 3.09 ± 0.31).

Significant differences in mean weight of prey were found between February and April (post hoc Tukey's N HSD test, $p < 0.001$). Mean fullness and mean number of prey in stomachs did not differ significantly (ANOVA, $p > 0.05$).

Table 5. *Hymenocephalus italicus*. Percentage contributions of typifying species/taxon (cut-off for low contribution at 80%) to within-group similarity for the identified prey in stomach contents of *H. italicus* throughout the sampling period

Prey species/taxon	Average abundance	Average similarity	Contribution (%)	Cumulative (%)
August				
Average similarity, 27.19				
Boreomysis arctica	1.86	19.48	71.62	71.62
Cyprinidae	0.59	2.58	9.49	81.11
September				
Average similarity, 22.76				
Boreomysis arctica	1.07	7.35	32.27	32.27
Cyprinidae	1.09	6.32	27.78	60.06
Copepoda	0.79	3.87	17	77.06
November				
Average similarity, 22.94				
Cyprinidae	1.46	13.15	57.31	57.31
Boreomysis arctica	1.03	5.48	23.88	81.2
February				
Average similarity, 18.33				
Boreomysis arctica	1.41	11.96	65.23	65.23
Andaniexis mimonectes	0.52	1.74	9.5	74.73
Natatolana borealis juvenile	0.58	1.47	8.02	82.75
April				
Average similarity, 24.89				
Natatolana borealis juvenile	1.11	7.01	28.17	28.17
Copepoda	1	5.9	23.7	51.87
Mysidacea	0.92	4.78	19.2	71.08
Boreomysis arctica	1	4.42	17.74	88.82
June				
Average similarity, 18.14				
Mysidacea	1.04	6.91	38.1	38.1
Cyprinidae	0.8	3.98	21.97	60.07
Boreomysis arctica	0.73	2.23	12.27	72.34
Copepoda	0.59	2.05	11.3	83.64

PERMANOVA provided evidence for significant differences among samples as a function of month ($F_{5,184}$ = 1.35, p < 0.05) and depth ($F_{9,184}$ = 1.38, p < 0.001). The pairwise comparison performed on the factor Month showed 2 periods of dietary change, one between February and April and the other between June and August (Table 4c). Separation was mainly attributed to high consumption on benthopelagic resources (mysids and suprabenthic gammarids) in spring (April), contrasting with increasing predation on polychaetes from August (contribution to diet 23%) to February (41%) when, according to SIMPER analysis, polychaetes mostly typified the *Nezumia aequalis* diet (Table 6).

Depth appeared to be a strong factor separating samples; the major discontinuity in diet occurred between 580 m and 660 to 750 m of depth (PERMANOVA pairwise comparison, between 580 and 660 m: t = 1.43, p < 0.01; between 580 and 750 m: t = 1.29, p < 0.05; between 660 and 750 m: t = 1.07, p >

0.05). The SIMPER routine showed that at 580 m *Nezumia aequalis* preyed mainly on suprabenthic gammarids (e.g. *Rhachotropis* spp., Stegocephalidae), which contributed more than 24% to similarity, and mysids (22.2%), whilst at 660 and 750 m depths polychaetes were the preferred prey items (38%).

Temporal variations in trophic position

For *Hoplostethus mediterraneus*, the mean (±SD) $\delta^{15}N$ value was 10.5 ± 0.4‰; however, non-significant (ANOVA, $F_{5,12}$ = 0.64, p = 0.68) seasonal differences occurred, with the lowest value observed in September and the highest in April (Fig. 2a). The correlation between $\delta^{13}C$ values of untreated (Fig. 2b) and normalised samples (Fig. 2c) was significant (R = 0.86, p = 0.03); 1-way ANOVA showed significant differences for $\delta^{13}C$ values for normalised samples among months ($F_{5,12}$ = 1.12, p < 0.05) but not for untreated samples ($F_{5,11}$ = 3.06, p = 0.06).

The seasonal trend of dietary $\delta^{15}N$ values obtained by Eq. (1), based on isotopic composition of ingested prey, fit well with simultaneous values observed from fish tissues (= assimilated food) (r = 0.73, p > 0.05; see also Fig. 3). Based on Eq. (2) the trophic position of *Hoplostethus mediterraneus* is 4.95.

The results of IsoSource, for the periods when diet was well established based on stomach contents, are shown in Fig. 4a. To easily read the graphs some rules regarding graphical representation provided by Philips & Gregg (2003) should be taken into account: (1) if the mixture is outside the convex polygon bounded by all sources, there is no possible solution to the linear mixing model; (2) mixtures near the periphery of the mixing polygon have well constrained solutions; (3) mixtures near the centre have more diffuse solutions and (4) narrow mixing polygons with small differences between sources have diffuse solutions. In summer the mixing polygon was narrow with the isotopic signature of *Hoplostethus mediterraneus* ('the mixture') falling in the middle (Fig. 4a, Summer). Myctophids (50th to 99th percentile: 31 to 44%), Pasiphaeidae (50th to 99th percentile: 12 to 53%, not shown in the graph) and Pandalidae (50th to 99th percentile: 14 to 60%, not

