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Inter-specific and ontogenic differences in $\delta^{13}C$ and $\delta^{15}N$ values and Hg and Cd concentrations in cephalopods

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ABSTRACT: Chemical tracers (e.g. stable isotopes or trace metals) are increasingly used to study trophic ecology and feeding habits of marine organisms and various factors can affect their values. The aim of the present study was to provide information on ontogenic effects on stable carbon and nitrogen values ($\delta^{13}C$ and $\delta^{15}N$) and on cadmium (Cd) and mercury (Hg) concentrations in 5 cephalopod species from the Bay of Biscay (North-East Atlantic Ocean). To this end, individuals belonging to 3 species showing the widest range of sizes were analysed for muscle $\delta^{13}C$ and $\delta^{15}N$ values and muscle Hg, and digestive gland Cd concentrations. Results showed that stable isotope ratios allowed discrimination of specific feeding strategies during ontogenesis. Segregation between 5 cephalopod species in terms of trophic ecology was also evidenced (different isotopic niches). In contrast, Hg concentrations varied over the same order of magnitude in these 5 cephalopod species, despite higher levels in the benthic octopus *Eledone cirrhosa*. Consistently, Hg concentrations followed the same ontogenic pattern and increased with increasing body size/age of cephalopods. Finally, Cd concentrations varied over 3 orders of magnitude among the 5 species. Despite possible effects of physiology in terms of metal bioaccumulation, Cd concentrations were likely to reflect specific feeding preferences or feeding zones and ontogenic variability within a single species. Thus, ontogenic effects have to be taken into account when stable isotope ratios of carbon and nitrogen or trace metals are used as ecological tracers; the best recommendation being to focus on a given class age.

KEY WORDS: Stable isotopes · Metals · Trace elements · Ontogenesis · Prey · Ecological tracer · Northeastern Atlantic

INTRODUCTION

A fundamental requirement to understand the structure and functioning of marine ecosystems is the knowledge of trophodynamics or trophic linkages among species of these ecosystems (Cury et al. 2003). Moreover, the definition of species trophic level is a key aspect of many ecosystem models, providing a valuable indicator to assess trophic structure and ecosystem changes, e.g. due to overfishing (Gascuel et al. 2005).

Until recently, the traditional method for investigating diet and trophic level of organisms was the study of stomach or gut contents. Such a method allows a precise taxonomic description of the prey consumed and of their size range. However, it also requires a lot of time, it may be skewed by the differential digestion of the different prey, and it only represents the food ingested (and not assimilated) in the short-term (Hobson & Wassenaar 1999, Santos et al. 2001). Therefore, the use of tracers has dramatically increased in trophic ecology in the last decades (e.g. Lahaye et al. 2005, Michener & Kaufman 2007). Such tracers, which are generally chemical parameters, encompass stable isotope ratios of carbon and nitrogen ($\delta^{13}C$ and $\delta^{15}N$), lipids, or some trace elements (either stable or radioactive). These tracers are investigated in biological tis-

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sues of consumers to complement traditional methods of dietary studies (Hobson & Wassenaar 1999).

Stable isotopes of carbon are often used as an indicator of the feeding zone or habitat, since ratios vary little between those of the primary producers of the local food chain or a prey and those of the consumers (≤1‰) (De Niro & Epstein 1978, Hobson 1999). Moreover, in the marine environment, pelagic and/or oceanic primary producers are generally depleted in ^{13}C compared to those in benthic and/or neritic environments, discriminating the pelagic vs. benthic or inshore vs. offshore contribution to food intake (France 1995, Sherwood & Rose 2005). In contrast, consumers are enriched in ^{15}N relative to their food (between 2.5 and 5‰) (De Niro & Epstein 1981, Vanderklift & Ponsard 2003), so stable isotopes of nitrogen are generally used as an indicator of the trophic level (Hobson & Welch 1992).

Trace metals such as cadmium (Cd) and mercury (Hg) are released in the environment from both natural and anthropogenic sources (e.g. zinc ore for Cd, volcanism and waste incineration for Hg). They reach the ocean through river inputs and atmospheric depositions (Cossa & Lassus 1989, Cossa et al. 1990). Trophic transfer is then the main pathway for the intake of both Cd and Hg, and these metals bioaccumulate in higher trophic level consumers (Eisler 1987, Cossa et al. 1990, Koyama et al. 2000, Lahaye et al. 2005). Thus, these non-essential elements may also be potential tracers of the habitat or of the feeding zone of consumers. In the case of Hg, its bioaccumulation is enhanced in biota from mesopelagic environments (Monteiro et al. 1996, Thompson et al. 1998). Seabirds feeding on mesopelagic fish thus exhibit higher Hg concentrations in their feathers than epipelagic feeders (Thompson et al. 1998, Ochoa-Acuña et al. 2002). Furthermore, Hg could represent a potential indicator of the trophic level, given its known biomagnification within food webs (Eisler 1987, Cossa et al. 1990). Cd shows enhanced bioaccumulation in various phyla (i.e. crustaceans, molluscs and vertebrates) from various marine environments (Bargagli et al. 1996, Bustamante et al. 1998, 2004, Zauke et al. 1999). In this way, cephalopods constitute a vector of Cd for their predators such as seabirds and marine mammals (Caurant & Amiard-Triquet 1995, Bustamante et al. 1998, Lahaye et al. 2005). Thus, Cd concentrations in short-beaked common dolphin *Delphinus delphis* identified long-term segregation between oceanic and neritic dolphin populations in the Bay of Biscay (Lahaye et al. 2005).

The signature (stable isotopes) or bioaccumulation (metals) of ecological tracers in a consumer's tissues greatly depends on its diet. The use of these chemical parameters as tracers in predators thus relies on a good knowledge of the factors influencing the values in prey, such as spatio-temporal variations, the chemical form of metals, or biological factors such as sex, species or taxa, or even those linked to ontogeny (age, growth, dietary shifts) (Vanderklift & Ponsard 2003, Lahaye et al. 2005, Perga & Gerdeaux 2005). In this respect, ontogenic effects have been poorly and especially unequally treated as a function of the potential tracers presented above. While Hg is well-known to bioaccumulate in muscle tissue with size or age in various taxa (Cossa et al. 1990, Rossi et al. 1993), Cd bioaccumulation patterns with age are less well-established and seem to differ between tissues, species, and (Cossa & Lassus 1989, Cronin et al. 1998, Miramand et al. 2006, Pierce et al. 2008). Finally, studies on ontogenic effects on stable isotope ratios in marine organisms are relatively scarce and mainly concern fish (Jennings et al. 2001, Perga & Gerdeaux 2005). The question of ontogenic changes on all these tracers has thus far been poorly treated in cephalopods. Such a consideration is very important as cephalopods are both active predators and prey, and thus constitute an important link within food webs (Piatkowski et al. 2001).

To study the feeding ecology of cephalopods, the use of tracers such as stable isotope ratios is particularly suitable (Cherel & Hobson 2005, Ruiz-Cooley et al. 2006, Parry 2008), notably because prey identification from the gut is often difficult (Jackson et al. 2007). In addition, these voracious predators can accumulate high metal concentrations in their tissues, especially in the digestive gland (Martin & Flegal 1975, Miramand & Bentley 1992). Food is generally considered as a major source for several metals in cephalopods, especially for Cd and Hg (Koyama et al. 2000, Bustamante et al. 2002a, Lacoue-Labarthe et al. 2009). Finally, as prey, cephalopods can transfer these metals to their predators, as described previously in the case of Cd in the Northeast Atlantic Ocean (Caurant & Amiard-Triquet 1995, Bustamante et al. 1998, Lahaye et al. 2005).

