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1 Species and ontogenic-related differences in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values and Hg and
2 Cd concentrations of cephalopods

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

44

45 **Keywords:** stable isotopes; metals; trace elements; ontogenesis; prey; ecological tracer;
46 Northeastern Atlantic.

47

48 **Introduction**

49 A fundamental requirement to understand the structure and functioning of marine ecosystems
50 is the knowledge of trophodynamics or trophic linkages among species of these ecosystems
51 (Cury et al. 2001). Moreover, the definition of species trophic level is a key aspect of many
52 ecosystem models, providing a valuable indicator to assess trophic structure and ecosystem
53 changes, due to overfishing, for example (Gascuel et al. 2005).

54 Until recently, the traditional method for investigating diet and trophic level of organisms was
55 the study of stomach or gut contents. Such a method allows a precise taxonomic description
56 of the prey consumed and of their size range. However, it also requires a lot of time, it may be
57 skewed by the differential digestion of the different prey, and it only represents the food
58 ingested (and not assimilated) in the short-term (Hobson & Wassenaar 1999, Santos et al.
59 2001). Therefore, the use of tracers has dramatically increased in trophic ecology in the last
60 decades (e.g. Lahaye et al. 2005, Michener & Kaufman 2007). Such tracers, which are
61 generally chemical parameters, encompass stable isotope ratios of carbon and nitrogen ($\delta^{13}\text{C}$
62 and $\delta^{15}\text{N}$), lipids, or some trace elements (either stable or radioactive). These tracers are
63 investigated in biological tissues of consumers to complement traditional methods of dietary
64 studies (Hobson & Wassenaar 1999).

65 Stable isotopes of carbon are often used as an indicator of the feeding zone or habitat, since
66 ratios vary little between those of the primary producers of the local food chain or a prey, and
67 those of the consumers ($\leq 1\%$) (De Niro & Epstein 1978, Hobson 1999). Moreover, in the
68 marine environment, pelagic and/or oceanic primary producers are generally depleted in ^{13}C
69 compared to benthic and/or neritic ones, discriminating the pelagic vs. benthic, or inshore vs.
70 offshore contribution to food intake (France 1995, Sherwood & Rose 2005). In contrast,
71 consumers are enriched in ^{15}N relative to their food (between 2.5 and 5%) (De Niro &

72 Epstein 1981, Vanderklift & Ponsard 2003), so stable isotopes of nitrogen are generally used
73 as an indicator of the trophic level (Hobson & Welch 1992).

74 Trace metals such as cadmium (Cd) and mercury (Hg) are released in the environment from
75 both natural and anthropogenic sources (e.g., zinc ore for Cd, volcanism and waste
76 incineration for Hg). They reach the ocean through river inputs and atmospheric depositions
77 (Cossa et al. 1990, Cossa & Lassus 1989). The trophic transfer is then the main pathway for
78 the intake of both metals and they bioaccumulate in higher trophic level consumers (Eisler
79 1987, Cossa et al. 1990, Koyama et al. 2000, Lahaye et al. 2005). Thus, these non-essential
80 elements may also be potential tracers of the habitat or of the feeding zone of consumers. In
81 the case of Hg, its bioaccumulation is enhanced in biota from mesopelagic environments
82 (Monteiro et al. 1996, Thompson et al. 1998). Seabirds feeding on mesopelagic fish thus
83 exhibit higher Hg concentrations in their feathers than epipelagic-feeders (Thompson et al.
84 1998, Ochoa-Acuña et al. 2002). Furthermore, Hg could represent a potential indicator of the
85 trophic level, given its known biomagnification within food webs (Eisler 1987, Cossa et al.
86 1990). Regarding Cd, this metal shows enhanced bioaccumulation in various phyla (i.e.,
87 crustaceans, molluscs and vertebrates) from various marine environments (Bargagli et al.
88 1996, Bustamante et al. 1998, 2004, Zauke et al. 1999). In this way, cephalopods constitute a
89 vector of Cd for their predators such as seabirds and marine mammals (Caurant & Amiard-
90 Triquet 1995, Bustamante et al. 1998, Lahaye et al. 2005). Thus, Cd concentrations in short-
91 beaked common dolphin *Delphinus delphis* allowed at identifying long-term segregation
92 between oceanic and neritic populations in the Bay of Biscay (Lahaye et al. 2005)

93 For ecological tracers, their signature (stable isotopes) or their bioaccumulation (metals) in a
94 consumer's tissues greatly depends on its diet. The use of these chemical parameters as
95 tracers in predators thus relies on a good knowledge of the factors influencing the values in
96 prey: for instance, spatio-temporal variations, the chemical form of metals, or biological

97 factors such as sex, species or taxa, or even those linked to ontogeny (age, growth, dietary
98 shifts) (Vanderklift & Ponsard 2003, Lahaye et al. 2005, Perga & Gerdeau 2005). In this
99 respect, ontogenic effects have been poorly and especially unequally treated as a function of
100 the potential tracer presented above. While Hg is well-known to bioaccumulate in muscle
101 tissue with size or age in various taxa (Cossa et al. 1990, Rossi et al 1993), Cd
102 bioaccumulation patterns with age are less well-established and seem to differ between
103 tissues, species and taxa (Cossa & Lassus 1989, Cronin et al. 1998, Miramand et al. 2006,
104 Pierce et al. 2008). Finally, studies on ontogenic effects on stable isotope ratios in marine
105 organisms are relatively scarce, and mainly concern fish taxa (Jennings et al. 2001, Perga &
106 Gerdeau 2005). The question of ontogenic changes on all these tracers has thus been poorly
107 treated in cephalopods. Such a consideration is very important as cephalopods play at the
108 same time the role of active predators and prey and thus constitute an important link within
109 food webs (Piatkowski et al. 2001).

110 To study cephalopod's feeding ecology, the use of tracers like stable isotope ratios is
111 particularly suitable (Cherel & Hobson 2005, Ruiz-Cooley et al. 2006, Parry 2008), notably
112 because prey identification from the gut is often difficult (Jackson et al. 2007). In addition,
113 these voracious predators can accumulate high metal concentrations in their tissues, especially
114 in the digestive gland (Martin & Flegal 1975, Miramand & Bentley 1992). Food is generally
115 considered as a major source for several metals in cephalopods, especially for Cd and Hg
116 (Koyama et al. 2000, Bustamante et al. 2002a, Lacoue-Labarthe et al. 2009). Finally, as prey,
117 cephalopods can transfer these metals to their predators, as described previously in the case of
118 Cd in the North-East Atlantic Ocean (Caurant & Amiard-Triquet 1995, Bustamante et al.
119 1998, Lahaye et al. 2005).

120 In this context, the aim of this study was to assess the effects of ontogenic changes on 1)
121 stable isotope ratios ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$), and 2) Cd and Hg bioaccumulation in several species of

122 cephalopods from a same area, to assess to what extent these ontogenic changes may affect
123 the use of these tracers as feeding ecology indicators in cephalopod's community or
124 ecosystem-scale studies.