Table 6. *Nezumia aequalis*. Percentage contributions of typifying species/taxon (cut-off for low contribution at 50%) to within-group similarity for the identified prey in stomach contents of *N. aequalis* throughout the sampling period

Prey species/taxon	Average abundance	Average similarity	Contribution (%)	Cumulative (%)
August				
Average similarity, 21.00				
Gammaridaea ind.	0.91	3.33	15.87	15.87
Nepthydae	0.92	2.93	13.98	29.84
Maera schmidti	0.88	2.37	11.31	41.15
Ostracoda	0.55	1.42	6.75	47.9
Rhachotropis spp.	0.6	1.25	5.97	53.87
Polychaeta ind.	0.62	1.24	5.92	59.79
September				
Average similarity, 22.84				
Nepthydae	1.46	6.89	30.16	30.16
Gammaridaea ind.	0.87	3.52	15.4	45.56
Boreomysis arctica	0.88	2.77	12.14	57.69
November				
Average similarity, 30.69				
Munnupsurus atlanticus	1.26	7.39	24.09	24.09
Nepthydae	1.43	6.99	22.76	46.85
Gammaridaea ind.	0.88	3.27	10.64	57.49
February				
Average similarity, 22.45				
Eunicidae	1.16	4.39	19.57	19.57
Gammaridaea ind.	0.99	4.36	19.44	39.02
Munnupsurus atlanticus	0.79	3.11	13.84	52.86
Polychaeta ind.	0.66	1.55	6.89	59.74
April				
Average similarity, 29.08				
Munnupsurus atlanticus	1.49	9.6	33.02	33.02
Mysidacea ind.	1.26	6.35	21.86	54.88
June				
Average similarity, 27.35				
Mysidacea ind.	1.23	6.9	25.23	25.23
Munnupsurus atlanticus	1.09	6.18	22.6	47.83
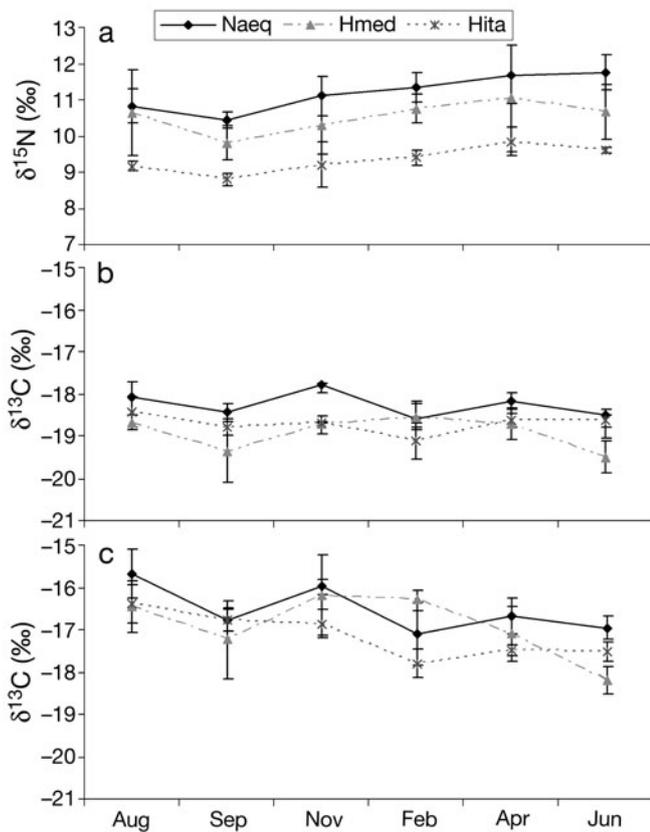
Fig. 2. *Hoplostethus mediterraneus, Hymenocephalus italicus* and *Nezumia aequalis.* Temporal variations of (a) δ^{15}N, (b) δ^{13}C and (c) normalised δ^{13}C values by month of *H. mediterraneus* (Hmed), *H. italicus* (Hita) and *N. aequalis* (Naeq). Error bars indicate ±SD
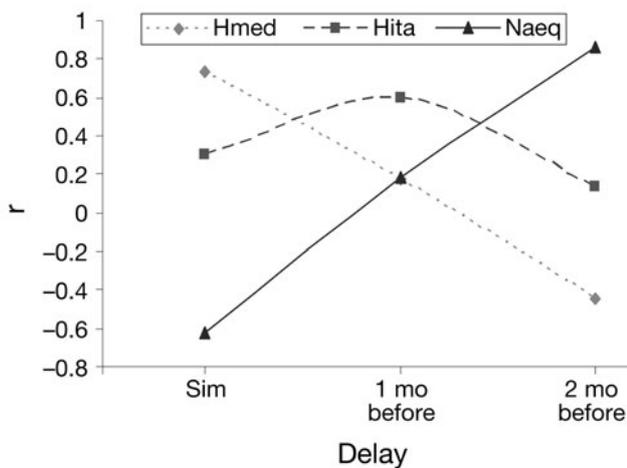
Fig. 3. *Hoplostethus mediterraneus, Hymenocephalus italicus* and *Nezumia aequalis.* Spearman's r correlations between expected (dietary) δ^{15}N values as calculated by Eq. (1) and observed values (Sim) as determined from direct tissue analysis for *H. mediterraneus* (Hmed), *H. italicus* (Hita) and *N. aequalis* (Naeq) and delayed by 1 (1 mo before) and 2 mo (2 mo before)