In this context, the aim of the present study was to assess the effects of ontogenic changes on (1) stable isotope ratios ($\delta^{13}C$, $\delta^{15}N$) and (2) Cd and Hg bioaccumulation in several species of cephalopods from a same area, to assess the extent to which these ontogenic changes may affect the use of these tracers as feeding ecology indicators in community or ecosystem-scale studies of cephalopods.

MATERIALS AND METHODS

Study area, sampling strategy and sample preparation. The Bay of Biscay (from 1 to 10° W and from 43 to 48° N) is a very large bay open to the Northeast Atlantic Ocean, with a vast continental shelf in its northern part (60 to 100 nautical miles wide), which is

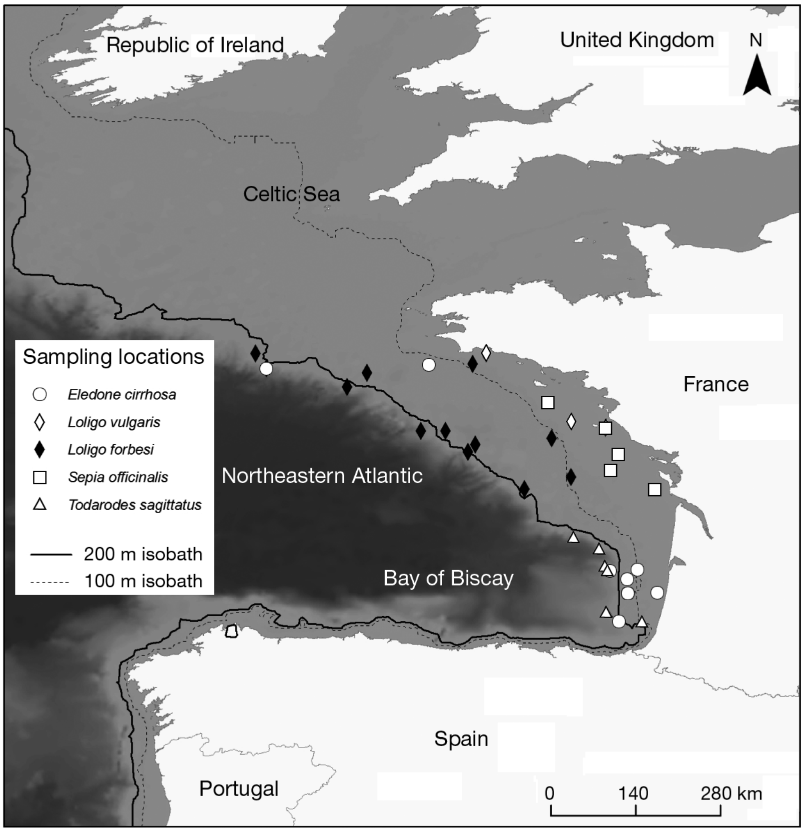

Fig. 1. Study area and sampling locations of cephalopod species (*Eledone cirrhosa*, *Loligo vulgaris*, *L. forbesi*, *Sepia officinalis*, *Todarodes sagittatus*) in the Bay of Biscay

reduced to only a few miles in its southern part (Fig. 1). We focused on 5 species of cephalopods that were caught in various habitats (inshore vs. offshore waters, pelagic vs. benthic or demersal domains): 3 squid species, the veined squid *Loligo forbesi*, the European squid *L. vulgaris*, and the European flying squid *Todarodes sagittatus*; 1 cuttlefish species, the common cuttlefish *Sepia officinalis*; and 1 octopus species, the horned octopus *Eledone cirrhosa*.

Loligo forbesi and *L. vulgaris* are benthopelagic species mainly feeding on fish and to a smaller extent on crustaceans, polychaetes, or other cephalopods (Roper et al. 1984, Guerra & Rocha 1994, Pierce et al. 1994). However, they differ in their distribution: *L. forbesi* is usually found in depths ranging from 100 to 400 m, whereas *L. vulgaris* generally occurs in depths shallower than 250 m (Roper et al. 1984, Lordan et al. 2001a). *Todarodes sagittatus* is a more oceanic species that is generally trawled beyond 200 m depth and may occur as deep as 1000 m. It is also mainly piscivorous (Roper et al. 1984, Lordan et al. 2001a,b). *Sepia officinalis* is a demersal and neritic species inhabiting waters from coastline to about 200 m depth but is most abundant in the upper 100 m. Its diet is generally composed of small molluscs, crabs, shrimps, other cephalopods and juvenile demersal fish (Roper et al. 1984). Finally, *Eledone cirrhosa* is a benthic species that may occur as deep as 500 m, but mostly occurs between 60 and 150 m, and feeds primarily on crustaceans such as shrimps, crabs, and lobsters (Roper et al. 1984, Lordan et al. 2001a).

Overall, 147 individuals were collected during the EVHOE groundfish surveys conducted by the Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER) from the continental shelf to the shelf-edge of the Bay of Biscay in the autumns of 2005 to 2008. Specimens were frozen at –20°C on board and then stored at the laboratory until dissection, during which each organism was weighed and measured (dorsal mantle length, DML) and a piece of mantle muscle and the whole digestive gland were removed. Characteristics (DML, weight, sex and average trawling depths) of the individuals are given in Table 1. After dissection, the samples were immediately placed in individual plastic bags, frozen again at –20°C and freeze-dried. Freeze-dried tissues were ground into a fine powder and stored in individual plastic vials until further analyses.

To assess ontogenic effects on $\delta^{13}C$ and $\delta^{15}N$ values and metal bioaccumulation in cephalopods, the 3 species presenting the widest size ranges were selected, i.e. *Loligo vulgaris*, *L. forbesi* and *Sepia officinalis*. Indeed, DML and age (or the number of increments on statoliths) are closely related in *Loligo* spp. and *S. officinalis*, despite some inter-individual variability in growth rates depending on hatching season (Rocha & Guerra 1999, Challier et al. 2002). Therefore, in the present study we assumed that several age classes were considered within the 3 species selected for ontogenic analyses. Also, juveniles with no visible distinct gonads were separated from males and females with known sex (immatures and matures considered together) because these species present a strong sexual dimorphism (Roper et al. 1984, Rocha & Guerra 1999). Juveniles, males, and females were compared as a 3 component categorical factor called 'sex'.

Isotopic analyses. Stable isotopes of carbon and nitrogen were analyzed in the mantle muscle, which is the tissue of reference in food web studies inferred

Table 1. *Loligo vulgaris*, *L. forbesi*, *Todarodes sagittatus*, *Sepia officinalis* and *Eledone cirrhosa.* Characteristics of individuals (N = sample size for each species), muscle stable isotope values, muscle Hg, and digestive gland Cd concentrations for 5 species of cephalopods from the Bay of Biscay. Values are means ± SD with ranges (minimum, maximum) for trawling depths and chemical parameter values, and ranges only for DML and TW. DML: dorsal mantle length; TW: total weight; M: males; F: females; J: juveniles (i.e. small individuals with no visible distinct gonads)