125

126 **Materials and methods**

127 ***Study area, sampling strategy and samples preparation***

128 The Bay of Biscay (from 1 to 10°W and from 43 to 48°N) is a very large bay opened on the
129 North-East Atlantic Ocean, with a vast continental shelf in its northern part (60 to 100
130 nautical miles wide), which is reduced to only a few miles in its southern part (Fig. 1). We
131 focused on 5 species of cephalopods that were caught in different habitats (inshore vs.
132 offshore waters, pelagic vs. benthic or demersal domains). They were three squid species, the
133 veined squid *Loligo forbesi*, the European squid *L. vulgaris* and the European flying squid
134 *Todarodes sagittatus*, one cuttlefish species, the common cuttlefish *Sepia officinalis*, and one
135 octopus, the horned octopus *Eledone cirrhosa*.

136 *L. forbesi* and *L. vulgaris* are benthopelagic species mainly feeding on fishes, and to a smaller
137 extent on crustaceans, polychaetes, or other cephalopods (Ropper et al. 1984, Guerra & Rocha
138 1994, Pierce et al. 1994). However, they differ in their distribution, *L. forbesi* being usually
139 found in depths ranging from 100 to 400 m, whereas *L. vulgaris* generally occurs in depths
140 much lower than 250 m (Ropper et al. 1984, Lordan et al. 2001b). *T. sagittatus* is a more
141 oceanic species that is generally trawled beyond 200 m depth and may occur up to 1000 m
142 depth. It is also mainly piscivorous (Ropper et al. 1984, Lordan et al. 2001a and b). *S.*
143 *officinalis* is a demersal and neritic species inhabiting waters from coastline to about 200 m
144 depth, but is most abundant in the upper 100 m. Its diet is generally composed of small
145 mollusks, crabs, shrimps, other cephalopods and juvenile demersal fishes (Ropper et al.
146 1984). Finally, *E. cirrhosa* is a benthic species that may occur up to 500 m depth, but mostly

147 between 60 and 150 m, and feeds primarily on crustaceans such as shrimps, crabs, and
148 lobsters (Ropper et al. 1984, Lordan et al 2001b).

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

159 To assess ontogenic effects on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values and metal bioaccumulation in
160 cephalopods, the 3 species presenting the widest size ranges were selected, i.e. *L. vulgaris*, *L.*
161 *forbesi* and *S. officinalis*. Indeed, DML and age (or the number of increments on statoliths)
162 are closely related in *Loligo* spp. and *S. officinalis*, despite some inter-individuals variability
163 in growth rates, depending on their hatching season (Rocha & Guerra 1999, Challier et al.
164 2002). In our study, we therefore assume that several age classes were considered within the 3
165 species selected for ontogenic analyses. Also, juveniles with no visible distinct gonads, males
166 and females (immatures and matures considered together) were separated, as these species
167 present a strong sexual dimorphism (Roper et al. 1984, Rocha & Guerra 1999). Juveniles,
168 males and females will be then compared as a 3 components categorical factor called “sex”.

169

170 ***Isotopic analyses***

171 Stable isotopes of carbon and nitrogen were analyzed in the mantle muscle, which is the
172 tissue of reference in food web studies inferred from stable isotope analyses (Hobson &
173 Welch 1992, Pinnegar & Polunin 1999). It allows comparisons of isotopic signatures between
174 individuals and taxa, minimizing inter-tissue differences in terms of biochemical and
175 physiological properties like protein turnover rate and metabolic routing (Cherel et al. 2009).

176 Lipids being highly depleted in ^{13}C relative to other tissue components (De Niro &
177 Epstein 1977), they were extracted from muscle samples using cyclohexane. To this end, an
178 aliquot of approximately 100 mg of fine powder was agitated with 4 mL of cyclohexane for 1
179 h. Next, the sample was centrifuged for 5 min at 4000 g, and the supernatant containing lipids
180 was discarded. Then, the sample was dried in an oven at 45°C for 48 h, and 0.35 ± 0.05 mg
181 subsamples of lipid-free powder were finally weighed in tin cups for stable isotope analyses.
182 These analyses were performed with an elemental analyser coupled to an Isoprime
183 (Micromass) continuous-flow isotope-ratio mass spectrometer (CF IR-MS). The results are
184 presented in the usual δ notation relative to the deviation from standards (Pee Dee Belemnite
185 for $\delta^{13}\text{C}$ and atmospheric nitrogen for $\delta^{15}\text{N}$), in parts per thousand (‰). Based on replicate
186 measurements of internal laboratory standards, experimental precision is of ± 0.15 and ± 0.20
187 ‰ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively.

188

189 *Metal analyses*

190 Total Hg analyses were conducted on the mantle muscle of all species, while Cd was analysed
191 in the digestive gland. Indeed, Hg is known to mainly accumulate under its organic form in
192 the muscle tissue of cephalopods (Cossa et al. 1990, Bloom 1992, Bustamante et al. 2006),
193 whereas the role of the digestive gland in the storage and detoxification of Hg in cephalopods
194 is not well defined (Bustamante et al. 2006, Pierce et al. 2008, Lacoue-Labarthe et al. 2009).
195 In contrast, the digestive gland is well recognized as the key organ in bioaccumulation and

196 detoxification of Cd in cephalopods (Miramand & Bentley 1992, Bustamante et al. 2002a,
197 Pierce et al. 2008).

198 Hg analyses were carried out with an Advanced Mercury Analyser (ALTEC AMA 254) as
199 described in Bustamante et al. (2006), on at least 2 homogenized dry muscle tissue
200 subsamples for each individual. Hg analyses were run according to a thorough quality control
201 program including the analysis of a Certified Reference Material (CRM) TORT-2 (lobster
202 hepatopancreas, National Research Council, Canada). CRM aliquots were treated and
203 analysed in the same conditions as the samples. CRM results were in good agreement with the
204 certified values, with an average recovery rate of 97%. The detection limit was 5 ng g⁻¹ dry
205 weight (dwt). All Hg concentrations in tissues reported below are expressed in ng g⁻¹ dwt.

206 Cd analyses were performed by ICP-OES as described in Hédouin et al. (2009) for two
207 subsamples of each digestive gland. Blanks and CRM samples were treated and analysed in
208 the same way as the cephalopod samples. The CRMs were dogfish liver DOLT-3 (NRCC)
209 and lobster hepatopancreas TORT-2 (NRCC). CRMs results were in good agreement with the
210 certified values with an average recovery rate of 92 and 98% for DOLT-3 and TORT-2,
211 respectively. The detection limit was 0.15 µg g⁻¹ dwt. All Cd concentrations given below are
212 expressed in µg g⁻¹ dwt.

