Does the trophic habitat influence the biochemical quality of the gonad of *Octopus vulgaris*? Stable isotopes and lipid class contents as bio-indicators of different life cycle strategies

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Abstract

Octopus vulgaris is a merobenthic octopus with high growth rates, and an opportunistic predator of a wide range of species which adapts the reproductive cycle to the oceanographic characteristics of their habitats. Trophic habitat and gonad quality of two neighbour populations of the Portuguese coast, subject to different oceanographic regimes, one inhabit a productive system with strong seasonality, and the other a oligotrophic system with punctual upwelling events, were compared using the digestive gland and beaks as recorders of trophic and habitat preferences, and gonads as indicators of egg quality. Cholesterol and phospholipids content, fatty acid (FA) profile of the digestive gland and stable isotopes, δ15N and δ13C, in the flesh and beaks were used as indicators of possible differences in the trophic habitat between populations. For gonad quality, the same bio-indicators were used to identify differences during maturation. The populations present similar trophic positions, with the south population presenting higher $\delta^{15}N$ signature. The cholesterol content is significantly different between populations as the FA profile of the digestive glands mainly in the EFA 14:0, 16:1n-7, 20:1n-9, ARA, EPA and DHA. The lipid classes' content in the gonad doesn't show a clear influence of environment although the major FA profile of the gonads is dependent of both maturity and region, showing that both timing of maturity and feeding influences the quality of gonads as proxies of eggs.

Keywords: *Octopus vulgaris*, trophic habitat, gonad quality, bio-indicators

Introduction

The common octopus *Octopus vulgaris* (Cuvier, 1797) is a target species by demersal trawl fleets and in numerous local fisheries in southern Europe and northwestern Africa waters, using hand jigs, pots, traps and trammel nets (Hastie et al. 2009). In the Iberian peninsula, *O. vulgaris* is the most important fisheries resource considering the relationship between first sale price and landings volume, particularly for Portuguese fleet, were the landings volume have increased by 50% in the last 20 years with considerable variation on landing weight in recent years (INE, 2012; ICES, 2012). Here, it is captured mainly with traps (~90%), thus having a major importance in the artisanal fishery, particularly on the south coast, where it accounts for more than 20% of the income of the fishing activity (INE, 2012)

Octopus vulgaris is defined as "r" life-history strategy organism with the production of numerous small eggs that hatch into planktonic free-swimming paralarvae. Those are not truly lecitotrophic, combining endogenous feeding (yolk) with exogenous (prey) feeding (Villanueva & Norman, 2008). As paralarvae, *O. vulgaris* is a specialized predator of crustacean (Roura et al, 2012), whereas as a juvenile and adult, it is an opportunistic predator of crustaceans, mollusks and fish (Quetglas et al, 1998; Smith, 2003; Rosa et al, 2004b). With high food consumption and conversion rates (Semmens et al, 2004), the diet of *O. vulgaris* depends on the life-cycle stage, size, depth of occurrence, habitat and seasonal availability of their prey (Nixon, 1985; Smith, 2003), thus having a very variable life cycle within the species**.** The reproductive season of *O. vulgaris* depends of the local oceanographic regime, presenting a long reproductive season in productive systems, frequently with two reproductive peaks, and with shorter reproductive seasons of one to two months in oligotrophic system (Lourenço et al, 2012). In this system, females direct energy and biomass largely to spawning, presenting significantly higher gonad-somatic indexes during this period in comparison with the females of the more productive areas (Lourenço et al, 2012).

The formation of the yolk is extremely important during maturation and egg development in *O. vulgaris*. Newly hatched paralarvae depend to some extent on the yolk reserves to survive (Villanueva & Norman, 2008). The nutritional content of the yolk is mainly protein, but lipids are also important for the membrane formation and energetic supply (11-14% of dry weight), due to the paralarvae nutritional requirements of polyunsaturated fatty acids (PUFA), phospholipids (phos)and cholesterol (chol) (Navarro and Villanueva, 2000).

The cephalopod hepatopancreas or digestive gland has different functions in the *Octopus* spp. physiology, including the synthesis and secretion of digestive enzymes, the reabsorption and metabolism of nutrients; synthesis and storage of lipids like chol, lipoproteins, glycogen, pigments, vitamins and protein-bound Fe, Cu, Ca and non-physiological heavy metals; and excretion and rejection of waste products of the digestion and cell metabolism (Blanchier & Boucaud-Camou, 1984; Budelmann et al. 1997; Moltschaniwskyj & Johnston, 2006). Its function, as a storage organ, indicates its utility as the ideal source of dietary tracers such as the essential fatty acids (EFA), particularly polyunsaturated fatty acids (PUFA), or other dietary lipids (e.g. chol and Triacilglycerol, tag) that are deposited in this organ with little or no modification of the lipid content (Philips et al, 2001).

Lipids are important dietary constituents, providing energy, vitamins and essential fatty-acids (EFA). Chol is the predominant sterol in the cephalopods lipids reserves (Sieiro et al, 2006) and proxy of the production of hormones in marine invertebrates (Kanazawa, 2001). The endogenous synthesis of chol seems to be absent in cephalopods, suggesting that it is an essential dietary nutrient (Villanueva & Norman, 2008). The Triacylglycerol (tag) is a neutral lipid involved in the fatty acids (FA) storage and metabolism. The FA are incorporated within the phos, as the building blocks for the membrane lipid bilayer (Dalsgaard et al, 2003, Bergé & Barnathan, 2005, Athenstaedt & Daum, 2006). The lipids, especially the TAG, display wide variability during certain periods of the annual cycle and changed living conditions, and thus possess great potential as "markers" of condition. The phos also characterize the state of the organism, particularly of their cell membrane (Shulman & Love, 2006). The most important EFA are the arachindonic acid 20:4n6 (ARA), the eicosapentaenoic acid 20:5n-3 (EPA) and the docosahexaenoic acid 22:6n-3 (DHA). Requirements of these EFA and the balance between them are important mainly to growth of the individuals, as those EFA are related with the energy channelling, cellular membrane structure and function as integrating the phos in the lipids bilayers (Tocher, 2010). In this study, we are particularly interested in the EFA, tag as reserve and a proxy of the FA metabolism, chol as a proxy of steroid hormones, and phos as a indicator of membrane production in the gonad.