Family	Species	N	Trawling depth (m)	Sex	DML (mm)	TW (g)	Muscle δ^{13}C (‰)	Muscle δ^{15}N (‰)	Muscle Hg (ng g^{-1} dry wt)	Digestive gland Cd (µg g^{-1} dry wt)
Loliginidae	*Loligo vulgaris*	28	32 ± 14 (25, 58)	16 M, 6 F, 6 J	73, 290	13, 562	–16.4 ± 0.5 (–17.5, –15.6)	14.5 ± 1.0 (12.1, 15.4)	142 ± 40 (65, 200)	5.2 ± 2.6 (2.3, 17.2)
	Loligo forbesi	50	171 ± 100 (96, 492)	21 M, 9 F, 20 J	39, 490	4, 1585	–17.8 ± 0.4 (–18.9, –16.5)	12.5 ± 0.7 (11.3, 14.4)	188 ± 126 (47, 547)	6.2 ± 2.8 (1.3, 12.0)
Ommastrephidae	*Todarodes sagittatus*	17	449 ± 99 (92, 536)	1 M, 16 F	195, 305	150, 627	–17.7 ± 0.5 (–18.7, –17.1)	12.2 ± 0.8 (10.7, 13.4)	248 ± 68 (139, 394)	18.6 ± 12.0 (7.7, 45.8)
Sepiidae	*Sepia officinalis*	30	32 ± 12 (25, 66)	14 M, 10 F, 6 J	48, 235	14, 1436	–16.4 ± 0.3 (–17.0, –15.8)	13.2 ± 0.7 (12.2, 14.8)	201 ± 72 (83, 380)	9.6 ± 9.4 (2.5, 44.7)
Octopodidae	*Eledone cirrhosa*	22	136 ± 54 (43, 337)	12 M, 8 F, 2 J	64, 123	97, 653	–16.7 ± 0.5 (–17.8, –16.0)	11.8 ± 0.3 (11.1, 12.3)	340 ± 72 (222, 561)	16.3 ± 9.6 (6.1, 40.0)

from stable isotope analyses (Hobson & Welch 1992, Pinnegar & Polunin 1999). The use of mantle muscle allows comparisons of isotopic signatures between individuals and taxa, minimizing inter-tissue differences in terms of biochemical and physiological properties like protein turnover rate and metabolic routing (Cherel et al. 2009).

Because lipids are highly depleted in ^{13}C relative to other tissue components (De Niro & Epstein 1977), they were extracted from muscle samples using cyclohexane. To accomplish this, an aliquot of approximately 100 mg of fine powder was agitated with 4 ml of cyclohexane for 1 h. Next, the sample was centrifuged for 5 min at 4000 × *g*, and the supernatant containing lipids was discarded. Then, the sample was dried in an oven at 45°C for 48 h, and 0.35 ± 0.05 mg subsamples of lipid-free powder were weighed in tin cups for stable isotope analyses. These analyses were performed with an elemental analyser coupled to an Isoprime (Micromass) continuous-flow isotope-ratio mass spectrometer (CF IR-MS). The results are presented in the usual δ notation relative to the deviation from standards (Pee Dee Belemnite for δ^{13}C and atmospheric nitrogen for δ^{15}N), in parts per thousand (‰). Based on replicate measurements of internal laboratory standards, experimental precision is of ±0.15 and ±0.20‰ for δ^{13}C and δ^{15}N, respectively.

Metal analyses. Total Hg analyses were conducted on the mantle muscle of all species, while Cd was analysed in the digestive gland. Hg is known to mainly accumulate in its organic form in the muscle tissue of cephalopods (Bustamante et al. 2006), whereas the role of the digestive gland in the storage and detoxification of Hg in cephalopods is not well defined (Bustamante et al. 2006, Pierce et al. 2008, Lacoue-Labarthe et al. 2009). In contrast, the digestive gland is well recognized as the key organ in bioaccumulation and detoxification of Cd in cephalopods (Miramand & Bentley 1992, Bustamante et al. 2002a, Pierce et al. 2008).

Hg analyses were carried out with an Advanced Mercury Analyser (ALTEC AMA 254) as described in Bustamante et al. (2006) on at least 2 homogenized dry muscle tissue subsamples for each individual. Hg analyses were run according to a thorough quality control program including the analysis of a certified reference material (CRM) TORT-2 (lobster hepatopancreas; National Research Council, Canada [NRCC]). CRM aliquots were treated and analysed in the same conditions as the samples. CRM results were in good agreement with the certified values, with an average recovery rate of 97%. The detection limit was 5 ng g^{-1} dry weight (dry wt). All Hg concentrations in tissues reported below are expressed in ng g^{-1} dry wt.

Cd analyses were performed by inductively coupled plasma optical emission spectroscopy as described in Hédouin et al. (2009) for 2 subsamples of each digestive gland. Blanks and CRM samples were treated and analysed in the same way as the cephalopod samples. The CRMs were dogfish liver DOLT-3 (NRCC) and lobster hepatopancreas TORT-2 (NRCC). CRM results were in good agreement with the certified values with an average recovery rate of 92 and 98% for DOLT-3 and TORT-2, respectively. The detection limit was 0.15 µg g^{-1} dry wt. All Cd concentrations given below are expressed in µg g^{-1} dry wt.

Data treatment. We first analyzed the relationships between the potential tracers (muscle δ^{13}C and δ^{15}N values, muscle Hg concentrations, and digestive gland Cd concentrations), with regard to cephalopod trawling depths and DML using a principal component analysis (PCA). The PCA was based on the correlation matrix and normalised data for each variable (i. e. centred and divided by the standard deviation). Then, all data submitted to statistical tests (or residuals in the case of analysis of variance, ANOVA) were checked for normality (Shapiro-Wilks test) and for homogeneity of variances (Bartlett's test). When these conditions were fulfilled, parametric tests were used in the subsequent analyses; otherwise, non-parametric analogues were used. Thus, a Kruskal-Wallis (KW) test followed by a multiple comparison test with Holm's adjustment method was performed to test inter-specific differences in muscle $\delta^{13}C$ and $\delta^{15}N$ values. For each of the 3 species selected for ontogenic analyses (*Loligo vulgaris*, *L. forbesi* and *Sepia officinalis*), the Pearson or Spearman correlation coefficient test was used to analyze the correlation between $\delta^{15}N$ values and DML, between log-transformed muscle Hg concentrations and DML or $\delta^{15}N$ values, and between log-transformed digestive gland Cd concentrations and DML or $\delta^{15}N$ values. On scatterplots of statistically significant relationships, a smoother LOWESS (locally weighted polynomial regression) was added to help interpretation of correlation coefficients. Finally, 1-way ANOVAs were performed to test between-sex differences in $\delta^{15}N$ values, muscle Hg concentrations, and digestive gland Cd concentrations in the same 3 species (ANOVA or KW test, followed by Tukey's HSD test in the case of ANOVA or a multiple comparison test with Holm's adjustment method in the case of KW). The level of significance for statistical analyses was always set at $\alpha = 0.05$. All statistical analyses were performed using the free software R (R Development Core Team 2010).

RESULTS

Inter-specific differences

The first 2 principal components accounted for 68% of the total variation present in the dataset (39 and 29% for axis 1 and 2, respectively). Depth and stable isotope values were the variables that contributed most to the first axis, whereas muscle Hg concentrations and DML contributed most to the second axis (i.e. contribution of the variable ≥25%; Fig. 2). Principal component 1 indicated that increased digestive gland Cd concentrations were associated with important trawling depths but with low $\delta^{13}C$ and $\delta^{15}N$ values (Fig. 2). Nevertheless, Cd concentrations in the digestive gland contributed more to the formation of the third axis, not represented here (contribution of 39%). Principal component 2 showed that increased muscle Hg concentrations are highly correlated with DML (Fig. 2). Finally, when individuals were grouped by species or by sex, it emerged that species were more segregated by principal component 1 (i.e. by muscle $\delta^{13}C$ and $\delta^{15}N$ values and to a lesser extent digestive gland Cd concentrations), while juveniles strongly differed from adults in their muscle Hg concentrations on principal component 2 (males and females generally not segregated) (Fig. 2).