213

214 ***Data treatment***

215 We first analyzed the relationships between the potential tracers (muscle δ¹³C and δ¹⁵N
216 values, muscle Hg concentrations, digestive gland Cd concentrations), with regard to
217 cephalopods trawling depths and DML using a principal component analysis (PCA). The
218 PCA was based on the correlation matrix and normalised data for each variable (i. e. centred
219 and divided by the standard deviation). Then, all data submitted to statistical tests (or
220 residuals in the case of analyse of variance, ANOVA) were checked for normality (Shapiro–

221 Wilk test) and for homogeneity of variances (Bartlett test). When these conditions were
222 fulfilled, parametric tests were used in the subsequent analyses; otherwise, non-parametric
223 analogues were used. Thus, a Kruskal-Wallis (KW) test followed by a multiple comparison
224 test with Holm adjustment method was performed to test inter-specific differences in muscle
225 $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. For each of the 3 species selected for ontogenic analyses (*L. vulgaris*,
226 *L. forbesi* and *S. officinalis*), the Pearson or Spearman correlation coefficient test was used to
227 analyze the correlation between $\delta^{15}\text{N}$ values and DML, between log-transformed muscle Hg
228 concentrations and DML or $\delta^{15}\text{N}$ values, and between log-transformed digestive gland Cd
229 concentrations and DML or $\delta^{15}\text{N}$ values. On scatterplots of statistically significant
230 relationships, a smoother LOWESS (Locally weighted polynomial regression) was added to
231 help interpretation of correlation coefficients. Finally, 1-way analyses of variance were
232 performed to test between-sex differences in $\delta^{15}\text{N}$ values, muscle Hg concentrations and
233 digestive gland Cd concentrations in the same 3 species (ANOVA or KW test, followed by
234 Tukey HSD test in the case of ANOVA, or a multiple comparison test with Holm adjustment
235 method in the case of KW). The level of significance for statistical analyses was always set at
236 $\alpha = 0.05$. All statistical analyses were performed using the free software R (R Development
237 Core Team, 2010).

238

239 **Results**

240 ***Inter-specific differences***

241 The first two principal components accounted for 68% of the total variation present in the
242 data set (39% and 29% for axis 1 and 2, respectively). Depth and stable isotopes values were
243 the variables most contributing to the first axis, whereas muscle Hg concentrations and DML
244 most contributed to the second one (i. e. contribution of the variable $\geq 25\%$, Fig. 2). Principal
245 component 1 indicated that increased digestive gland Cd concentrations were associated with

246 important trawling depths, but with low $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (Fig. 2). Nevertheless, Cd
247 concentrations in the digestive gland contributed more to the formation of the third axis, not
248 represented here (contribution of 39%). Principal component 2 showed that increased muscle
249 Hg concentrations are highly correlated with DML (Fig. 2). When individuals were finally
250 grouped by species or by sex, it emerged that species were more segregated by the principal
251 component 1 (i. e. by muscle $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values and to a lesser extent digestive gland Cd
252 concentrations), while juveniles strongly differed from adults in their muscle Hg
253 concentrations on the principal component 2 (males and females generally not segregated)
254 (Fig. 2).

255 Regarding inter-specific differences in carbon and nitrogen measurements in particular (Fig.
256 3), the 5 species of cephalopods from the Bay of Biscay differed significantly by both their
257 average $\delta^{13}\text{C}$ ($p_{\text{KW}} < 0.001$) and $\delta^{15}\text{N}$ values ($p_{\text{KW}} < 0.001$). Post hoc multiple comparison test
258 showed that *L. forbesi* and *T. sagittatus* had significantly lower $\delta^{13}\text{C}$ values ($p < 0.05$) than *L.*
259 *vulgaris*, *S. officinalis* and *E. cirrhosa* (Table 1, Fig. 3). As for nitrogen, *T. sagittatus* and *L.*
260 *forbesi* (more oceanic species) did not differ significantly ($p > 0.05$) on the one hand, whereas
261 *L. vulgaris*, *S. officinalis* and *E. cirrhosa* (neritic species) presented significantly different
262 $\delta^{15}\text{N}$ values on the other hand ($p < 0.05$) (Table 1, Fig. 3).

263

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

265 *Ontogenic effects on stable isotope ratios*

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

269 In *L. vulgaris*, the correlation coefficient was low between $\delta^{15}\text{N}$ values and DML
270 ($R^2_{\text{Spearman}}=0.233$, $p=0.010$), reflecting the non-linear relationship existing between both

271 variables (Fig. 4a). Indeed, $\delta^{15}\text{N}$ values were very similar for individuals larger than
272 approximately 100 mm DML, corresponding to adult individuals that could be sexed and
273 strongly differed from juvenile ones (Table 2).

274 In *L. forbesi*, there was a positive correlation between $\delta^{15}\text{N}$ values and DML ($R^2_{\text{Pearson}}=0.439$,
275 $p<0.001$), but variability between similar-sized individuals was high (Fig. 4b). There was a
276 significant difference in $\delta^{15}\text{N}$ values between not juveniles and females on the one hand, and
277 males on the other hand (Table 2). This contributed to the increase in $\delta^{15}\text{N}$ values with
278 increasing size as males are considerably larger than females in this species (Fig. 4b).

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

282

283 *Ontogenic effects on metal bioaccumulation*

284 In the 3 species, log-transformed Hg concentrations in the muscle were strongly and
285 positively correlated with DML ($R^2_{\text{Spearman}}=0.600$, $R^2_{\text{Spearman}}=0.836$ and $R^2_{\text{Pearson}}=0.596$, all
286 $p<0.001$, in *L. vulgaris*, *L. forbesi* and *S. officinalis* respectively) (Fig. 5). Hg concentrations
287 in the muscle and $\delta^{15}\text{N}$ values were also correlated ($R^2_{\text{Spearman}}=0.361$, $R^2_{\text{Spearman}}=0.404$, both
288 $p<0.001$, and $R^2_{\text{Pearson}}=0.182$, $p=0.019$, in *L. vulgaris*, *L. forbesi* and *S. officinalis*
289 respectively) (Fig. 5).

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

293 Regarding Cd, there was a significant negative correlation between log-transformed
294 concentrations and DML on the one hand ($R^2_{\text{Spearman}}=0.416$, $p<0.001$), and between log-
295 transformed concentrations and $\delta^{15}\text{N}$ values on the other hand ($R^2_{\text{Pearson}}=0.253$, $p<0.001$), in *L.*

296 *forbesi* only (Fig. 6). In fact, males (comprising larger individuals with the highest $\delta^{15}\text{N}$
297 values) significantly differed from juveniles and females (Table 2, Fig. 6). No significant
298 correlation between log-transformed digestive gland Cd concentrations and DML or $\delta^{15}\text{N}$
299 values, neither difference between sexes were found in *L. vulgaris* and *S. officinalis* (Fig. 6,
300 Table 2).

301

302 **Discussion**

303 *Inter-specific differences in trophic ecology and metal bioaccumulation*

304 From a community point of view, isotopic signatures (carbon and nitrogen combined) were
305 the chemical parameters that best segregated five cephalopod species from the Bay of Biscay
306 (Fig. 2 and 3). Four distinct isotopic niches (as described by Newsome et al. 2007) emerged,
307 with *L. forbesi* and *T. sagittatus* sharing the same isotopic niche (Fig. 3). The different
308 isotopic niches suggest clear segregations in terms of trophic ecology, and agree with what is
309 currently known about the spatial distribution and feeding habits of the 5 species. Indeed, *L.*
310 *forbesi* and *T. sagittatus* generally show a more offshore distribution that fit well with their
311 low $\delta^{13}\text{C}$ values relative to *L. vulgaris* and *S. officinalis* (Roper et al. 1984, Lordan et al.
312 2001b). The enriched $\delta^{13}\text{C}$ values of *E. cirrhosa* may be explained by the benthic habitat of
313 this octopus, where baseline $\delta^{13}\text{C}$ values are higher than in pelagic ecosystems (France 1995).
314 As for muscle $\delta^{15}\text{N}$ values, they increased with the probability for a species to feed more on
315 fish than on pelagic or benthic crustaceans in the neritic species, with *L. vulgaris* > *S.*
316 *officinalis* > *E. cirrhosa* (Roper et al. 1984, Pierce et al. 1994, Pinczon du Sel et al. 2000,
317 Neves et al. 2009). This is in accordance with $\delta^{15}\text{N}$ values of the potential prey available for
318 the Bay of Biscay. Small pelagic crustaceans such as the euphausiid *Meganyctiphanes*
319 *norvegica* or those constituting zooplankton (e.g., other euphausiids, copepods, mysids)
320 present considerably lower $\delta^{15}\text{N}$ values than small pelagic fish in the Bay of Biscay (between