The $\delta^{13}C$ and the $\delta^{15}N$ signature in different tissues of a predator like *O. vulgaris* reflects its habitat and trophic position respectively (Stowasser, 2004; Cherel & Hobson, 2005). Consumers or predators are enriched in ^{15}N relatively to their food and consequently $\delta^{15}N$ measurement is an indicator of the consumer trophic position (Vander Zanden & Rasmussen, 2001; Cherel and Hobson, 2005). ^{13}C varies little with along the food chain and $\delta^{13}C$ is used to determine primary sources in the trophic web indicating the habitat of the organism, and the inshore *vs* offshore, or pelagic *vs* benthic contribution to the food intake (Cherel & Hobson, 2005; Jackson et al, 2007). The study of the $\delta^{13}C$ and $\delta^{15}N$ stable isotope signatures both in the muscle and in the cephalopod beaks, are complementary approaches to the stomach content analysis and EFA profile in studies of trophic dynamic and feeding ecology, permitting the identification of ontogenic migration and feeding shift events (Stowasser, 2004; Jackson et al, 2007). In species like *O. vulgaris*, for which diet studies are difficult to carry out due to the diversity of preys (Smith, 2003) and high prey digestion rate (Boucaud-Camou et al, 1976), a high number of empty stomachs, and the influence of capture methods using bait, this analytic approach combining fatty acids and stable isotopes analyses, provides an average diet information regarding both feeding regime and habitat of a particular population (Stowasser, 2004).

This is the first simultaneous study on the effect of the diet on the nutritional quality of the lipid reserves in the digestive gland and in the mature oocytes of the gonad in two populations of common octopus *Octopus vulgaris* subjected to distinct environmental conditions, one in the northwest Portuguese coast, a productive system integrated in the Western Iberia Upwelling System, and the other one in the south portuguese coast integrated in the Gulf of Cadiz System influenced by the oligotrophic and warmer waters of the Huelva front were downwelling and upwelling events are weaker and not seasonal. The study aims to use the profile of different lipid classes as EFA, sterols, specifically chol, tag and phos as bio-indicators of variations on diet and eggs quality on immature and fully developed gonads, using as

control the lipid content in the digestive gland. Additionally, from the feeding ecology perspective, it is interesting to identify and quantify the EFA as well as the chol profile in two populations with different diets. The $\delta^{13}C$ and $\delta^{15}N$ stable isotope signatures are determined in the upper and lower beaks of immature and mature individuals of the same populations in order to identify the average feeding regime and habitat of both populations.

Material and Methods

Sampling

Digestive gland (DG) and gonad (OV) samples were collected from animals captured in the small-scale *O. vulgaris* fisheries captures landed in the ports of Peniche (northwest coast) and Olhão (south coast) (Figure 1). Considering both area and maturity stage as explanatory factors, sampling was conducted during March and April 2011 in both sampling sites, collecting the digestive gland and the gonad of 5 immature and 5 mature females in each sampling site with a total of 5x2(mat)x2(site)x2(tissue)=40 samples. The DG and OV samples were freeze dried and stored at -20 ºC. Cleaned upper and lower beaks and bucal mass samples were also collected from frozen animals and kept in 70% ethanol for isotopic analysis. The dorsal mantle length (DML in mm), and individual weight (W in g) were measured to the nearest 5 mm and 0.1 g, respectively. Immature and maturing females were classified as immature, and fully mature and spawning females were classified as mature (according to Guerra 1975).

Stable isotope analyses

Beaks and flesh samples were freeze-dried and homogenized prior to analysis. To avoid the depletion of $\delta^{13}C$ values due to lipids presence, flesh samples rinsed successively in a 2 : 1 chloroform-methanol solution (Cherel et al. 2005). Nitrogen and carbon isotope ratios were determined via Finningan conflo II interface to a Thermo Delta V S mass spectrometer coupled to a Flash EA1112 Series elemental analyser. Approximately 0.3 mg of each sample was combusted in a tin cup for the simultaneous determination of nitrogen and carbon isotope ratios. Isotope ratios are presented in the usual δ notation based on the PeeDee Belemnite (PDB) for carbon and atmospheric N_2 (AIR) for nitrogen, and expressed as ‰. Replicate measurements of internal laboratory standards (acetanilide) indicate precision < 0.2‰ both for $\delta^{13}C$ and $\delta^{15}N$.

The results were analyzed statistically considering the explanatory factors tissue, area and maturity. The assumption of normality for each explanatory factor was tested by the Shapiro-Wilk's test, and sample homogeneity was tested by Bartlett´s test The factorial analysis ANOVA was applied to explore the effects of each factor by itself and the interaction of factors, area and maturity. The hypothesis test for the one-way ANOVA was performed under the assumption of H_0: no effect of the factor (area or maturity) in the stable isotope ($\delta^{13}C$ or $\delta^{15}N$) mean value. The hypothesis test for the two-way ANOVA was perfomed under the assumption of H_0: there is no effect of the interaction between the factors area and maturity in the stable isotope (δ13C or δ15N) mean value.