Regarding inter-specific differences in carbon and nitrogen measurements in particular (Fig. 3), the 5 species of cephalopods from the Bay of Biscay differed significantly by both their average $\delta^{13}C$ ($p_{KW} < 0.001$) and $\delta^{15}N$ values ($p_{KW} < 0.001$). Post hoc multiple comparison test showed that *Loligo forbesi* and *Todarodes sagittatus* had significantly lower $\delta^{13}C$ values ($p < 0.05$) than *L. vulgaris*, *Sepia officinalis* and *Eledone cirrhosa* (Table 1, Fig. 3). As for nitrogen, *T. sagittatus* and *L. forbesi* (more oceanic species) did not differ significantly ($p > 0.05$), whereas *L. vulgaris*, *S. officinalis* and *E. cirrhosa* (neritic species) presented significantly different $\delta^{15}N$ values ($p < 0.05$) (Table 1, Fig. 3).

Ontogenic effects in *Loligo vulgaris*, *L. forbesi* and *Sepia officinalis*

Ontogenic effects on stable isotope ratios

As a positive correlation between $\delta^{13}C$ and $\delta^{15}N$ values was evidenced by the PCA (Fig. 2), correlation coefficient tests between stable isotope values and DML were only performed on $\delta^{15}N$ values to avoid redundant analyses and figures.

In *Loligo vulgaris*, the correlation coefficient was low between $\delta^{15}N$ values and DML ($R^2_{Spearman} = 0.233$, $p = 0.010$), reflecting the non-linear relationship existing between both variables (Fig. 4a). Indeed, $\delta^{15}N$ values were very similar for individuals larger than approximately 100 mm DML, corresponding to adult individuals that could be sexed, and strongly differed from juvenile ones (Table 2).

In *Loligo forbesi*, there was a positive correlation between $\delta^{15}N$ values and DML ($R^2_{Pearson} = 0.439$, $p < 0.001$), but variability between similar-sized individuals was high (Fig. 4b). There was no significant difference in $\delta^{15}N$ values between juveniles and females, but both were significantly different from males (Table 2). This contributed to the increase in $\delta^{15}N$ values with increasing size, as males are considerably larger than females in this species (Fig. 4b).

In *Sepia officinalis*, there was also a positive relationship between $\delta^{15}N$ values and DML, but the correlation coefficient was lower ($R^2_{Pearson} = 0.273$, $p = 0.003$); $\delta^{15}N$ values varied greatly between similar-sized individuals at all stages (Fig. 4c).

Ontogenic effects on metal bioaccumulation

In the 3 species, log-transformed Hg concentrations in the muscle were strongly and positively correlated with DML ($R^2_{Spearman} = 0.600$, $R^2_{Spearman} = 0.836$, and $R^2_{Pearson} = 0.596$, in *Loligo vulgaris*, *L. forbesi* and *Sepia officinalis*, respectively; all $p < 0.001$) (Fig. 5). Hg concentrations in the muscle and $\delta^{15}N$ values were also correlated ($R^2_{Spearman} = 0.361$, $R^2_{Spearman} = 0.404$ in *L. vulgaris* and *L. forbesi*, respectively, both $p < 0.001$; $R^2_{Pearson} = 0.182$ in *S. officinalis*, $p = 0.019$) (Fig. 5).

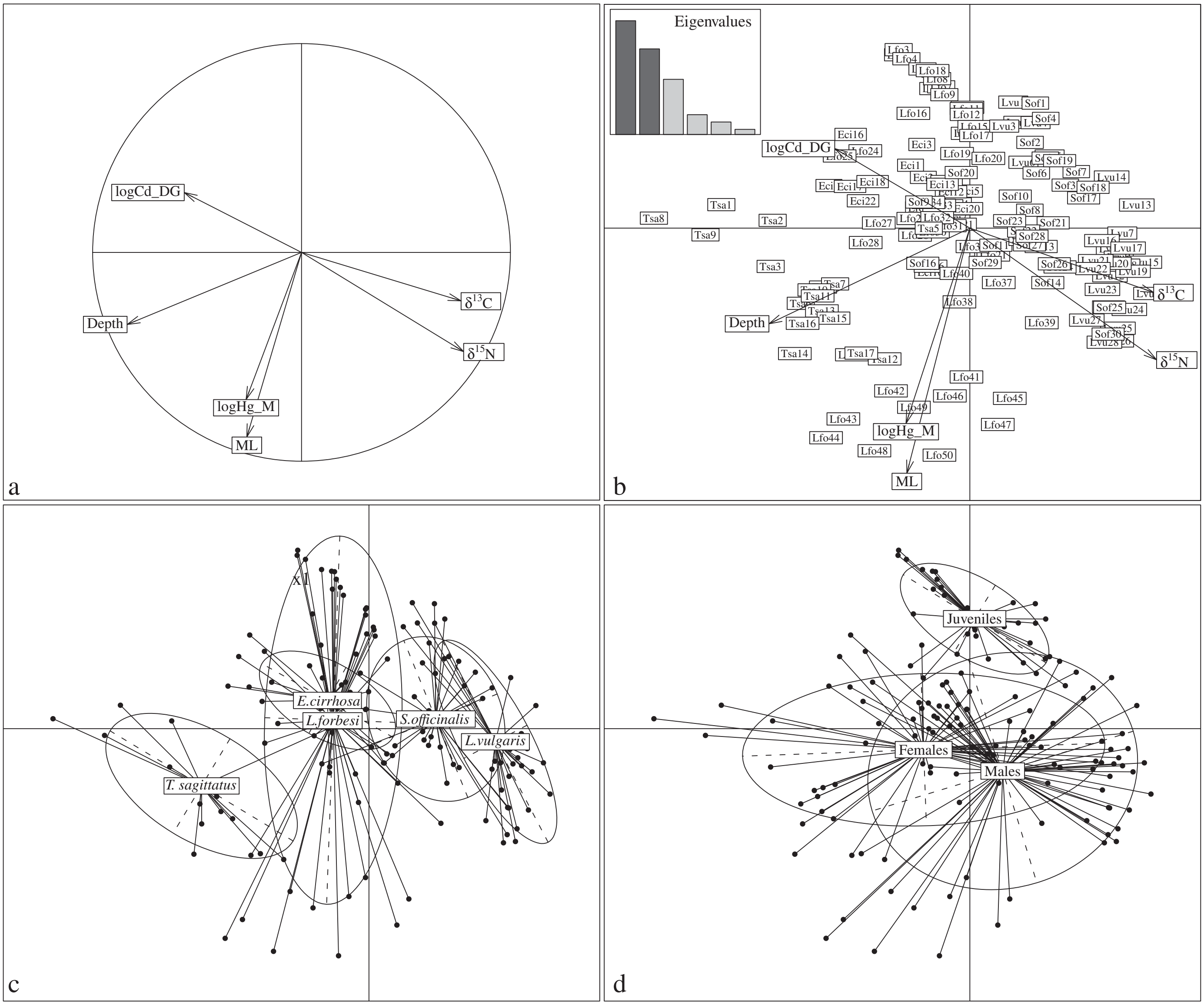

Fig. 2. *Eledone cirrhosa*, *Loligo vulgaris*, *L. forbesi*, *Sepia officinalis* and *Todarodes sagittatus.* Projection of variables (Cd_DG: Cd in digestive gland; Hg_M: Hg in muscle; ML: mantle length) and individuals on the first 2 components resulting from the principal component analysis (PCA). (a) Correlation biplot showing the distribution of the variables. The length of the line for a variable shows how well it is represented by the 2-dimensional approximation, and reflects its contribution to the first 2 principal components. Horizontal axis: principal component 1 (eigenvalue = 2.3, that is 39% of the variability explained by axis 1); vertical axis: principal component 2 (eigenvalue = 1.8, that is 29% of the variability explained by axis 2). Variables pointing in the same direction display a high positive correlation. Variables pointing in the opposite direction have a high negative correlation. Variables with an angle of 90° have a small correlation close to 0. (b) Projection of individuals on the correlation biplot, and histogram of eigenvalues of the different components resulting from the PCA. The black shaded bars refer to the first 2 components with higher eigenvalues, represented on the correlation biplot. The grey shaded bars refer to the other components. Eigenvalues = 2.3, 1.8, 1.1, 0.4, 0.3, 0.1 for the components resulting from the PCA, from 1 to 6 respectively. (c) Grouping of individuals by species. (d) Grouping of individuals by sex (juveniles: small individuals with no visible distinct gonads)