321 2 and 5‰ difference in average) (Chouvelon et al., unpublished data). Moreover, while
322 benthic crustaceans should present higher $\delta^{15}\text{N}$ values due to their scavenger behaviour, they
323 actually exhibit lower average $\delta^{15}\text{N}$ values also (about 1‰) compared to those of small
324 pelagic or demersal coastal fish such as Gobiidae, Callyonimidae, Ammodytidae or
325 Atherinidae in the Bay of Biscay (Le Loc'h et al. 2008, Chouvelon et al., unpublished data).
326 *L. vulgaris*, and to lesser extent *S. officinalis* that consume those kind of fish (Pierce et al.
327 1994, Pinczon du Sel et al. 2000) thus exhibit higher $\delta^{15}\text{N}$ values than the benthic crustacean
328 feeder *E. cirrhosa* (Ropper et al. 1984).

329 Concerning metals, average Hg concentrations in the muscle tissue varied over the same order
330 of magnitude in the 5 species (Table 1) and did not contribute significantly in the segregation
331 of the species in the PCA (Fig. 2). In contrast to Hg, average Cd concentrations in the
332 digestive gland varied over different orders of magnitude between species (Table 1). Cd
333 concentrations tend to increase with the proportion of benthic crustaceans compared to fish in
334 the diet of the neritic species, with *E. cirrhosa* > *S. officinalis* > *L. vulgaris* (Fig. 2, Table 1).
335 Indeed, food is the major source of Cd intake by cephalopods (Koyama et al. 2000,
336 Bustamante et al. 2002a), and benthic invertebrates generally exhibit higher Cd
337 concentrations relative to fish (Cossa & Lassus 1989, Cabrera et al. 1994). In the species
338 more related to the oceanic domain, the higher Cd concentrations in *T. sagittatus* compared to
339 *L. forbesi* may be more related to physiological features than to diet or habitat, as both species
340 had a similar isotopic niche (Fig. 3). As discussed above, both squids are mainly piscivorous
341 and present a relative offshore distribution (Pierce et al. 1994, Lordan et al. 2001a and b), but
342 their digestive gland cells strongly differ in terms of structure. The digestive gland cells of *T.*
343 *sagittatus* contain a “boule” structure typical of many cephalopods species, which are non-
344 existent in Loliginidae (Boucau-Camou & Yim 1980, Boucher-Rodoni & Boucaud-Camou
345 1987). Thus, “boules” may contribute to store larger amounts of Cd in *T. sagittatus*

346 (Bustamante et al. 2002b). Therefore, the influence of such a physiological difference cannot
347 be excluded for the species more related to the coastal domain, *E. cirrhosa* and *S. officinalis*
348 having "boule" structures while they are absent in *L. vulgaris* (Table 1).

349

350 ***Ontogenic changes as an important source of intra-specific variations***

351 In cephalopod species presenting the widest size ranges, the analysis of the relationship
352 between $\delta^{15}\text{N}$ values and DML allowed at distinguishing different feeding profiles during
353 ontogenesis (Zimmer et al. 2007, Parry 2008). $\delta^{15}\text{N}$ values of *L. vulgaris* increased drastically
354 between juvenile and adult stages, from which it remained relatively constant (females and
355 males not differing) (Fig. 4, Table 2). By contrast, in *L. forbesi*, only larger males (> 300 mm
356 DML) showed significantly higher $\delta^{15}\text{N}$ values (Fig. 4). This may be due to a difference in
357 prey composition or prey size range in those larger individuals that may have greater energy
358 needs (Rocha et al. 1994, Pierce et al. 1994). Nevertheless, the variability in $\delta^{15}\text{N}$ values over
359 life stages for males, females and juveniles strongly suggest that this species has an important
360 dietary plasticity during ontogenesis (Fig. 4). As previously commented, *L. vulgaris* likely
361 remains closely related with coastal waters throughout its life cycle, whereas *L. forbesi* is a
362 more oceanic species that carry out relatively long offshore displacements throughout its life
363 cycle (Rocha & Guerra 1999). These different ontogenic patterns between both *Loligo* species
364 are confirmed by the analyses of their isotopic signatures (Fig. 3 and Fig. 4). While *L.*
365 *vulgaris* may always feed on the same prey species in the coastal food web (i.e. mainly small
366 pelagic coastal fish), *L. forbesi* may feed on different food webs with different baseline
367 isotopic signatures during ontogenesis (e.g. coastal vs. upper slope or oceanic food web). This
368 could contribute to the greater trophic plasticity of *L. forbesi* in comparison with *L. vulgaris*.
369 Furthermore, the contribution of crustaceans is slightly higher in the diet of *L. forbesi* than in
370 those of *L. vulgaris* (Guerra and Rocha 1994, Pierce et al. 1994). Finally, $\delta^{15}\text{N}$ values recorded

371 for juveniles, males and females in *S. officinalis* suggest that this species also displays a
372 strong dietary plasticity at all stages of its life cycle (Fig. 4, Table 2). Thus, isotopic
373 signatures were in agreement with the stomach contents data that related a general
374 opportunistic behaviour and flexible diet of *S. officinalis* (Pinczon du Sel et al. 2000, Neves et
375 al. 2009).

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

393 Finally, the bioaccumulation patterns of Cd in the digestive gland during ontogenesis differed
394 between *L. vulgaris*, *L. forbesi* and *S. officinalis* (Fig. 6, Table 2). While no clear pattern of
395 digestive gland Cd levels with DML nor with $\delta^{15}\text{N}$ values was revealed in *L. vulgaris*, Cd