shown in the graph) seemed to be more abundant prey than the preferred prey, *Natatolana borealis*, as indicated by stomach contents analysis (50th to 99th percentile: 7 to 25%). In autumn the situation was similar (Fig. 4a, Autumn), although the mixing polygon was broad and the mixture fell close to the signatures of myctophids (50th to 99th percentile: 43 to 60%) and *N. borealis* (50th to 99th percentile: 18 to 53%), which represented the bulk of the diet, followed by Pasiphaeidae (50th to 99th percentile: 8 to 33%), in good agreement with the results based on the diet and Eq. (1). The results of IsoSource, for the 2 seasons for which only a few stomachs were available, are shown in Fig. 4b. In winter the mixing polygon was broad with the mixture falling near one end, close to myctophid signatures (Fig. 4b, Winter). Fish appeared to constitute the majority of the diet (50th to 99th percentile: 40 to 63%), with Sergestidae as an important secondary food source (50th to 99th percentile: 17 to 41%), and the other potential food sources making up the remainder of the diet (Fig. 4b). In spring (Fig. 4b, Spring) fish again appeared to be important in the diet (26 to 54%) together with *N. borealis* (4 to 26%), while the other species (*Tryphosites longipes*, *Boreomysis arctica*, *Pasiphaea* spp. and *Plesionika martia*) had somewhat less precise, small to medium, dietary contributions (between 0 and 15% and 0 and 45%; only *T. longipes* was reported in Fig. 4b).

Temporal changes occurred in δ^{15}N values of *Hymenocephalus italicus* (Fig. 2a) with the lowest value observed in September and the highest in April, although the differences were not significant. The correlation between δ^{13}C values of untreated and normalised samples was not significant (Spearman's r = 0.67, p = 0.19); although a more depleted δ^{13}C value occurred in February (Fig. 2b), δ^{13}C values of untreated samples did not show significant differences by month, but the high variability among values (as showed by the wide confidence limits) may have influenced the test result. The δ^{13}C values of normalised samples did differ significantly among months ($F_{5,12}$ = 9.43, p < 0.001), with more depleted values evident from February to June compared with those from August to November.

For *Hymenocephalus italicus*, the highest correlation between dietary δ^{15}N and tissue values was obtained for tissue values from 1 mo before (Spearman's r = 0.62, p < 0.05; see also Fig. 3). Overall, the mean δ^{15}N value was 9.34 ± 0.37‰, corresponding to a trophic level of 4.89, based on Eq. (2).