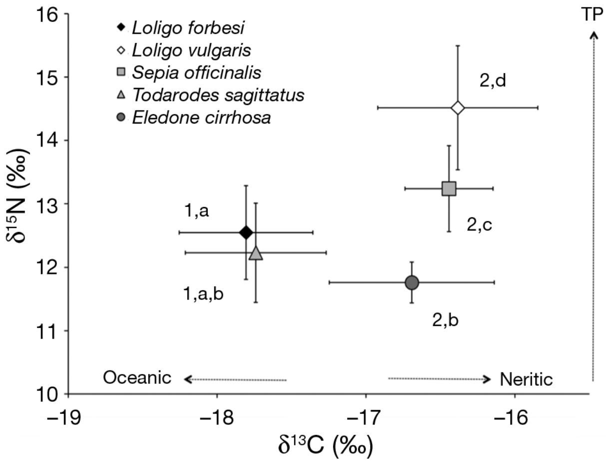

Fig. 3. *Loligo vulgaris*, *L. forbesi*, *Sepia officinalis*, *Todarodes sagittatus* and *Eledone cirrhosa.* Muscle $\delta^{13}C$ and $\delta^{15}N$ values (‰) of 5 species of cephalopods from the Bay of Biscay. Species not sharing the same numbers ($\delta^{13}C$) and letters ($\delta^{15}N$) are significantly different ($\delta^{13}C$: Kruskal-Wallis $\chi^2 = 97.5$, df = 4, $p < 0.001$; $\delta^{15}N$: Kruskal-Wallis $\chi^2 = 80.9$, df = 4, $p < 0.001$). Values are means ± SD. TP = Trophic position

In the 3 species, adults significantly differed from juveniles in their muscle Hg concentrations (Table 2). Among adults, average Hg concentrations were significantly different between males and females for *Loligo vulgaris* and *L. forbesi* but not for *Sepia officinalis* (Table 2).

Regarding Cd, there was a significant negative correlation between log-transformed concentrations and DML on the one hand ($R^2_{\text{Spearman}} = 0.416$, $p < 0.001$), and between log-transformed Cd concentrations and $\delta^{15}N$ values on the other hand ($R^2_{\text{Pearson}} = 0.253$, $p < 0.001$), in *Loligo forbesi* only (Fig. 6). In fact, males (comprising larger individuals with the highest $\delta^{15}N$ values) significantly differed from juveniles and females (Table 2, Fig. 6). No significant correlation was found between log-transformed digestive gland Cd concentrations and DML or $\delta^{15}N$ values, neither were differences found between sexes in *L. vulgaris* and *Sepia officinalis* (Fig. 6, Table 2).

DISCUSSION

Inter-specific differences in trophic ecology and metal bioaccumulation

From a community point of view, isotopic signatures (carbon and nitrogen combined) were the chemical parameters that best segregated 5 cephalopod species from the Bay of Biscay (Figs. 2 & 3). Four distinct isotopic niches (as described by Newsome et al. 2007) emerged, with *Loligo forbesi* and *Todarodes sagittatus* sharing the same isotopic niche (Fig. 3). The different isotopic niches suggest clear segregations in terms of trophic ecology, and these results agree with what is currently known about the spatial distribution and feeding habits of the 5 species. Indeed, *L. forbesi* and

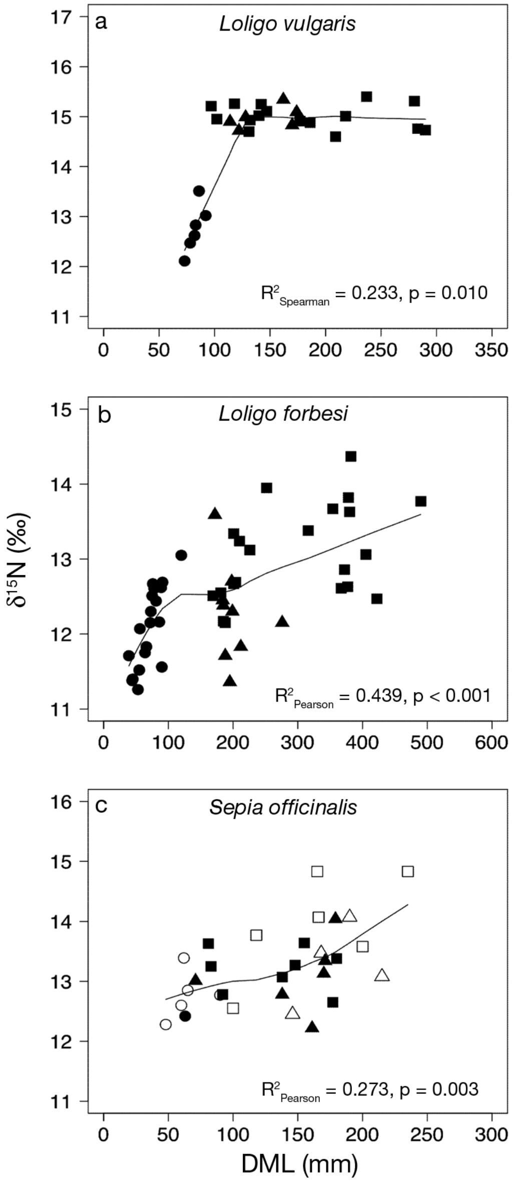

Fig. 4. *Loligo vulgaris*, *L. forbesi* and *Sepia officinalis.* Relationships between muscle $\delta^{15}N$ values and dorsal mantle length (DML) in (a) *Loligo vulgaris*, (b) *L. forbesi*, and (c) *Sepia officinalis*; individuals are separated by sex and by year. Squares = males; triangles = females; circles = juveniles; black = 2008; white = 2006. Smoothing lines (robust, locally weighted scatterplot smoothing system based on the LOWESS algorithm with the software R) represent the fitted non-linear trend of the values when the correlation coefficient test is significant

Table 2. *Loligo vulgaris*, *L. forbesi* and *Sepia officinalis*. Muscle δ^{15}N values, muscle Hg, and digestive gland Cd concentrations in individuals separated by sex (juveniles: small individuals with no visible distinct gonads). Groups with the same letter indicate that sexes are not significantly different (post hoc Tukey's test in the case of ANOVA, multiple comparison test [MPC] with Holm's adjustment method in the case of Kruskal-Wallis [KW]). N = sample size