396 concentrations in the digestive gland of *L. forbesi* significantly decreased with DML and to
397 lesser extent with $\delta^{15}\text{N}$ values (Fig. 6). Such a decrease in Cd concentrations with increasing
398 body size was already observed for *L. forbesi* sampled around the United Kingdom (Pierce et
399 al. 2008). These authors explained that decrease as a consequence of a dietary shift with
400 increasing body size, strengthened by a dilution of the metal in the digestive gland due to the
401 very fast growth of this squid. The dilution hypothesis seems to be confirmed here in *L.*
402 *forbesi*. Indeed, the decrease in Cd concentrations is linked to larger males presenting the
403 lowest Cd concentrations, and also the lowest $\delta^{15}\text{N}$ values (Fig. 4 and Fig. 6, Table 2). By
404 contrast with *L. forbesi*, Cd concentrations in the digestive gland of *S. officinalis* from the Bay
405 of Biscay did not show clear relationships with DML nor with $\delta^{15}\text{N}$ values or sex (Fig 6,
406 Table 2). Thus, our results for *S. officinalis* do not match those from Miramand et al. (2006)
407 who found a clear increase of Cd concentrations with size in the digestive gland of the
408 cuttlefish from the English Channel. Such an increase was explained by the very long
409 biological half-life of Cd in cuttlefish following its assimilation from food (Bustamante et al.
410 2002a). The strong retention capacity of Cd in the digestive gland of *S. officinalis* is probably
411 related to its cells containing the typical “boule” structures (Boucau-Camou & Yim 1980,
412 Boucher-Rodoni & Boucaud-Camou 1987), as commented above for *T. sagittatus*.
413 Nevertheless, in our study, values of Cd levels varied greatly between individuals of *S.*
414 *officinalis* (ranging from 2.5 to 44.7 $\mu\text{g g}^{-1}$ dwt). In absence of clear relationships of these
415 values neither with DML nor with $\delta^{15}\text{N}$ values, we can only speculate in explaining this
416 variability. Migration patterns of this species for reproduction may explain such a difference
417 between individuals that could come from different sites with different degrees of
418 contamination of potential prey. Further investigations on a possible geographical influence
419 on Cd concentrations should be carried out.

420

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

422 Stable isotopes of carbon and nitrogen appeared to offer great possibilities for describing the
423 trophic ecology of species at the community-scale (resource partitioning, trophic segregation),
424 as well as changes of dietary habits during ontogenesis (Cherel & Hobson 2005, Zimmer et al.
425 2007, Parry 2008, Cherel et al. 2009, this study). Also, if information derived from isotopic
426 signatures often need to be validated by available data on diet (e.g., from stomach contents
427 analyses), the stable isotope approach presents the advantage to reflect a longer-term average
428 diet compared to the stomach contents method (Ruiz-Cooley et al. 2006). Nevertheless, there
429 is a dramatic lack of information on specific differences in metabolic turn-over and isotopic
430 fractionation in cephalopods, whereas it could greatly improve our understanding and
431 interpretation of isotopic data for inter-specific differences in trophic ecology.

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

440 Finally, despite direct ontogenic effects related to growth in some species (i.e., dilution
441 effect), digestive gland Cd concentrations in cephalopods are likely to reflect 1) diet
442 preferences between species (i.e., species mainly feeding on benthic crustaceans vs. pelagic
443 fish prey), despite possible individuals specialization during ontogenesis; 2) differences in the
444 physiology of metal accumulation between species (Bustamante et al. 2002b). Also, in case
445 cephalopods would be considered as prey in diet studies of their consumers such as marine

446 mammals or seabirds, only a limited size range of individuals (potential size range consumed)
447 should be taken into account (Lahaye et al. 2005). Then, ontogenic effects should not cause
448 any bias.

449

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461

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Table 1. Characteristics of individuals, muscle stable isotopes values, muscle Hg and digestive gland Cd concentrations of 5 species of cephalopods from the Bay of Biscay. Values are means \pm SD with ranges (minimum; maximum) for trawling depths and chemical parameters values, and only the range for DML and TW.

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

DML = Dorsal Mantle length; TW = Total Weight; M = males; F = females; J = juveniles.

Table 2. Muscle $\delta^{15}\text{N}$ values, muscle Hg and digestive gland Cd concentrations in individuals of *Loligo vulgaris*, *L. forbesi* and *Sepia officinalis* separated by sex. Groups (same letter) indicate that sexes are not significantly different (post hoc Tukey test in the case of ANOVA, multiple comparison test (MPC) with Holm adjustment method in the case of KW).

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

1 **Caption to figures**
2

3 **Fig. 1.** Map of the study area and sampling locations of each cephalopod species in the Bay of Biscay.
4

5 **Fig. 2.** Projection of variables and individuals on the first two components resulting from the principal
6 component analysis (PCA). A) Correlation biplot showing the distribution of the variables. The length of the
7 line for a variable shows how well it is represented by the two-dimensional approximation, and reflects its
8 contribution to the first two principal components. Horizontal axis: principal component 1 (eigenvalue = 2.3,
9 that is 39% of the variability explained by axis 1); vertical axis: principal component 2 (eigenvalue = 1.8, that is
10 29% of the variability explained by axis 2). Variables pointing in the same direction display a high positive
11 correlation. Variables pointing in the opposite direction have a high negative correlation. Variables with an
12 angle of 90° have a small correlation close to 0. B) Projection of individuals on the correlation biplot, and
13 histogram of eigenvalues of the different components resulting from the PCA. The black shaded bars refer the
14 first two components with heigher eigenvalues, represented on the correlation biplot. The grey shaded bars refer
15 to the other components. Eigenvalues = 2.3, 1.7, 1.1, 0.4, 0.3, 0.1 for the components resulting from the PCA,
16 from 1 to 6 respectively. C) Grouping of individuals by species. D) Grouping of individuals by sex.
17
18

19 **Fig. 3.** Muscle $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (‰) of 5 species of cephalopods from the Bay of Biscay. Species not
20 sharing the same numbers ($\delta^{13}\text{C}$) and letters ($\delta^{15}\text{N}$) are significantly different ($\delta^{13}\text{C}$: Kruskal-Wallis $\chi^2 = 97.5$,
21 $\text{df} = 4$, $p < 0.001$; $\delta^{15}\text{N}$: Kruskal-Wallis $\chi^2 = 80.9$, $\text{df} = 4$, $p < 0.001$). Values are means \pm SD. TP = Trophic
22 position.
23