147 Trophic position (TP) in the food web of both populations were determined as (Vander Zanden
148 & Rasmussen, 2001): $TP = 2 + (\delta^{15}N_{oct} - \delta^{15}N_{zoop})/3.4$; where $\delta^{15}N_{oct}$ is the mean $\delta^{15}N$ ratio determined for
149 the flesh by study area, and $\delta^{15}N_{zoop}$ was determined as the mean $\delta^{15}N$ value for 200-500 μm zooplankton
150 collected in the northwest coast and, as the mean $\delta^{15}N$ value for zooplankton with size > 200μm for the
151 south coast. The zooplankton samples and base $\delta^{15}N$ data were collected by Bode et al. (2007) during
152 spring time between of 2002-2004.

153 **Lipid classes analyses**

154 The total lipid (TL) fraction was extracted by the Bligh & Dyer (1959) method. Samples of ≈ 1 g
155 and ≈ 2 g of dry tissue of DG and OV were used respectively. The results were expressed as g lipid/100 g
156 dry weight. Lipid classes were determined by different spectrophotometric methods. The phos fraction
157 was purified from the total lipid extract according to Auborg et al. (1996). Total phos were quantified by
158 measuring the organic Phophorus in total lipid extracts according to the Raheja et al (1973) method based
159 on a complex formation with ammonium molybdate. Results are expressed as g PL/100 g dry weight.
160 Total chol was determined in the total lipids extracts by the method of Huang et al. (1961) based on the
161 Liebermann-Buchardt reaction. Results are expressed in g chol/100 g dry weight. FA composition of
162 lipids extracted from the Bligh & Dyer extract, was determined converting total lipids into fatty acid
163 methyl esters (FAME), according to the Lepage & Roy (1984) method. FAME were analysed by gas
164 chromatography. Peaks corresponding to fatty acids were identified by comparison of their retention
165 times with standard mixtures. Peak areas were automatically integrated, 19:0 fatty acid being used as an
166 internal standard for quantitative analysis. The concentration of each fatty acid or fatty acid group was
167 expressed as g/100g total FAME.

168 The results were analysed statistically considering the explanatory factors tissue, area and
169 maturity. The assumption of normality for each explanatory factor was tested by the Shapiro-Wilk's test,
170 and sample homogeneity was tested by Bartlett´s test. For the lipid classes analysis, differences between
171 mean content of TL , chol, tag and phos by factor was tested by the Kruskal-Wallis test. The hypothesis
172 for the Kruskal-Wallis test was formulated under the assumption of H_0: there is no effect of the factor
173 (area or maturity) on the mean lipid class content in the tissue (GD or OV). The interaction effect between
174 factors tissue x area, tissue x maturation and area x maturation were tested applying the Friedman's Two-
175 Way Analysis of Variance by Ranks. The factors tissue and area were used as blocks and the factors area
176 and maturity were used as treatments. The Hypothesis test was defined as H_0:The levels of each block
177 (tissue or area) are statistically identical, even within the different levels of treatment (area or maturity);
178 H_1: The levels of each block (tissue or area) are statistically different, beyond the differences found within
179 each level of treatment (area or maturity) (Gibbons & Chakraborti, 2003).

180 The fatty acid profile was compared by explanatory factor, tissue, area and maturity and the
181 interaction between area and maturity by means of Multivariate ANOVA, followed by the Principal
182 Component Analysis of the majority FA, aiming to identify the FA which drive the differences between
183 areas and between maturity stages (Zuur et al, 2007). For each fatty acid, the assumption of sample
184 normality and homogeinity for each explanatory factor was tested by the Shapiro-Wilk's and Bartlett´s

185 respectively. Differences between mean concentration between area and maturity stage were tested by *t*-
186 test or the non-parametric Mann-whitney test depending on whether the sample was normally distributed
187 or not (Zar, 1999). Major fatty acids content was normalized before PCA.

188 **Results**

189 **Diet and Habitat**

190 The mean individual weight of the females collected was 2340.30 ± 979.45 g in the northwest
191 coast and 2637.40 ± 479.00 g for the south coast. The bio-indicators used to assess the nutritional
192 requirements of each population were the total lipid, tag , chol and phos contents in the digestive gland in
193 the two studied areas. Table 1 presents the mean lipid content determined by maturity stage and by area,
194 while Figure 3 displays the mean content of the lipid classes in the digestive gland (DG) and in the gonad
195 (OV) by study area and by maturity stage. The mean content of total lipids in the digestive gland in the
196 northwest coast is 15.00 ± 4.11 % DW, lower than in the south coast, 20.69 ± 6.20 % DW (Table 1). In
197 this tissue, the mean content in chol depends of area with digestive glands collected in the northwest coast
198 present a significantly higher content of chol than in the south coast (Figure 2, Table 2). Considering the
199 effect of maturity, independently of the area of collection, there seems to be no effect in the total lipid
200 content or in the specific lipid classes studied, with the exception of phos (Table 2). However, total lipid,
201 chol and tag content, tend to increase from immature to mature females (Figure 2). The two-way
202 Friedman test for the interaction between the two factors, area and maturity, shows a certain degree of
203 interaction between those, in the case of total lipids content and tag, chol and phos *per se* (Table 2). On
204 the other hand, analyzing the independent effect of area on the lipid classes content, reveals that these
205 vary with area: TAG and Chol content are higher in the northwest coast while the DG of the south coast
206 are richer in Pho than the DG of northwest coast.