Species	Sex	N	Mean ± SD	Range	Test and characteristics	Group
Muscle δ^{15}N (‰)						
Loligo vulgaris	Juveniles	6	12.8 ± 0.5	12.1, 13.5	1-way ANOVA (+ Tukey's test)	a
	Females	6	15.0 ± 0.2	14.7, 15.3	$F = 130.7$, df = 2	b
	Males	16	15.0 ± 0.2	14.6, 15.4	$p < 0.001$	b
Loligo forbesi	Juveniles	20	12.1 ± 0.5	11.3, 13.1	1-way ANOVA (+ Tukey's test)	a
	Females	9	12.3 ± 0.6	11.4, 13.6	$F = 14.8$, df = 2	a
	Males	21	13.1 ± 0.6	12.2, 14.4	$p < 0.001$	b
Sepia officinalis	Juveniles	6	12.7 ± 0.4	12.3, 13.4	1-way ANOVA (+ Tukey's test)	a
	Females	10	13.2 ± 0.6	12.2, 14.1	$F = 3.6$, df = 2	a, b
	Males	14	13.5 ± 0.7	12.6, 14.8	$p = 0.041$	b
Muscle Hg (ng g^{-1} dry wt)						
Loligo vulgaris	Juveniles	6	83 ± 18	65, 113	KW + MPC tests (Holm's)	a
	Females	6	161 ± 14	136, 173	$\chi^2 = 12.2$, df = 2	b
	Males	16	156 ± 31	81, 200	$p = 0.002$	c
Loligo forbesi	Juveniles	20	84 ± 20	47, 142	KW + MPC tests (Holm's)	a
	Females	9	199 ± 68	99, 302	$\chi^2 = 34.7$, df = 2	b
	Males	21	282 ± 128	109, 547	$p < 0.001$	c
Sepia officinalis	Juveniles	6	124 ± 34	83, 169	1-way ANOVA (+ Tukey's test)	a
	Females	10	238 ± 82	126, 380	$F = 6.7$, df = 2	b
	Males	14	209 ± 52	108, 290	$p = 0.004$	b
Digestive gland Cd (µg g^{-1} dry wt)						
Loligo vulgaris	Juveniles	6	4.4 ±1.0	3.5, 6.0	KW + MPC tests (Holm's)	a
	Females	6	4.8 ± 1.2	2.3, 5.9	$\chi^2 = 1.3$, df = 2	a
	Males	16	5.6 ± 3.3	3.3, 17.2	$p = 0.530$	a
Loligo forbesi	Juveniles	20	8.0 ± 2.1	4.9, 12.0	KW + MPC tests (Holm's)	a
	Females	9	7.4 ± 3.2	1.7, 11.0	$\chi^2 = 26.7$, df = 2	a
	Males	21	4.0 ± 1.3	1.3, 6.7	$p < 0.001$	b
Sepia officinalis	Juveniles	6	7.0 ± 1.7	5.5, 9.8	KW + MPC tests (Holm's)	a
	Females	10	13.0 ± 13.9	2.5, 44.7	$\chi^2 = 0.8$, df = 2	a
	Males	14	8.4 ± 7.0	3.3, 30.1	$p = 0.670$	a

T. sagittatus generally show a more offshore distribution that fit well with their low δ^{13}C values relative to *L. vulgaris* and *Sepia officinalis* (Roper et al. 1984, Lordan et al. 2001a). The enriched δ^{13}C values of *Eledone cirrhosa* may be explained by the benthic habitat of this octopus, where baseline δ^{13}C values are higher than in pelagic ecosystems (France 1995). Muscle δ^{15}N values increased with the probability of a species to feed more on fish than on pelagic or benthic crustaceans in the neritic species, with *L. vulgaris* > *S. officinalis* > *E. cirrhosa* (Roper et al. 1984, Pierce et al. 1994, Pinczon du Sel et al. 2000, Neves et al. 2009). This is in accordance with δ^{15}N values of the potential prey available for the Bay of Biscay. Small pelagic crustaceans such as the euphausid *Meganyctiphanes norvegica* or those constituting zooplankton (e.g. other euphausids, copepods, mysids) present considerably lower δ^{15}N values than small pelagic fish in the Bay of Biscay (between 2 and 5‰ difference in average) (authors' unpubl. data). Moreover, while benthic crustaceans should present higher δ^{15}N values due to their scavenger behaviour, they actually also exhibit lower average δ^{15}N values (about 1‰) compared to those of small pelagic or demersal coastal fish such as Gobiidae, Callyonimidae, Ammodytidae or Atherinidae in the Bay of Biscay (Le Loc'h et al. 2008, authors' unpubl. data). *Loligo vulgaris*, and to lesser extent *S. officinalis*, consume those kind of fish (Pierce et al. 1994, Pinczon du Sel et al. 2000), and thus exhibit higher δ^{15}N values than the benthic crustacean feeder *E. cirrhosa* (Roper el al. 1984).

Concerning metals, average Hg concentrations in the muscle tissue varied over the same order of magnitude in the 5 species (Table 1) and did not contribute significantly to the segregation of the species in the PCA (Fig. 2). In contrast to Hg, average Cd concentrations in the digestive gland varied over different orders of magnitude between species (Table 1). Cd concentrations tend to increase with the proportion of benthic crustaceans compared to fish in the diet of the neritic species, with *Eledone cirrhosa* > *Sepia officinalis* > *Loligo vulgaris* (Fig. 2, Table 1). Indeed, food is the major source of Cd intake by cephalopods (Koyama et al. 2000, Bustamante et al. 2002a), and benthic invertebrates generally exhibit higher Cd concentrations relative to fish (Cossa & Lassus 1989, Cabrera et al. 1994). In the species more associated with the oceanic domain, the higher Cd concentrations in *Todarodes sagittatus* compared to *L. forbesi* may be related to physiological features more than to diet or habitat, as both species had a similar isotopic niche (Fig. 3). As discussed above, both squids are mainly piscivorous and present an offshore distribution (Pierce et al. 1994, Lordan et al. 2001a,b), but their digestive gland cells