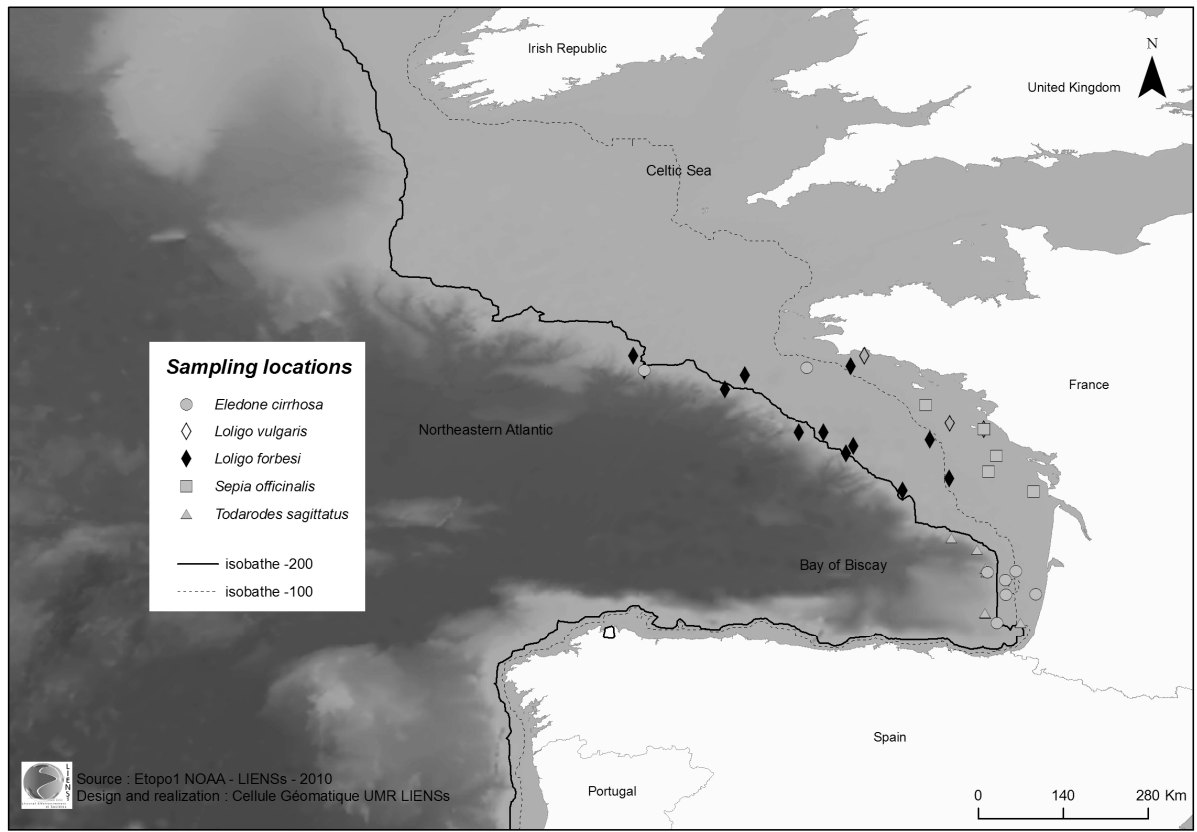
24 **Fig. 4.** Relationships between muscle $\delta^{15}\text{N}$ values and DML in a) *Loligo vulgaris*, b) *L. forbesi* and c) *Sepia*
25 *officinalis*, individuals being separated by sex and by year. Squares = males; triangles = females; circles =

26 juveniles; black = 2008; white = 2006. Smoothing lines (robust, locally weighted scatterplot smoothing system
27 based on the LOWESS algorithm with the software R) represent the fitted non-linear trend of the values.

28
29 **Fig. 5.** Relationships between log-transformed muscle Hg concentrations and DML (on the left) or $\delta^{15}\text{N}$ values
30 (on the right) in a) and b) *Loligo vulgaris*, c) and d) *L. forbesi*, and e) and f) *Sepia officinalis*, individuals being
31 separated by sex and by year. Squares = males; triangles = females; circles = juveniles; black = 2008; white =
32 2006. Smoothing lines (robust, locally weighted scatterplot smoothing system based on the LOWESS algorithm
33 with the software R) represent the fitted non-linear trend of the values.

34
35 **Fig. 6.** Relationships between log-transformed digestive gland Cd concentrations and DML (on the left) or $\delta^{15}\text{N}$
36 values (on the right) in a) and b) *Loligo vulgaris*, c) and d) *L. forbesi*, and e) and f) *Sepia officinalis*, individuals
37 being separated by sex and by year. Squares = males; triangles = females; circles = juveniles; black = 2008;
38 white = 2006. Smoothing lines (robust, locally weighted scatterplot smoothing system based on the LOWESS
39 algorithm with the software R) represent the fitted non-linear trend of the values.