207 The $\delta^{15}N$ signature is considered a bio-indicator of the consumer trophic position. The δ15N
208 value determined in the flesh samples is identical between areas and maturity stages (table 2) ranging
209 between and 11.44 ± 0.67‰ in mature females of the northwest coast and 12.09 ± 0.87 ‰ in mature
210 females of the south coast (Table 1, Figure 3). This bio-indicator presents values 4‰ higher in the flesh
211 than in both upper and lower beaks. The δ15N values in the upper and lower beaks are statistically
212 identical ($t = 1.12$, $df = 72.674$, p-value > 0.05), and are not affected by the maturity stages (Table 2).
213 Despite that, the beaks present different δ15N considering the study areas, with the beaks of south coast
214 presenting higher values of this isotope than northwest coast in app 1‰. The trophic position determined
215 based on the flesh δ15N signature was 3.40 for the northwest coast and 3.66 for the south coast (Figure
216 3), slightly higher than expected by the zooplanktonic base δ15N signature for the south coast.

217 The $\delta^{13}C$ ratio is a bio-indicator of habitat preference. The mean $\delta^{13}C$ determined for the flesh is
218 identical between areas and maturity stages (Table 2, Figure 3), and significantly different from the mean
219 $\delta^{13}C$ determined in the beaks. The upper beak presents in average higher δ13C values than the lower
220 beak, although these differences are not significative. The factorial analysis shows that there is no
221 statistical differences between beaks of different study areas and different maturity stages for this isotopic

222 signature (t = -1.89, df = 61.19, p-value > 0.05). Although, the δ13C in lower beaks seems to be affected
223 by maturity stage (Table 2).

224 In the DG, the fatty acids (FA) represent 31µg/mg and 46 µg/mg of the total lipid content in the
225 northwest coast and the south coast, respectively. The most abundant FA are the saturated fatty acids
226 (SFA), followed by the polyunsaturated fatty acids (PUFA) and the monounsaturated fatty acids (MUFA)
227 (Table 3). Results of the MANOVA analysis of the major FA profile in the digestive gland shows that
228 content levels depend both on area (F = 7.96, p-value < 0.05) and maturity (F = 5.84, p-value < 0.05),
229 with no effect of the interaction between both factors (F = 1.02, df = 10, p-value > 0.05). The mean
230 content of the myristic acid, C14:0, the palmitoleic acid, C16:1n7, the eiconenoic acid C20:1n9,
231 arachidonic acid (ARA) , C20:4n6 and the Docosahexaenoic acid (DHA) C22:6n3 is significantly
232 statistically different between areas, as well as the ratio DHA/EPA mainly due to the increase in DHA
233 content in digestive glands of the south coast (Table 3).

234 The principal component analysis was conducted based on the correlation matrix of the
235 normalized observations of the major fatty acids contents determined in the 20 digestive glands sampled.
236 For the digestive gland, the first component explains 37.86 % of the variation observed and the second
237 component explains 19.89 % of the variation observed, with both axis explaining 57.75 % of the variation
238 associated observed. Nevertheless, the biplot for the digestive gland (Figure 4, Digestive gland) shows
239 that the SFA C16:0 and the MUFA C16:1n7 and C18:1n9 are positively related with the first component
240 and the PUFA ARA, DHA and EPA are negatively related with the same component.The SFA C14:0 and
241 18:0 are positively related with the second component while the PUFA EPA and DHA present negative
242 signal in relation to this component. The graphical position of observations scores in relation to the first
243 component show that northwest immature digestive glands are related with higher content of the C16:0
244 and MUFA fatty acids, while south immature digestive glands are related with higher content of PUFA
245 fatty acids. Regarding mature digestive glands, the PCA is not able to reduce the dimension of the
246 variability.

247 **Maturity**

248 In order to study the effect of differences at the nutritional level on the maturation of the oocytes
249 in the gonad, we followed the same bio-indicators used to study the nutritional effect in the digestive
250 gland, total lipid content, the tag content, the chol content and the phos content in the gonad of immature
251 and mature females.

252 Mature gonads of the northwest coast present higher contents in tag, chol and phos content,
253 although no statistically significant differences were found on the mean content of those bio-indicators
254 between immature and mature individuals (Figure 2, Table 2). In the south coast, the gonads of mature
255 individuals present higher content of tag and phos but only the latter are significant. Mature females of
256 the northwest coast are not significantly different from mature females of south coast in the total lipids
257 mean content ($\chi^2_{Kruskal}$ = 0.88, p-value > 0.05) although the gonads of females in the northwest coast seem
258 to present higher contents of tag (1.96 ± 1.09 % dw) and chol (1.00 ± 0.45 % dw) and lower contents of

phos (0.146 ± 0.04 % dw) than the females of the south coast (tag = 1.31 ± 0.93 % dw; chol = 0.74 ± 0.39 % dw and pho = 0.19 ± 0.04 % dw) (Figure 6).

Stable isotope signatures between maturity stages show that the $\delta^{15}N$ signature is independent of maturity stage but not $\delta^{13}C$ (Table 2), for which significant differences were found in the this isotope signature between immature and mature females from the south coast ($t= 2.93$ p-value < 0.05).

The FA profile in the gonads is affected by area (MANOVA, $F = 5.62$, p-value < 0.05) and by maturity (MANOVA, $F = 4.09$, p-value < 0.10). Mature gonads are rich in SFA, showing the capacity to produce saturated fatty acids namely palmitic acid C16:0, with a significant increase from immature to mature females in the northwest coast. Another FA that presents significant differences between immature and mature gonads is the stearic acid C18:0 in the northwest coast, decreasing from immature to mature gonads (Table 3). MUFA are the second most abundant fatty acids in the gonads, with the presence vaccenic acid C18:1n7 as minority FA, presenting significant differences between the northwest coast and the south coast namely in the FA C17:1 and the C20:1n9. The FA C16:1n7 is significantly more abundant in immature than in mature gonads of northwest coast. The PUFA represent between 9% and 13% of the FAME present in the total lipid content of the gonads, with the content in ARA, DHA and EPA decreasing significantly from immature gonads to mature in both study areas. Particularly the significant decrease in DHA in both regions leads to significant differences between the DHA/EPA ratio of immature and mature females in that area (Table 3).