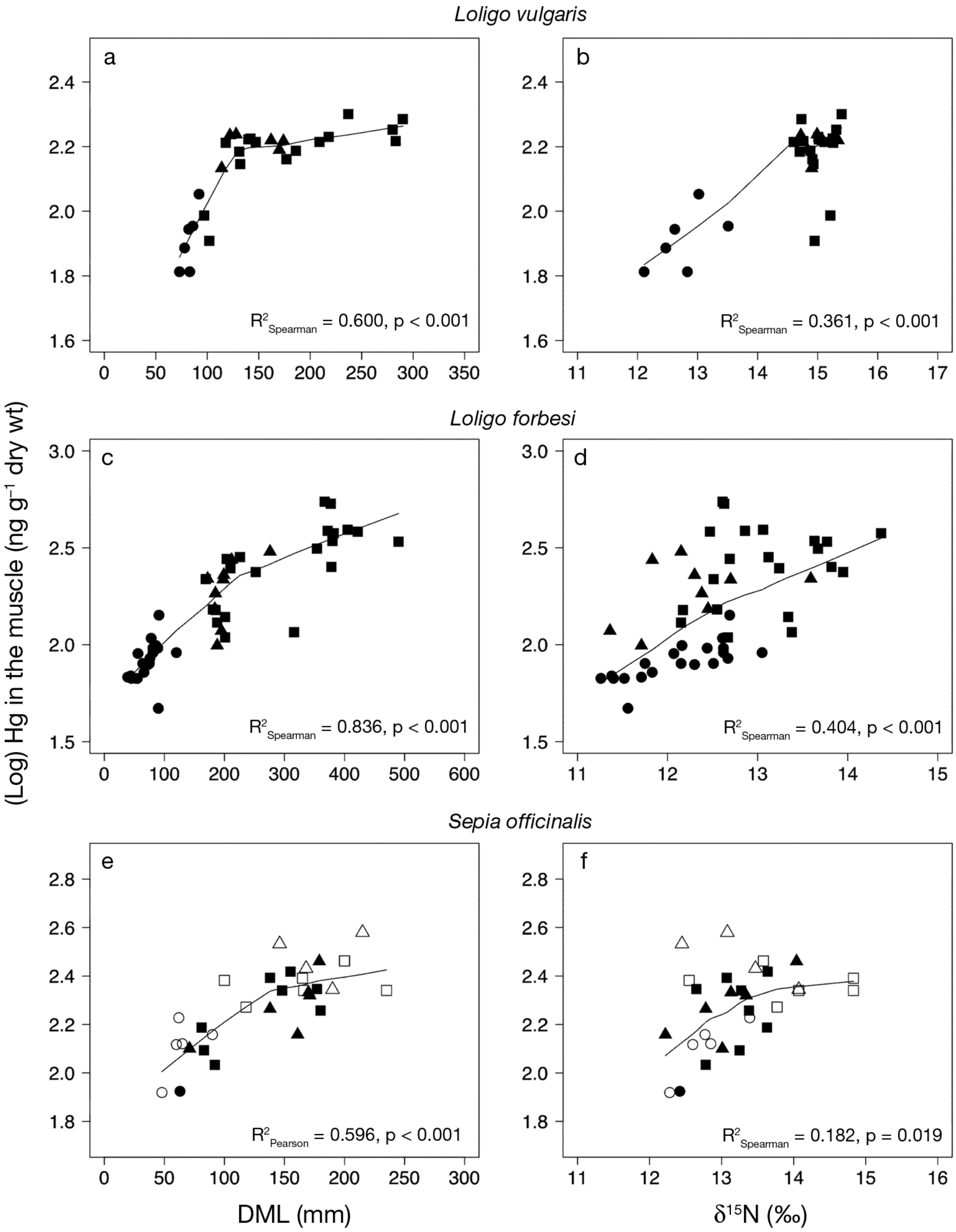

Fig. 5. *Loligo vulgaris*, *L. forbesi* and *Sepia officinalis.* Relationships between log-transformed muscle Hg concentrations and both dorsal mantle length (DML) (left panels) and $\delta^{15}N$ values (right panels) in (a,b) *Loligo vulgaris*, (c,d) *L. forbesi*, and (e,f) *Sepia officinalis*; individuals are separated by sex and by year. Squares = males; triangles = females; circles = juveniles; black = 2008; white = 2006. Smoothing lines (robust, locally weighted scatterplot smoothing system based on the LOWESS algorithm with the software R) represent the fitted non-linear trend of the values when the correlation coefficient test is significant

strongly differ in terms of structure. The digestive gland cells of *T. sagittatus* contain a 'boule' structure typical of many cephalopod species that are non-existent in Loliginidae (Boucaud-Camou & Yim 1980, Boucher-Rodoni & Boucaud-Camou 1987). 'Boules' may contribute to storage of larger amounts of Cd in *T. sagittatus* (Bustamante et al. 2002b). Therefore, the influence of such a physiological difference cannot be excluded for the species that inhabit the coastal domain; *E. cirrhosa* and *S. officinalis* have 'boule' structures, while they are absent in *L. vulgaris* (Table 1).

Ontogenic changes as an important source of intra-specific variations

In cephalopod species that presented the widest size ranges, the analysis of the relationship between $\delta^{15}N$ values and DML allowed us to distinguish different feeding profiles during ontogenesis (Zimmer et al. 2007, Parry 2008). $\delta^{15}N$ values in *Loligo vulgaris* increased drastically between juvenile and adult stages and then remained relatively constant (females and males not differing) (Fig. 4, Table 2). In contrast, in *L. forbesi*, only larger males (>300 mm DML) showed significantly higher $\delta^{15}N$ values (Fig. 4). This may be due to a difference in prey composition or prey size range in those larger individuals that may have greater energy needs (Pierce et al. 1994, Rocha et al. 1994). Nevertheless, the variability in $\delta^{15}N$ values over life stages for males, females, and juveniles strongly suggest that this species has an important dietary plasticity during ontogenesis (Fig. 4). As previously commented, *L. vulgaris* likely remains in coastal waters throughout its life cycle, whereas *L. forbesi* is a more oceanic species that has relatively long offshore displacements throughout its life cycle (Rocha & Guerra 1999). These different ontogenic patterns between both *Loligo* species are confirmed by the analyses of their isotopic signatures (Figs. 3 & 4). While *L. vulgaris* may always feed on the same prey species in the coastal food web (i.e. mainly small pelagic coastal fish), *L. forbesi* may feed on different food webs with different baseline isotopic signatures during ontogenesis (e.g. coastal vs. upper slope or oceanic food web). This could contribute to the greater trophic plasticity of *L. forbesi* in comparison with *L. vulgaris*. Furthermore, the contribution of crustaceans is slightly higher in the diet of *L. forbesi* than in those of *L. vulgaris* (Guerra & Rocha 1994, Pierce et al. 1994). Finally, $\delta^{15}N$ values recorded for juveniles, males, and females in *Sepia officinalis* suggest that this species also displays a strong dietary plasticity at all stages of its life cycle (Fig. 4, Table 2). Thus, isotopic signatures were in agreement with the stomach content data that reflected the general opportunistic behaviour and flexible diet of *S. officinalis* (Pinczon du Sel et al. 2000, Neves et al. 2009).

Ontogenic effects were also found for Hg in the mantle muscle. Hg concentrations varied greatly within each species, with concentrations up to 10 times greater in the individuals showing the highest values compared to those displaying the lowest ones (e.g. in *Loligo forbesi*, Table 1). Hg levels increased with DML in the 3 species, and correlations between Hg concentrations and $\delta^{15}N$ values were considerably lower than those with DML (Fig. 5). This result highlights the ability for Hg to bioaccumulate with age in the muscle of cephalopods (Rossi et al. 1993, Pierce et al. 2008). However, the relationship between muscle Hg levels and $\delta^{15}N$ values in *L. vulgaris*, in particular, also reflected the trophic switch between juveniles and adults (Figs. 4 & 5) since food intake is the major source of Hg in cephalopods (Lacoue-Labarthe et al. 2009). Hg speciation has not been determined in this study, but it is likely that bioaccumulated Hg was the organic form methyl-Hg, which represents the most important form of Hg in cephalopod muscle (Bustamante et al. 2006, Raimundo et al. 2010). The role of the digestive gland in the storage and detoxification of Hg is still controversial (Bustamante et al. 2006, 2008, Pierce et al. 2008, Lacoue-Labarthe et al. 2009). A preferential redistribution of methyl-Hg from the digestive gland to the muscle is expected (Bustamante et al. 2006), where it may bind with sulphydryl groups of muscular proteins (Bloom 1992, Bustamante et al. 2006) and accumulate during ontogenesis.

Finally, the bioaccumulation patterns of Cd in the digestive gland during ontogenesis differed between *Loligo vulgaris*, *L. forbesi* and *Sepia officinalis* (Fig. 6, Table 2). While no clear pattern of digestive gland Cd concentrations with DML or $\delta^{15}N$ values was revealed in *L. vulgaris*; Cd concentrations in the digestive gland of *L. forbesi* significantly decreased with DML and to lesser extent with $\delta^{15}N$ values (Fig. 6). Such a decrease in Cd concentrations with increasing body size was already observed for *L. forbesi* sampled around the United Kingdom (Pierce et al. 2008). These authors explained the observed decrease as a consequence of a dietary shift with increasing body size, strengthened by a dilution of the metal in the digestive gland due to the very fast growth of this squid. The dilution hypothesis seems to be confirmed in the present study for *L. forbesi*. Indeed, the decrease in Cd concentrations is linked to larger males presenting the lowest Cd concentrations and also the lowest $\delta^{15}N$ values (Figs. 4 & 6, Table 2). By contrast with *L. forbesi*, Cd concentrations in the digestive gland of *S. officinalis* from the Bay of Biscay did not show clear relationships with DML nor with $\delta^{15}N$ values or sex (Fig 6, Table 2). Thus, our