For *Nezumia aequalis*, δ^{15}N values showed only slight, non-significant differences, with the lowest value observed in September and the highest in June (Fig. 2a). The correlation between δ^{13}C values of untreated and normalised samples was significant (Spearman's r = 0.85, p = 0.03); δ^{13}C values of
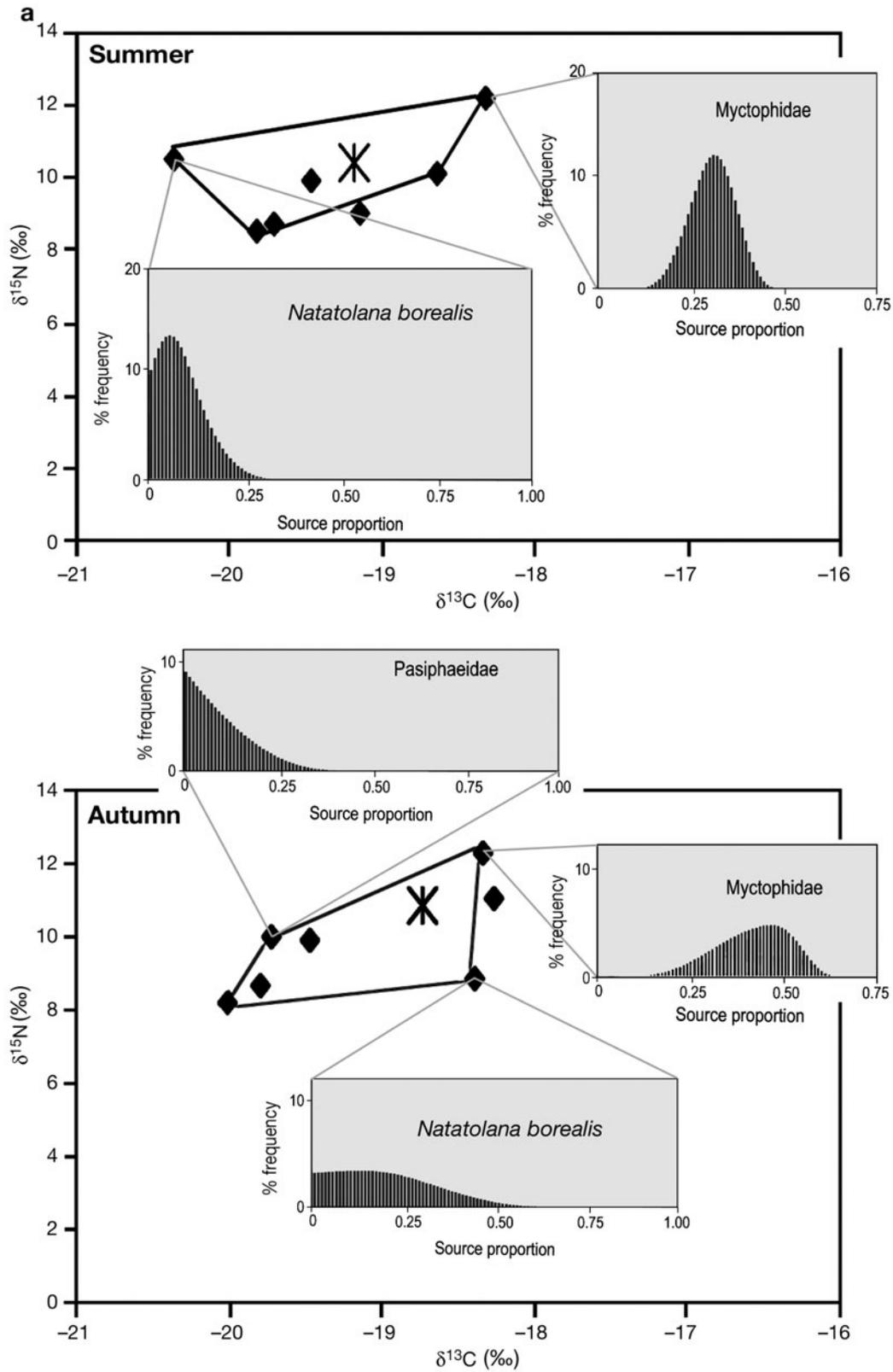
Fig. 4. *Hoplostethus mediterraneus*. Mixing polygon for δ^{13}C and δ^{15}N signatures of 7 food sources for (a) periods for which diet was well established (summer and autumn) and (b) periods for which diet was not well established (winter and spring, few specimens) for *H. mediterraneus* (after correcting for trophic fractionation). Histograms show the distribution of feasible contributions from the 3 most important sources of *H. mediterraneus* diet (indicated by asterisk [*]). Values shown in the boxes are 1 to 99 percentile ranges for these distributions
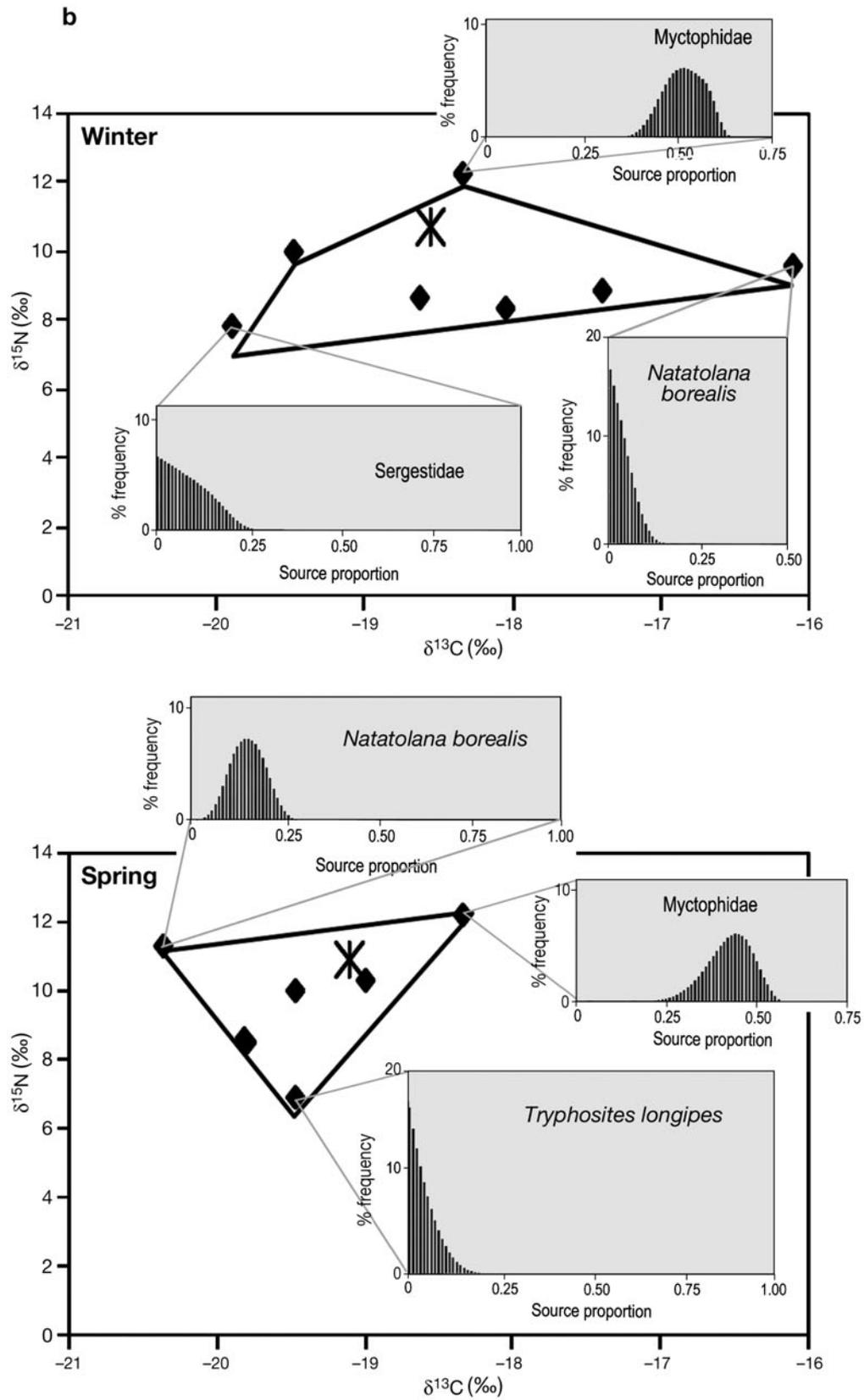
Fig. 4 (continued)