For the gonad FA profile, components one and two of the principal component analysis explain about 54.98 % of the observed variation. The first component explains 36,80% of the variation and is positively related with C18:0 and the MUFA C16:1n7, the C17:1, 18:1n9 and with the PUFA ARA, DHA and EPA, on the other and the SFA C14:0 and C16:0 correlates negatively with this component. The second component explains 18.18 % or variance and is positively correlated with C18:0 and the short-chain MUFA C17:1 and C18:1n9, while C14:0, C16:1n7 and the PUFA ARA, DHA and EPA correlates negatively with this component. The position of the scores in relation to the component axes shows that the variation explained by component one is related with maturity, immature individuals being related with lower than average content of DHA, ARA and EPA. It is noteworthy that immature gonads from the northwest coast present higher than average content of the MUFA 17:1, 18:1n9 and 16:1n7 and the immature gonads from the south coast are associated with higher content of the PUFA, ARA, DHA and EPA.

Discussion

This simultaneous study on the effect of the diet on the nutritional quality of the lipid reserves in the digestive gland (GD) and in the mature oocytes of the gonad (OV) in two populations of common octopus *Octopus vulgaris* allowed, firstly to understand the trophic role of two populations subject to different environmental conditions and secondly to evaluate which nutritional differences exist between those and how these affect the nutritional quality of gonad as proxy of the eggs.

295 The stable isotopic signatures of ^{13}C and ^{15}N are particularly good indicators of cephalopods
296 habitat and diet preferences (Jackson et al., 2007). In this study the $\delta^{13}C$ and $\delta^{15}N$ signatures were
297 assessed in the flesh and beaks. *O. vulgaris* is a fast growing organism with a high turnover rate of tissue,
298 with associated high turnover rates for the stable isotopes (Post. 2002), with the $\delta^{15}N$ and $\delta^{13}C$ signatures
299 in the flesh becoming good short term indicators of diet and habitat. On the other hand, the beaks grow by
300 accumulation of material with no turnover, and here the $\delta^{15}N$ and $\delta^{13}C$ signatures are indicators of the life
301 cycle history. The variation $\delta^{13}C$ signature observed between flesh and beaks is not significant, although
302 the $\delta^{15}N$ in the flesh is ≈ 4‰ higher than in the upper and lower beaks of *Octopus vulgaris*. This
303 variability is also observed elsewhere for *Octopus vulgaris* and by Cherel & Hobson (2005) in the muscle
304 and beaks of *Psychroteuthis glacialis.* Those authors suggested that the difference found in the nitrogen
305 signature between the flesh and beaks is related with the chitin synthesis and consequent N accretion in
306 the squid beaks. Considering this physiologic depletion in the $\delta^{15}N$ and the short term turnover of muscle
307 tissues in cephalopods, the flesh is the proper tissue to determine the population trophic position. The TP
308 is identical in both populations and places the *Octopus vulgaris* as third order consumer in the benthic
309 food web in those regions. These results are corroborated by a preliminary study on the stomach content
310 of both populations where the more abundant prey species were fish, crustacean and other molluscs,
311 although with some differences in the frequency of occurrence between regions, with the population of
312 northwest coast feeding more on fish and the south coast population of crustacean and bivalves. On the
313 other hand, studies published on trophodynamics of the Atlantic shelf of the Iberian Peninsula also places
314 pelagic and benthic cephalopod species in the third and forth consumers level (Bode et al., 2007; Mèndez-
315 Fernandez et al., 2007). Mèndez-Fernandez et al. (2007) actually presents a slightly higher trophic level
316 for *Octopus vulgaris* (TP = 3.8) and *Eledone cirrhosa* (TP = 3.6) in Galician waters (northern
317 neighbouring waters to this study), this corroborates the increase observed in the TP and in the $\delta^{15}N$ with
318 latitude, due to the increasing base $\delta^{15}N$ signature with latitude observed by Bode et al. (2007).

319 Nevertheless, the comparision between the $\delta^{15}N$ in common octopus beaks of different study
320 areas is possible. The higher $\delta^{15}N$ ratio in the south coast in relation to the northwest coast can indicate a
321 dominance of crustacea (crabs) in the diet of *O. vulgaris* in this area as indicated by Stowasser (2004) for
322 *Loligo forbesi,* a loliginid common in the northeast Atlantic that is also considered an opportunistic
323 predator. The upper/lower beaks present different $\delta^{15}N$ signatures between study areas, and $\delta^{13}C$ between
324 maturity stages (the lower beaks) of the southern population. This could indicate that even if the of
325 trophic level does not change, the dietary history between mature and immature females in the south coast
326 is different. Cherel and Hobson (2005) indicate that a variation of 4 ‰ (greater than we have shown)
327 indicates a change in the TP. Nevertheless cephalopod beaks grow by accretion of new molecules of
328 proteins and chitin and there is no turnover after synthesis, suggesting that a certain level of life-history is
329 being trapped. In fact, beaks retain molecules from early development to the time of death, and their
330 isotopic signature therefore integrates the feeding ecology of the animal over its whole life-history
331 (Cherel and Hobson, 2005). Similar signatures have been found for the antarctic giant squids
332 *Mesonychoteuthis hamiltoni* and *Architeuthis dux* (Cherel and Hobson, 2005), and for the squid *Loligo*
333 *forbesi* for the $\delta^{13}C$ in the muscle.