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Figure 1

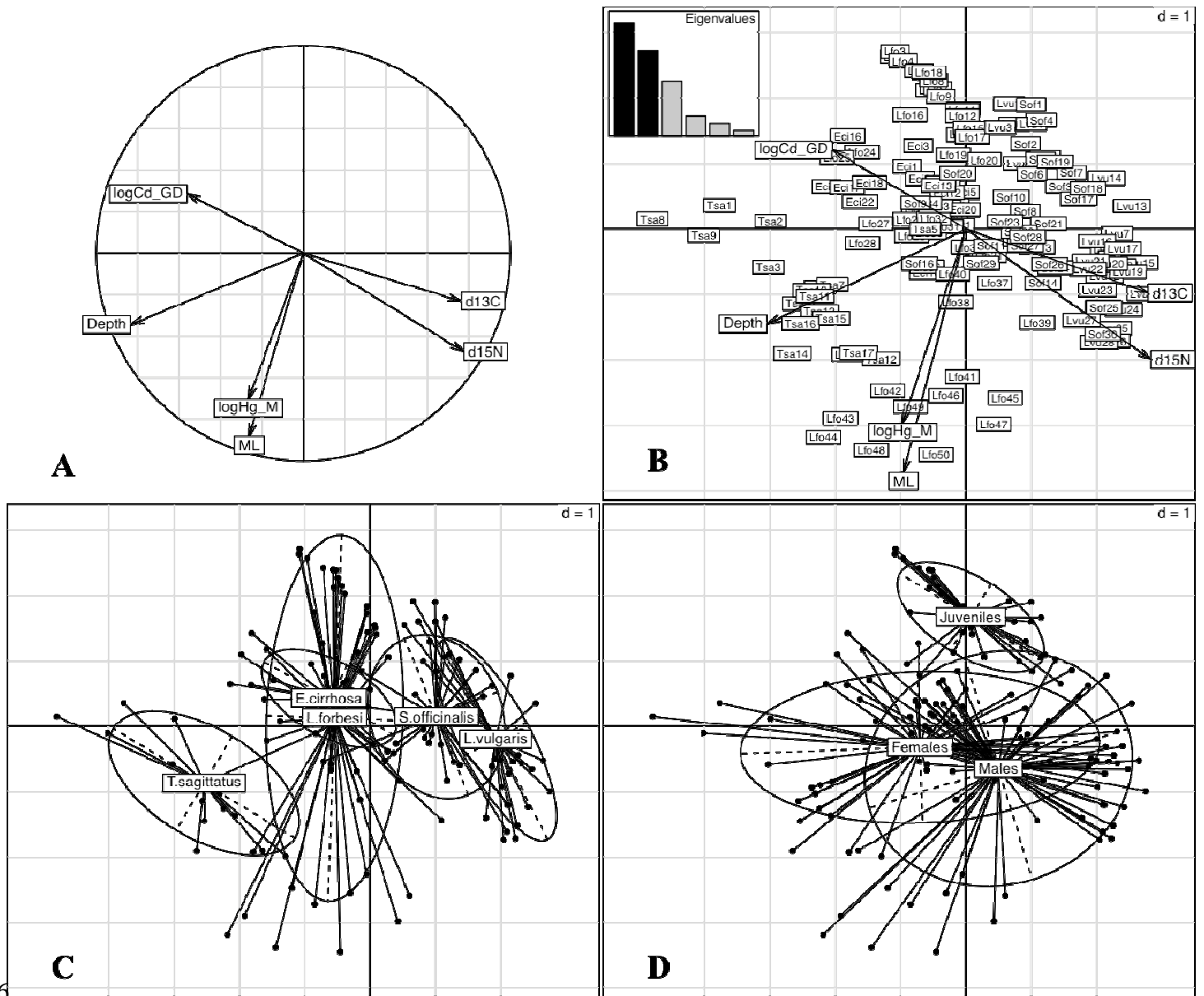


Figure 2

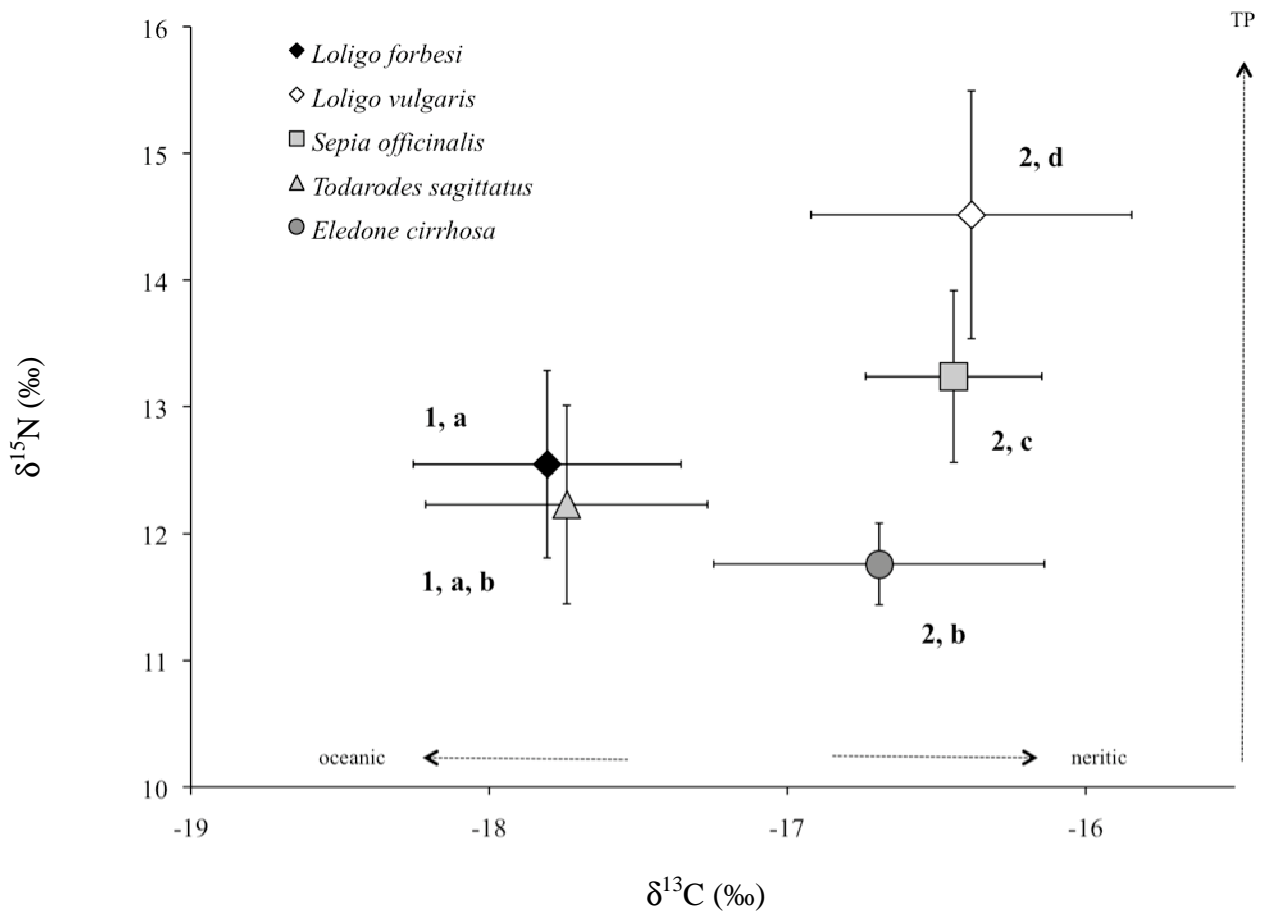