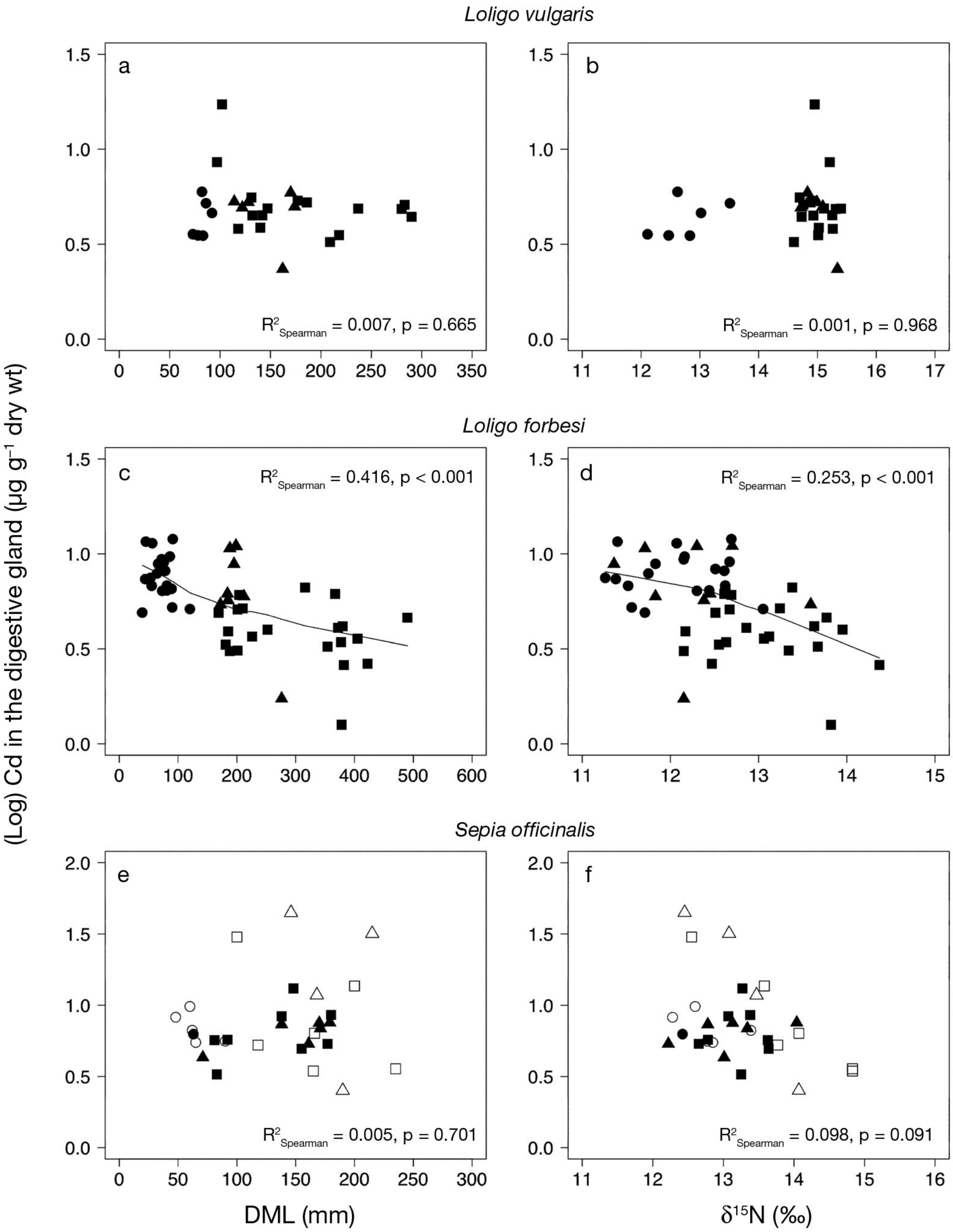

Fig. 6. *Loligo vulgaris*, *L. forbesi* and *Sepia officinalis.* Relationships between log-transformed digestive gland Cd concentrations and both dorsal mantle length (DML) (left panels) and $\delta^{15}N$ values (right panels) in (a,b) *Loligo vulgaris*, (c,d) *L. forbesi*, and (e,f) *Sepia officinalis*; individuals are separated by sex and by year. Squares = males; triangles = females; circles = juveniles; black = 2008; white = 2006. Smoothing lines (robust, locally weighted scatterplot smoothing system based on the LOWESS algorithm with the software R) represent the fitted non-linear trend of the values when the correlation coefficient test is significant

results for *S. officinalis* do not match those from Miramand et al. (2006) who found a clear increase of Cd concentrations with size in the digestive gland of the cuttlefish from the English Channel. Such an increase was explained by the very long biological half-life of Cd in cuttlefish following its assimilation from food (Bustamante et al. 2002a). The strong retention capacity of Cd in the digestive gland of *S. officinalis* is probably related to its cells containing the typical 'boule' structures (Boucaud-Camou & Yim 1980, Boucher-Rodoni & Boucaud-Camou 1987), as commented above for *T. sagittatus*. Nevertheless, in our study, values of Cd levels varied greatly between individuals of *S. officinalis* (ranging from 2.5 to 44.7 µg g^{-1} dry wt).

In the absence of clear relationships of Cd concentrations with DML and δ^{15}N values, we can only speculate in explaining this variability. Migration patterns of *S. officinalis* for reproduction may explain such a difference between individuals that come from different sites with different degrees of contamination of potential prey. Further investigations of the possible geographical influence on Cd concentrations should be carried out.

Implication for the use of stable isotopes, Hg and Cd as ecological tracers

Stable isotopes of carbon and nitrogen appear to offer great possibilities for describing the trophic ecology of species at the community scale (resource partitioning, trophic segregation), as well as changes of dietary habits during ontogenesis (Cherel & Hobson 2005, Zimmer et al. 2007, Parry 2008, Cherel et al. 2009, present study). Also, if information derived from isotopic signatures often need to be validated by available data on diet (e.g. from stomach contents analyses), the stable isotope approach presents the advantage of reflecting a longer-term average diet compared to the stomach contents method (Ruiz-Cooley et al. 2006). Nevertheless, there is a dramatic lack of information on specific differences in metabolic turn-over and isotopic fractionation in cephalopods, and such information could greatly improve our understanding and interpretation of isotopic data for inter-specific differences in trophic ecology.

Inter-specific comparisons (i.e. same order of magnitude in muscle Hg concentrations) as well as intra-specific ones (i.e. bioaccumulation with age in the muscle for all species) revealed that muscular Hg does not represent a good tracer of the feeding zone or habitat at the scale of the Bay of Biscay. Individuals from about the same size or age class must be considered for comparison (e.g. inter-specific or geographical comparisons). The same precaution should probably be taken to use this trace element as an indicator of the trophic level, as Hg (and particularly methyl-Hg) biomagnifies along food chains (Eisler 1987, Cossa et al. 1990).

Finally, despite direct ontogenic effects related to growth in some species (i.e. dilution effect), digestive gland Cd concentrations in cephalopods are likely to reflect (1) diet preferences between species (i.e. species mainly feeding on benthic crustaceans vs. pelagic fish prey) despite possible individual specialization during ontogenesis and (2) differences in the physiology of metal accumulation between species (Bustamante et al. 2002b). Also, in cases where cephalopods would be considered as prey in diet studies of their consumers such as marine mammals or seabirds, only a limited size range of individuals (potential size range consumed) should be considered (Lahaye et al. 2005), and ontogenic effects should not cause any bias.

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