Considering that the digestive gland is mainly a lipid reservoir and therefore with high lipid content, it is the ideal organ to trace fatty acids as diet bio-indicators (Phillips et al. 2001; Moltschaniwskyj and Johnston, 2006; García-Garrido et al. 2010). Here the total lipid content ranged between 15 % in northwest coast and 20.69 %, in line with the obtained by Rosa et al. (2004a) for females captured in the Portuguese northwest coast, and lower to have been found in pelagic squids as *Moroteuthis ingens* (Phillips et al., 2001) which accounts for 40% of lipids in the digestive gland (although measured in wet mass). Contrary to what one should expect for a nutrient rich area, the northwest coast population presents relatively low lipid content in comparison to the south coast population. This is probably related with timing. In March and April, the sampling months, the northwest coast is characterized by a prior upwelling conditions, and fish present low fat content as detected for sardine *Sardina pilchardus* (Bandarra et al. 1997) and horse-mackerel *Trachurus trachurus* (Bandarra et al., 2001). On the other hand, in the south Portuguese coast, east to Cape Sta Maria, phytoplankton peaks are common in early spring associated to the warmer and nutrient rich river plumes, which associated to the shallow waters, create good conditions for the development of these earlier phytoplankton blooms (Ruiz et al., 2006). This nutrient richer environment in south coast is corroborated by the relative high content in the essential fatty acids ARA, EPA and DHA, essential fatty acids for carnivorous species produced by the producers community (Dalsgaard et al., 2003).

The higher content in TAG, defined as storage lipid (Lee et al., 2006), in the south coast digestive glands can be related with fito and zooplankton peaks of early spring, but also with the dominance crustacean preys in this coast as detected in the diet preliminarily study and also by Rosa et al. (2004b) which is directly related with the higher content with TAG (Phillips et al., 2003).Nevertheless, the higher content in total lipids, chol, SFA and MUFA in the digestive gland of the northwest coast is likely the result of a dominance in detritivorous species in their diet, where the impact of primary production cycles is attenuated.

The high content of 18:1n-9 (≈ 11 % in both study areas), which is indicative of deeper waters preys, and 16:0 (≈ 20% in both study areas) indicative of the presence of herbivorous preys, in the digestive gland of *Octopus vulgaris* is indicative of its diverse diet (Piché et al 2010). On the other hand, the DHA/EPA ratio seems to be unbalanced in the northwestern population. For a carnivorous species as this one, the DHA content should be higher relatively to the EPA as it happens in the south coast (Dalsgaard et al., 2003). This is can indicate that probably during this period of time the northwest population is feeding in a detritivores habitat, although the study conducted by Rosa et al (2004a) in the same region didn't found this unbalanced DHA/EPA ratio.

In terms of lipid content, the gonad presents on average 12% dw of total lipids, the tag and cho contents being identical, at around 5% of dry weight. The phos in the gonad represented in this study around 0.5 % dw of total lipids, similarly to what was found by Rosa et al. (2004a). The fatty acid profile shows a decrease in the PUFA content in the gonads with maturity, which can be related with fact that during maturation, these FA are being seized by the phos to form the lipid bilayer of the oocyte walls (Tocher, 2010). As in the squids *Sepioteuthis lessoniana* and *Photololigo* sp. (Semmens, 1998), or the cuttlefish *Sepia officinalis* (Blanchier and Boucaud-Camou, 1984), the lipid class contents in the digestive

gland do not correlate directly with the lipid content of the oocits in the gonad. Although the females only reach maturity beyond an optimum body mass independently of the gonad size (Lourenço et al. 2012), they are income breeders (Houston et al. 2007; Quetglas et al. 2011) that channel the energy directly from food to the gonad during maturation. So, the different levels in tag and chol noted in the digestive glands between geographical areas should be expected of income breeders. Despite that, the histograms comparing the lipid classes content in the digestive gland and gonads between study areas (Figure 5) show that the lipid classes' content in mature gonads is quite similar between those areas, even thow there are differences between tag and chol content in the digestive glands.

On the other hand the fact that the FA profile is variable both with area and maturity is evidence that there are differences between the quality of the oocytes of the northwest and south coasts during the first semester of the year. The gonads of the south coast are richer in PUFA and in MUFA, those of the northwest are richer in SFA. No differences were found in the ratio DHA/EPA in either the gonads or the digestive gland between areas, as also noted by Faría et al. (2011) for the Patagonian red octopus *Enteroctopus megalocyathus*, there being a direct effect of feeding in the quality of the eggs, and the content of FA of the diet seeming to affect the FA profile of the eggs, mainly the DHA content and the DHA/EPA ratio.

The differences found in the $\delta^{13}C$ signature in lower beaks of immature and mature females in the south coast may be related with the fact that each animal may spend different parts of the life-cycle in different environments. Mature females migrate to rocky substrates to lay and care for the eggs (Boyle and Rodhouse, 2005; Quetglas et al. 2011) and in the south coast this migration represents a major changes in habitat.

In terms of stock management, this study reflects how stocks are coupled with the environment and the habitat that surrounds them, from changes in prey availability to the pattern of physical characteristics of the environment all of which necessarily have variable impacts on the parental generation. What we have been able to show is that at least some differences can be noted in the egg quality, with a potential impact on the offspring.

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Figure legends:

Figure 1 - Sampling ports and coastal characteristics.

Figure 2 – Mean lipid classes content in % dw (± SD) aggregated by tissue (digestive gland; gonad), by area (nw – Northwest coast; south – South coast) and by maturity stage (i - immature, m - mature). Different superscript letters indicate significant statistical differences ($p < 0.05$).