untreated samples did not show significant differences by month. As for *Hymenocephalus italicus*, more depleted values, although not significantly, occurred from February to June (Fig. 2b). Significant differences were found among δ^{13}C values of normalised samples ($F_{5,12}$ = 4.71, p < 0.05), with August being more enriched than the other months.

The highest correlation for *Nezumia aequalis* between dietary and tissue δ^{15}N values was obtained when dietary values were correlated with tissue values obtained 2 mo previously (Spearman's r = 0.86; p < 0.05; see also Fig. 3). Overall the mean δ^{15}N value was 11.19‰ (±0.39), corresponding to a trophic level of 5.64.

The trophic levels calculated by assuming a trophic enrichment of 3.25‰ were 4.31 for *Hoplostethus mediterraneus*, 4.26 for *Hymenocephalus italicus* and 4.85 for *Nezumia aequalis*.

Correlations with biotic and abiotic factors

Stomach fullness of *Hoplostethus mediterraneus* was significantly correlated with month (Spearman's r = 0.17, p < 0.05), total length (r = −0.21, p < 0.05) and weight (r = −0.29, p < 0.01) of specimens. Among the explanatory environmental variables, fullness was also correlated with salinity (r = −0.88, p = 0.01) and the concentration of surface chl *a* recorded 2 mo before sampling (r = −0.94, p < 0.001).

Fullness of *Hymenocephalus italicus* was correlated with PAL (r = −0.21, p < 0.01) and weight (r = −0.21, p < 0.01). Among the environmental variables explored, fullness of *H. italicus* was only correlated with surface chl *a* concentration recorded 3 mo before sampling began (r = 0.94, p < 0.001). Weight and PAL of *Nezumia aequalis* specimens were significantly correlated with fullness (r = −0.17, p = 0.01), but fullness was not correlated with any of the environmental variables, although it was negatively correlated with density of specimens (i.e. the total number of specimens collected in each month; r = −0.83, p = 0.04).

DISCUSSION

Methodological approach

Our results on the effect of formalin on muscle tissue partially confirmed previous findings. According to published studies (Arrington & Winemiller 2002, Sarakinos et al. 2002, Sweeting et al. 2004 and references cited therein) our results for the 3 species indicated a minimal effect of formalin on δ^{15}N values after 24 mo (mean formalin effect, 0.27 ± 0.03‰). Conversely formalin fixation affected δ^{13}C in all 3 species over the

period of study and the effect varied among species. In all species there was an increasing depletion with time of preservation (about 2‰ in *Hoplostethus mediterraneus* and *Hymenocephalus italicus* and 1‰ in *Nezumia aequalis*). This suggests a relationship between species behaviour (i.e. increasing depletion with increasingly pelagic diet and habits) and effects of preservative on δ^{13}C, which in turn may be related to the lipid content of the species. As observed by Drazen (2007), benthopelagic fishes had significantly larger livers with more lipid, less water and less protein than did benthic fishes; thus, lipid levels may be higher in *H. mediterraneus*, which have strongly pelagic habits, than in macrourids; similarly, lipid levels may be higher in *H. italicus*, a more pelagic feeder, than in *N. aequalis*. Sargent et al. (1983) found similar lipid levels in *Hoplostethus atlanticus* and *H. mediterraneus*, and Phleger & Grigor (1990) demonstrated that in *H. atlanticus* the high lipid content, mainly wax-esters, plays an important role in the buoyancy of the species during its vertical displacements. Our results concur with those of other authors (Hobson et al. 1997, Bosley & Wainright 1999, Arrington & Winemiller 2002), who found samples preserved in formalin to be more negative (i.e. depleted in δ^{13}C) with respect to controls.