Figure 3

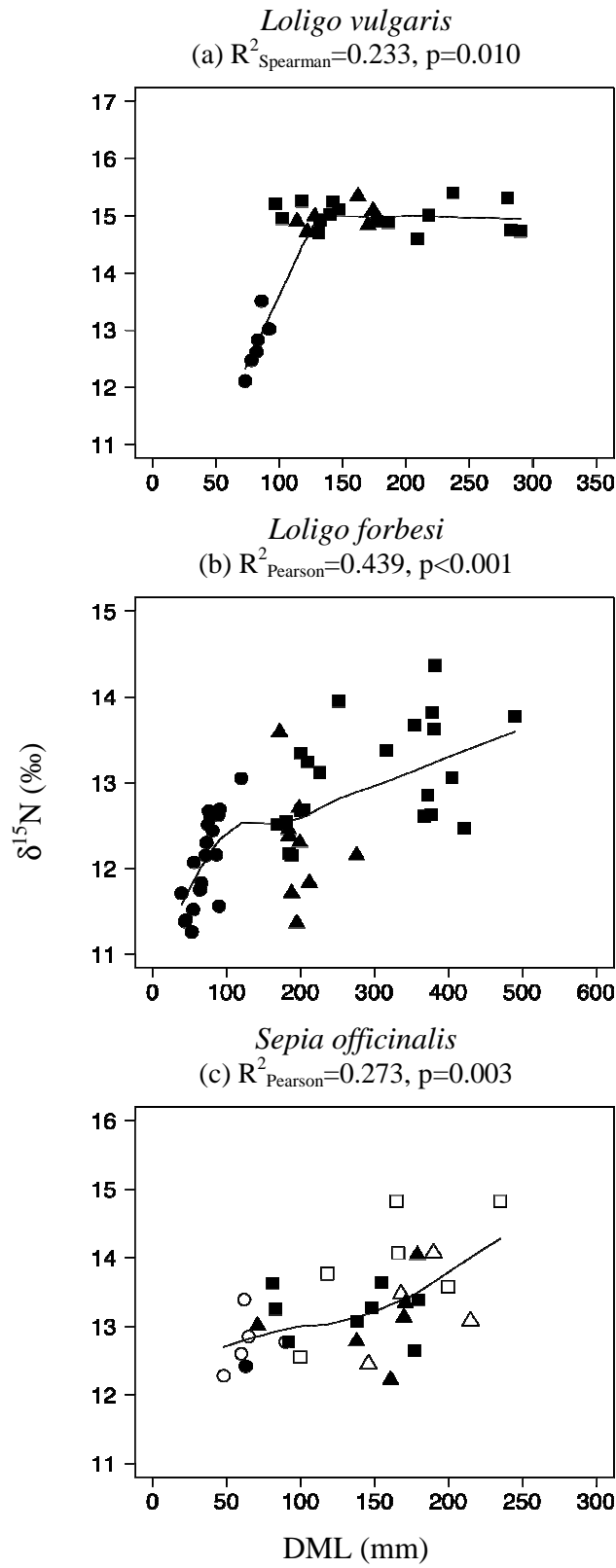


Figure 4

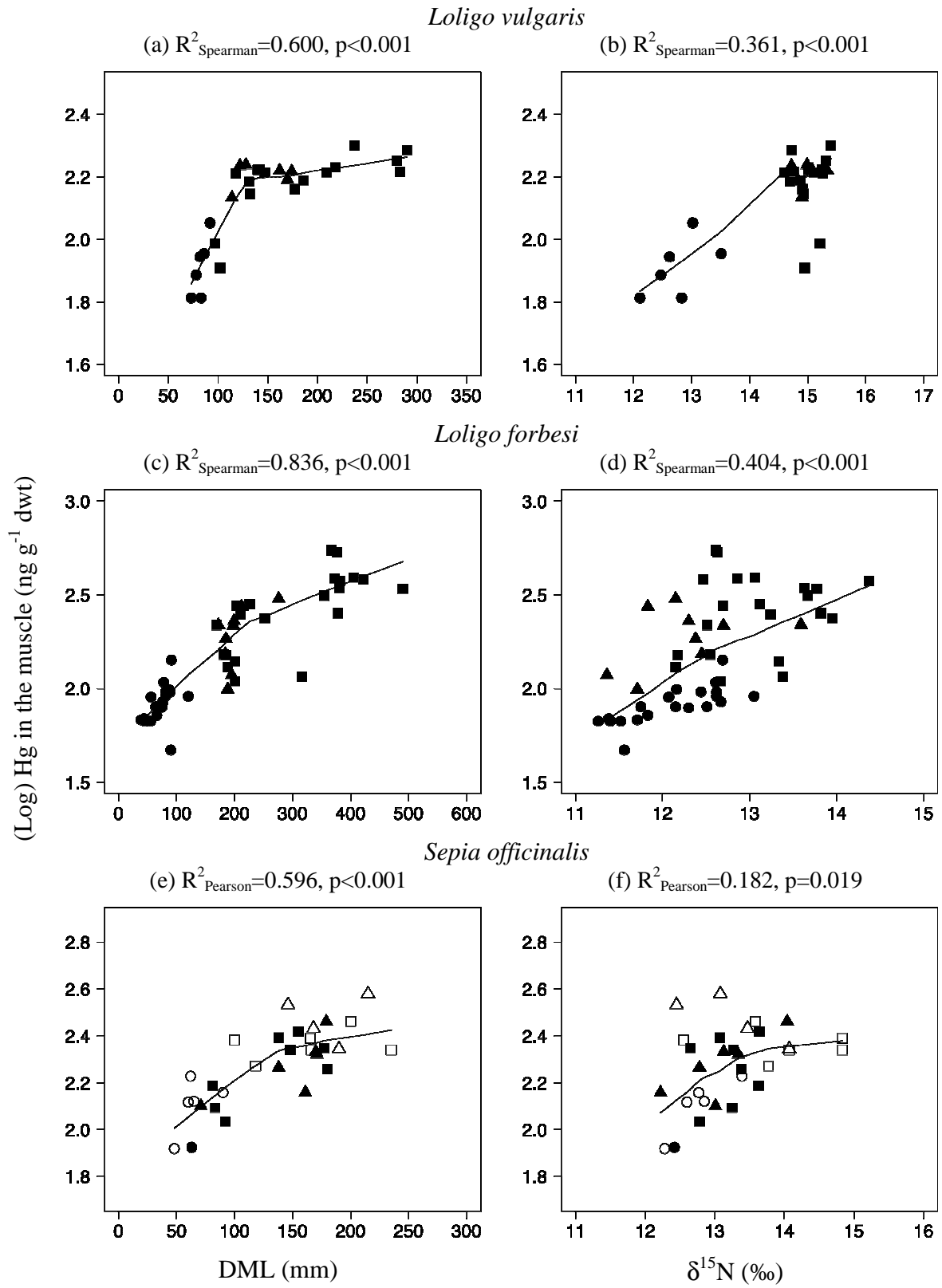


Figure 5

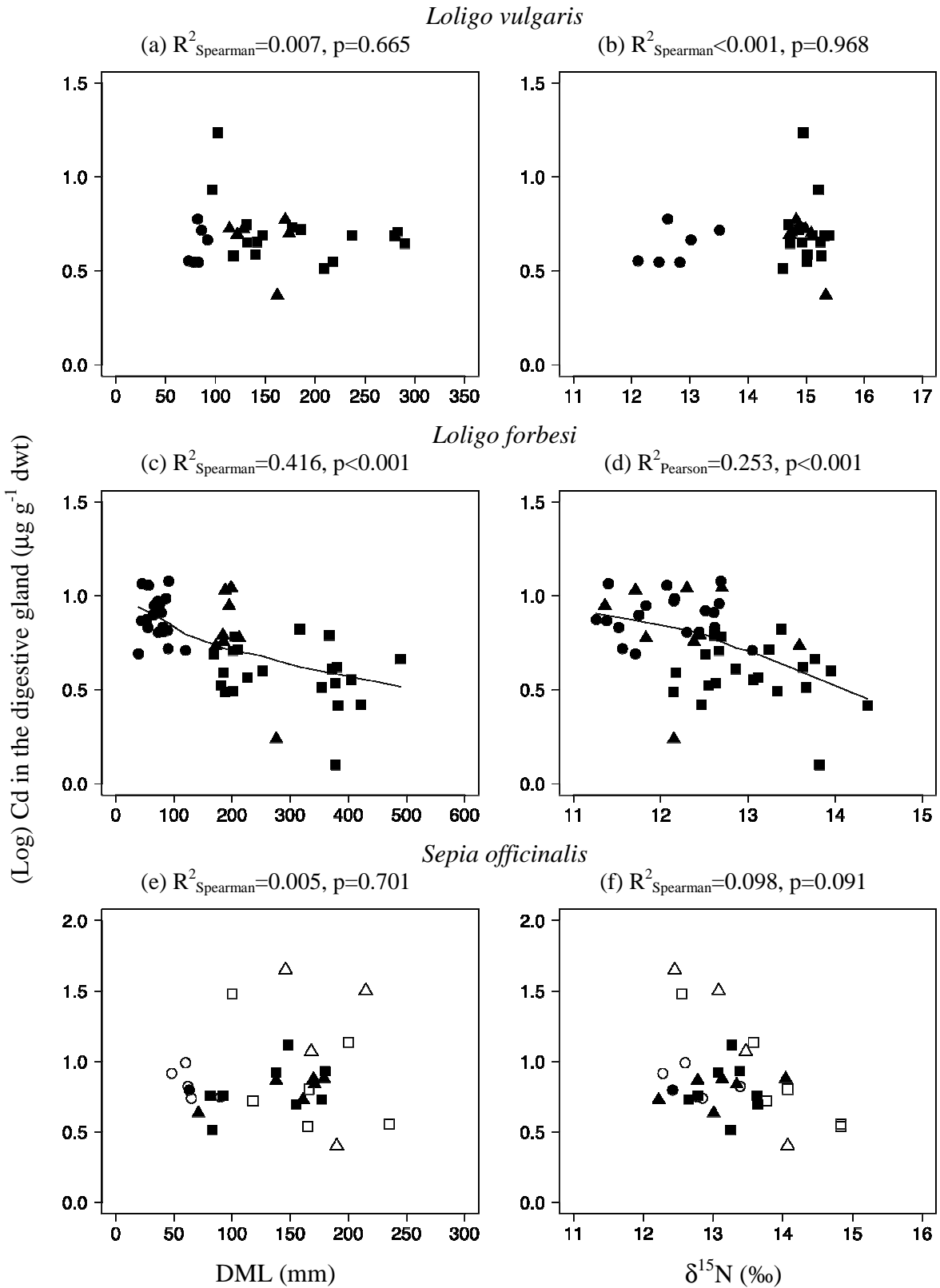


Figure 6