Figure3 – *Octopus vulgaris* stable isotope $\delta^{15}N$ and $\delta^{13}C$ signatures in the flesh and upper and lower beaks by explanatory factors area and maturity. The upper right and left graphics represent the stable isotope signatures for the northwest and south coast, and the lower left and right graphics represent the stable isotope signatures for immature and mature individuals.

Figure 4 – Principal component analysis byplots of the fatty acid profiles of the digestive gland (on the left) and gonad (on the right). The loadings correspond to the FA variables analysed and the scores in grey correspond to each one of the sample analysed.

Figure 5 – Comparison of the Lipid classes content in the digestive gland and gonad of mature females in the northwest coast and in the south coast. tag – Triacylglycerol, chol – Cholesterol and phos – Phospholipids in the digestive gland and in the gonad.

Table 1 – Carbon ($\delta^{13}C$), Nitrogen ($\delta^{15}N$) isotope ration (mean±SD, ‰) in the flesh and in the upper and lower beak, trophic level (±SD) and total lipid content (mean ± SD, %dw) in the digestive gland and gonad by study area (northwest coast and south coast), and maturity stage (immature and mature). The superscript * indicates significant statistical difference (p-value < 0.05) between maturity stages, and the superscript ** indicates significant statistical difference between study areas.

		Flesh		Upper beak		Lower beak		Trophic Level	DG Total Lipid (% dw)	OV Total Lipid (% dw)
		$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)			
Northwest coast	Immature	-16.55 ± 0.82	11.91 ± 0.71	-17.25 ± 0.69	7.29 ± 0.64	-17.01 ± 0.76	7.64 ± 0.94		14.60±4.44	15.39 ± 2.36
	Mature	-17.31 ± 0.37	11.44 ± 0.67	-17.84 ± 0.76	7.38 ± 0.91	-17.61 ± 0.60	7.78 ± 0.61	3.40 ± 0.21**	15.39 ± 4.22	12.53±3.27
	Mean	-16.85 ±0.76	11.72 ± 0.70	-17.53 ± 0.76	7.33 ± 0.75**	-17.29 ± 0.74	7.70 ± 0.78**		15.00±4.11	13.96±3.08
South coast	Immature	-16.57 ± 0.62	11.84 ± 0.46	-17.45 + 1.36	8.43 ± 0.56	-16.76 ± 0.67	8.69 ± 0.98		16.97 ± 4.30	12.16 ± 3.62
	Mature	-16.96 ±0.86	12.09 ± 0.87	-17.60 ± 0.72	8.74 ± 0.89	-17.38 ± 0.67	8.71 ± 0.70	3.66 ± 0.18**	24.42 ± 5.76	11.03 ± 0.99
	Mean	-16.70 ± 0.71	11.92 ± 0.61	-17.49 ± 1.19	8.52 ± 0.66**	-16.94 ± 0.71	8.70 ± 0.89**		20.69 ± 6.20	11.59 ± 2.57

Table 2 - One-way, two-way analysis of variance and hypothesis testing considering the applied bio-indicators, δ15N, δ13C, total lipids content % dw TL), Triacilglycerol content (% dw tag), Cholesterol content (% dw cho) and Phospholipids content (% dw phos) determined for flesh, beaks, digestive gland and gonad by studied region and maturity stage.