As observed by several authors, formalin may have affected preserved specimens through 2 different mechanisms. The first is selective loss of material from the specimen during preservation. The second is uptake of the preservative into the tissue. Formalin is a carbon-based chemical with a characteristic δ^{13}C signature, which is normally depleted with respect to fish tissues. Formalin hydrolyses protein (Hobson et al. 1997, Gloutney & Hobson 1998, Bosley & Wainright 1999, Arrington & Winemiller 2002); thus, we expected depletion of δ^{13}C in formalin-preserved specimens, consistent with loss of hydrolysed proteins. However, differences in protein content as well as in lipid content (Arrington & Winemiller 2002), among our 3 tested fishes, related to their swimming capacity, probably explain the different observed patterns.

Duration of sample preservation and preservative concentration also could affect isotope signatures, and the former appears to have an effect through the first 2 yr of preservation, contrary to other studies (Mullin et al. 1984, Sweeting et al. 2004 and references cited therein), where no effect of preservation duration was found. Although there are several studies on this topic, and almost all point to a clear depletion effect of formalin on δ^{13}C, certain differences were highlighted in our study compared with others. In our study, the R^2 of the observed trends of depletion were 0.48 and 0.66, excluding *Nezumia aequalis*. In brackish-water fish, Arrington & Winemiller (2002) found an R^2 value of 0.41, and in different marine organisms, including

kelp, Kaehler & Pakhomov (2001) found an R^2 of 0.19. This suggests that the patterns of variation in $\delta^{13}C$ depletion are species-specific (Kelly et al. 2006).

With respect to the value of per-step trophic enrichment, 2.54‰ as suggested by Vanderklift & Ponsard (2003) is the most appropriate value to use in this study instead of the widely accepted 3.4‰ (Minagawa & Wada 1984, Post 2002) or 3.25‰ (proposed by Vanderklift & Ponsard 2003 for carnivore vertebrates). In our own data, the per-step trophic enrichment between fish and their diet is <3‰. *Hoplostethus mediterraneus* has a $\delta^{15}N$ value of 10.9‰, while its preferred prey in this study (the isopod *Natatolana borealis*) has a $\delta^{15}N$ of 8.10‰; *Nezumia aequalis* has a $\delta^{15}N$ value of 11.5‰, while among its preferred prey the amphipods of the genus *Rhachotropis* have a mean $\delta^{15}N$ value of 9.13‰ (data published in Fanelli et al. 2009). In both cases it is evident that the per-step trophic enrichment is lower than 3.25‰ and also lower than 2.54‰ in some cases.

Diet and temporal variations

Hoplostethus mediterraneus actively foraged on natatory benthopelagic prey, which must be captured in the benthic boundary layer (Angel 1990). The isopod *Natatolana borealis* was the dominant prey ingested by *H. mediterraneus* on the Algerian Basin slope. This feeding behaviour seemed to be characteristic of the Algerian Basin and may be related to a particular abundance of *N. borealis* in the area (Cartes et al. 2008). Indeed, in other studies, mesopelagic, migrating prey (e.g. natantian decapods mainly belonging to Sergestidae and myctophid fish [Marshall & Merrett 1977, Du Buit 1978, Merrett & Marshall 1980, Madurell & Cartes 2005] and euphausiids [Pais 2002]) dominated the diet. Accordingly, mixing models indicated high consumption by *H. mediterraneus* on myctophids in winter and spring, when a limited number of *H. mediterraneus* were caught.

The overall seasonal changes observed in the relative importance of prey species appear to be related to fluctuations in available prey in the environment and/or to different energy requirements, as has been suggested in several studies of deep-sea fish (e.g. Gordon 1979, Blaber & Bulman 1987, Madurell & Cartes 2005, Cartes et al. 2009). Although limited to small specimens (5 to 6 mm, rarely 10 mm), our data on suprabenthos and mesozooplankton resources in the area (Cartes et al. 2008, authors' unpubl. data) indicate availability of large isopods throughout the year, even in February and April when they were consumed less, which is consistent with Kaim-Malka's (1997) data from an adjacent area of the Mediterranean slope.

Accordingly, mixing models carried out on stable isotope data for the main potential food sources indicated *Natatolana borealis* as one of the major food sources in summer and autumn, while mesopelagic fish (e.g. mycthophids) were consumed more in February and April, although these fish seemed to be important prey items throughout the year. Despite the occurrence of mysids (*Boreomysis arctica*), amphipods (*Tryphosites longipes*) and natantian decapods (e.g. Sergestidae, *Plesionika martia* and Pasiphaeidae) in stomach contents, mixing models confirmed they are accessory prey in the diet of *Hoplostethus mediterraneus* in the Algerian Basin. The shift from almost exclusive consumption of *N. borealis* to predation on mesopelagic fish may be attributed to increased energy requirements before reproduction (see below), since myctophids have a high energetic value (Blaber & Bulman 1987). *H. mediterraneus* reaches a reproductive peak in May to September (D'Onghia et al. 1998). This selective shift was confirmed by the large availability of *N. borealis*, which was recorded as abundant throughout the year, and also in February when it was consumed less (Cartes et al. 2008). *H. mediterraneus* may be defined as a specialist feeder.

The 2 macrourids inhabiting mid-bathyal depths in the Algerian Basin, *Hymenocephalus italicus* and *Nezumia aequalis*, showed diverse diets that were composed mainly of suprabenthic prey (i.e. amphipods, copepods, ostracods, isopods and cumaceans) and infauna (i.e. polychaetes and tanaids). Macrourid fishes are characteristically described as generalist feeders, with widely diversified diets (Pearcy & Ambler 1974, Macpherson 1979, Mauchline & Gordon 1984). This probably constitutes an adaptive advantage in the deep-water, low productivity environments inhabited by macrourids (Pearcy & Ambler 1974). Previous studies (McLellan 1977, Geistdoerfer 1978, Macpherson 1979) attributed the more benthic behaviour of *Nezumia* sp. compared with *H. italicus* to differences in mouth morphology (inferior in the former, apical in the latter), and we found in this study that *N. aequalis* preyed more on infaunal organisms (e.g. the tanaid *Apseudes spinosus*, polychaetes, and the amphipod *Maera schmidti*) than did *H. italicus*. Competition may also be reduced by the offset timing of peak abundance depths of the 2 species; *H. italicus* was more abundant at 550 m (mean ± SD, 1294 ± 655 ind. km^{-2}) than at 750 m (134 ± 88 ind. km^{-2}), while *N. aequalis* centred around 750 m (916 ± 253 ind. km^{-2}, Moranta et al. 2008).

Seasonality in feeding behaviour was correlated to the pelagic or benthic habits of the species. According to previous findings (Haedrich & Henderson 1974, Mauchline & Gordon 1984, Drazen et al. 2001) strong seasonal changes in the diet are due to larger seasonal

fluctuations in pelagic prey compared with benthic prey. Thus, the diet of *Hymenocephalus italicus* that fed on suprabenthic/pelagic resources showed a stronger seasonal trend than *Nezumia aequalis* that preyed mainly upon infauna. Infauna is less variable than suprabenthos (BIOMARE project unpubl. data). Changes in diet could be related, apart from seasonal variation in resource availability, to different energetic requirements for reproduction. In the Mediterranean Sea, *N. aequalis* spawns throughout the year (January to February, April to May and October; Carrassón & Matallanas 1998, Massutí et al. 1995), which could explain the low variation in its dietary composition compared with that of *H. italicus*. In contrast, although mature individuals of *H. italicus* were found throughout the year, ripe females were more abundant in spring and summer (Massutí et al. 1995). Changes in *H. italicus* mainly occurred between November and February, related to a pre-spawning period with different energetic requirements. From February to April, *H. italicus* fed on the more energetic isopods *Munnupsurus atlanticus* and *Natatolana borealis*, instead of on mysids, which are low in caloric content (see Cartes et al. 2008). In any case, seasonality was stronger in *Hoplostethus mediterraneus* than in the 2 macrourids, probably due to its stronger benthopelagic habits and to its more restricted reproductive period.

The relatively high trophic position of *Nezumia aequalis* (see next paragraph and also Polunin et al. 2001, who found a $\delta^{15}N$ of 14.18‰ for this species offshore from Ibiza) is probably due to the high consumption by *N. aequalis* of carnivorous polychaetes (Eunicidae, Aphroditidae, Nepthydae), as suggested by Badalamenti et al. (2002) as an explanation for the high trophic level determined for red mullet *Mullus barbatus*.

In general, trophic levels calculated by Eq. (2) were higher than those reported in Stergiou & Karpouzi (2002) based on the TrophLab estimation from the stomach contents analysis reported in literature for the same species. Differences were ca. 1 trophic level for *Hoplostethus mediterraneus* and *Hymenocephalus italicus* and up to 1.5 trophic levels for *Nezumia aequalis*. This may be attributable to the fact that TrophLab does not take into account the high complexity and lengthening of benthic food webs, assigning a high trophic level to prey fish while underestimating benthic carnivores such as polychaetes or suprabenthic amphipods (Fanelli et al. in press).

Temporal variations in trophic levels

Despite the different feeding behaviour displayed by these 3 deep-sea fishes, a general increase in $\delta^{15}N$ values was observed from September to April. For all 3

species, reproduction occurred throughout the year, but the highest percentages of mature individuals were observed in April for the 2 macrourids and in February for *Hoplostethus mediterraneus* (Massutí et al. 1995, D'Onghia et al. 1998, 1999). An analysis of caloric content of prey in stomachs (Madurell & Cartes 2005, 2006, Cartes et al. 2008) suggested a peak in energy requirements just before the main reproductive period, as also shown for some decapod crustaceans in the area (Fanelli & Cartes 2008, Cartes et al. 2008). The increase in $\delta^{15}N$ values in February to April could be correlated to predation on more energetic prey before the spawning period, which also corresponded to consumption of prey at higher trophic levels (e.g. decapods and fish in *H. mediterraneus* and isopods in *Hymenocephalus italicus*). The peak reproductive period is also accompanied by changes in metabolism and increased lipid levels in pre-spawning individuals. Apparently, variations in isotopic composition were not correlated with dietary changes (i.e. did not correspond to prey found in stomachs). However, the expected (dietary) $\delta^{15}N$ values were strongly correlated with values obtained from tissues collected after a delay of 1 to 2 mo.

Isotopic turnover rate can be divided into growth and metabolic tissue replacement components (Hesslein et al. 1993, MacAvoy et al. 2001, Maruyama et al. 2001). Maruyama et al. (2001) showed that the contribution of metabolic turnover is important, and hypothesised that the relative contribution of metabolic turnover might be higher in slow-growing than in fast-growing fish. Assuming similar growth, since the study was performed with adult specimens of approximately the same size all year round, the contribution of metabolism (and diet) accounted for most of the nitrogen isotopic change in our study. The turnover time of deep-sea fish that normally live at a temperature of ~13°C could be as long as that in some freshwater fishes, e.g., lacustrine *Oncorhynchus nerka*, in which it was estimated to be ca. 40 d (80% turnover rate, Sakano et al. 2005). Tissue turnover depends on the metabolism of each species, and it is expected that there will be higher metabolism (and assimilation of $\delta^{15}N$) among plankton feeders than in benthos feeders (Koslow 1996). Hence, the synchrony between dietary and tissue $\delta^{15}N$ values found in *Hoplostethus mediterraneus*, and the progressive delay found in more benthic, probably slower growing macrourids, with the highest delay evident in the most benthic species, *Nezumia aequalis*.

Changes in $\delta^{13}C$ were stronger in *Hoplostethus mediterraneus* than in the 2 macrourids. In accordance with high fluctuations in planktonic prey availability, ^{13}C signatures changed more markedly over the year in *H. mediterraneus* than in the macrourids. A similar

trend was observed for the more pelagic *Plesionika martia* compared with the more benthic *P. heterocarpus* (Fanelli & Cartes 2008), suggesting a general response for the entire deep-water community.

Influence of biological and environmental variables

In the 3 species, stomach fullness was negatively correlated with body length of the fish (and weight in the case of *Hoplostethus mediterraneus*), which implies that small fish eat relatively more than large fish, probably because of their higher metabolism and energy requirements for growth (Koslow 1996). Only in *H. mediterraneus* was fullness positively correlated to month, which corresponds with the stronger seasonality apparent in its diet and its more pelagic behaviour compared with the 2 macrourid species. The overall seasonal changes observed in the relative importance of preferred prey seem to be related not only to fluctuations in available prey in the environment, as has been suggested in several studies of deep-sea fish (e.g. Gordon 1979, Blaber & Bulman 1987), but with changes in prey selection (e.g. more energy-rich prey consumed before reproduction). A similar trend was found in deep-sea pandalids (Fanelli & Cartes 2008), e.g. an increasing influence of seasonality in the pelagic feeder *Plesionika martia* compared with the more benthic *P. heterocarpus*, and in juvenile *Merluccius merluccius* compared with adults (Cartes et al. 2009). Again, for the fish we analysed, fullness was correlated to surface primary production recorded during the previous 2 and 3 mo in *H. mediterraneus* and *Hymenocephalus italicus*, respectively, whilst such relationships were not found for *Nezumia aqualis*. Therefore, *H. mediterraneus* and *H. italicus*, which are more linked to planktonic prey, and in turn to primary production, were more closely coupled with blooms of primary production than was *N. aequalis*, a strictly benthophagous fish, which probably relates to aspects of their life histories (e.g. recruitment, swarming to spawn). The delay observed between high values of fullness and primary production on the surface may indicate that these deep-sea fishes, at least the benthopelagic species, were mainly linked to the seasonal arrival of particulate organic matter (POM) at the seabed from the surface around the Balearic Islands.

In contrast to the Catalonian slope (Cartes et al. 2009) where strong cascade fluxes occur and are channelled through submarine canyons, the Algerian Basin south of the Balearic Islands is an insular area with little influence from advective inputs (Cartes et al. 2008). Thus, POM arriving at the seabed is mainly of marine origin and vertically transported as phytodetritus from the surface (Fanelli et al. 2009). In the pelagic domain, a de-

lay between peaks of phytoplankton blooms and krill was suggested by different investigators in the North Sea (Noji 1991, Dam et al. 1993) and has also been observed in the bathyal domain of the Algerian Basin (Cartes et al. 2008, Fanelli & Cartes 2008). Fullness in *Hoplostethus mediterraneus* was also correlated with an increase in salinity, which could imply a relationship between this species and changes in water masses, i.e. it seems that the presence of this species in the Algerian Basin was mainly linked to Levantine intermediate waters. Consistently, *H. mediterraneus* abundance decreases dramatically from February to June. During August and September 2003, an anticyclonic gyre was present in the Gulf of Valencia, which produced a branch of the Balearic Current flowing inside the Mallorca Channel, with distinct winter intermediate water and Levantine intermediate water values (Lopez-Jurado et al. 2008). Correlations with salinity close to the seabed suggest a link between food consumption of *H. mediterraneus* (increment from August to November) and changes in water masses.

In conclusion, deep-sea fishes around the Balearic Islands are subjected to intra-annual variability in environmental conditions of both a trophic (primary production) and an oceanographic (water masses) nature. In addition, this environmental variability seems to have a higher and more rapid influence in species belonging to low trophic levels that consume pelagic prey species than among benthos feeders situated at the top of deep-sea trophic webs.

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