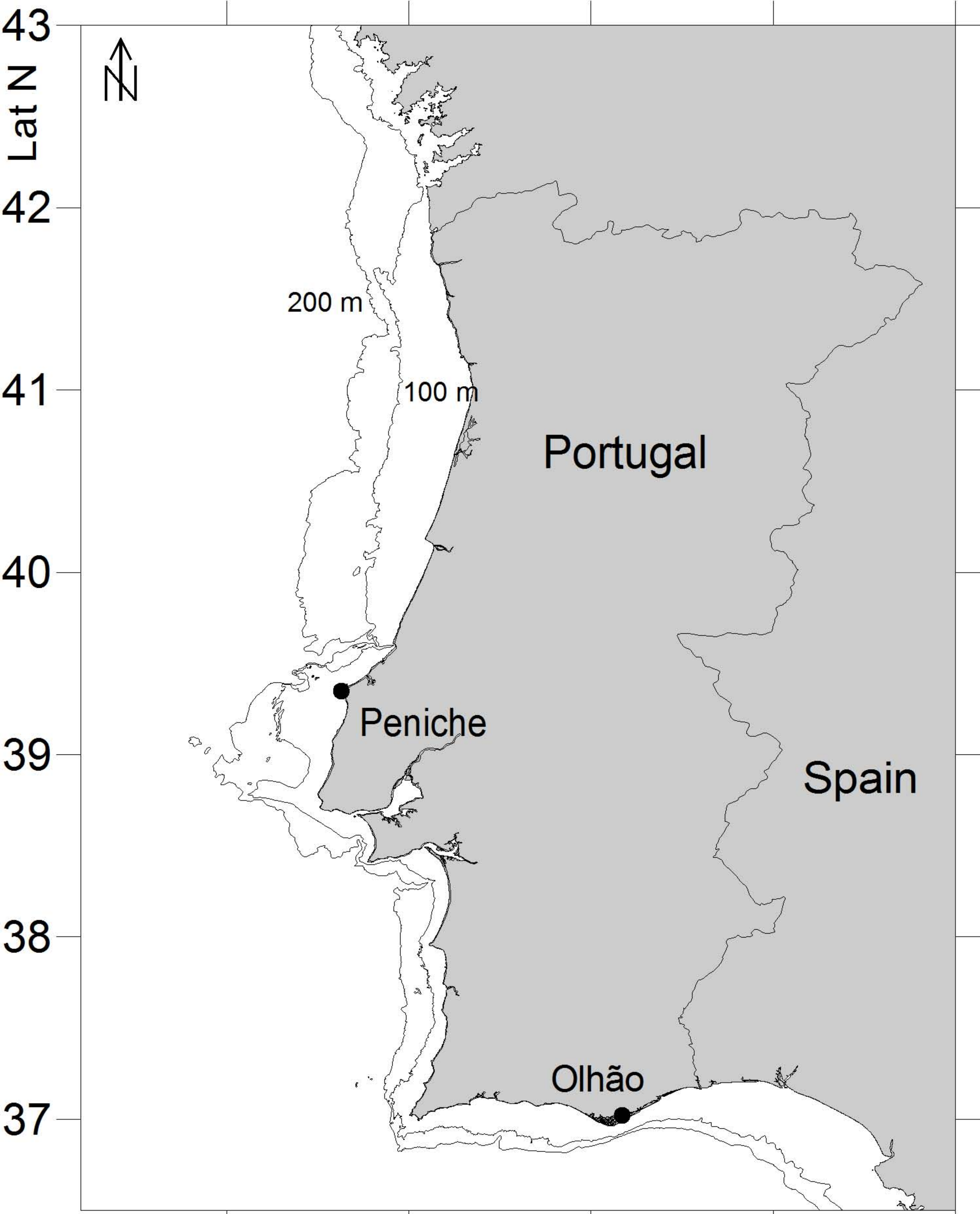
Tissue	Indicator	Test	Factor	df	Statistics	p-value	Conclusion
Flesh	δ13C	One-way ANOVA	area	26	F=0.28	>0.05	Accept H_0
			mat	26	F=4.00	>0.05	Accept H_0
		Two-way ANOVA	area*mat	24	F=0.44	>0.05	Accept H_0
	δ15N	One way ANOVA	area	26	F=0.62	>0.05	Accept H_0
			mat	26	F=0.02	>0.05	Accept H_0
		Two-way ANOVA	area*mat	24	F=1.83	>0.05	Accept H_0
Beaks	δ13C	One-way ANOVA	area	66	F=0.80	>0.05	Accept H_0
			mat	**66**	**F=5.93**	**<0.05**	**Reject H_0**
		Two-way ANOVA	area*mat	64	F=0.21	>0.10	Accept H_0
	δ15N	One-way ANOVA	area	**66**	**F=33.46**	**<0.05**	**Reject H_0**
			mat	**66**	**F=5.93**	**<0.05**	**Reject H_0**
		Two-way ANOVA	area*mat	64	F=0.21	>0.05	Accept H_0
Digestive gland	TL	One-way ANOVA	**area**	**1**	χ^2_K=3.57	>0.05	Accept H_0
			mat	1	χ^2_K=1.28	>0.05	Accept H_0
		Two-way ANOVA	**area*mat**	**1**	**χ^2_F=34.00**	**<0.05**	**Reject H_0**
	tag	One-way ANOVA	area	1	χ^2_K=1.66	>0.05	Accept H_0
			mat	**1**	χ^2_K=3.57	>0.05	Accept H_0
		Two-way ANOVA	**area*mat**	**1**	**χ^2_F=34.00**	**<0.05**	**Reject H_0**
	cho	One-way ANOVA	**area**	**1**	**χ^2_K=14.28**	**<0.05**	**Reject H_0**
			mat	1	χ^2_K=0.21	>0.05	Accept H_0
		Two-way ANOVA	**area*mat**	**1**	**χ^2_F=32.00**	**<0.05**	**Reject H_0**
	phos	One-way ANOVA	area	1	χ^2_K=3.35	>0.05	Accept H_0
			mat	1	**χ^2_K=3.93**	**=0.05**	**Reject H_0**
		Two-way ANOVA	**area*mat**	**1**	**χ^2_F=40.00**	**<0.05**	**Reject H_0**
Gonad	TL	One-way ANOVA	area	1	**χ^2_K=3.29**	**>0.05**	**Accept H_0**
			mat	1	χ^2_K=2.28	>0.05	Accept H_0
		Two-way ANOVA	**area*mat**	**1**	**χ^2_F=40.00**	**<0.05**	**Reject H_0**
	tag	One-way ANOVA	area	1	χ^2_K=2.29	>0.05	Accept H_0
			mat	1	χ^2_K=1.85	>0.05	Accept H_0
		Two-way ANOVA	**area*mat**	**1**	**χ^2_F=40.00**	**<0.05**	**Reject H_0**
	cho	One-way ANOVA	area	1	χ^2_K=1.65	>0.05	Accept H_0
			mat	1	χ^2_K=0.02	>0.05	Accept H_0
		Two-way ANOVA	**area*mat**	**1**	**χ^2_F=40.00**	**<0.05**	**Reject H_0**
	phos	One-way ANOVA	area	1	χ^2_K=0.76	>0.05	Accept H_0
			mat	1	**χ^2_K=8.73**	**<0.05**	**Reject H_0**
		Two-way ANOVA	**area*mat**	**1**	**χ^2_F=40.00**	**<0.05**	**Reject H_0**

Table 3 - Fatty acids (mean ± SD, g/100 g FAME) profile in the digestive gland and gonad of immature and mature females collected in the northwest and south portuguese coast. * indicates significant statistical difference (p-value < 0.05) between immature and mature individuals; ** indicates significant statistical difference (p-value < 0.05)in the fatty acid content between sampling area.

	Digestive Gland						Gonad					
Fatty acid	**northwest coast**			**south coast**			**northwest coast**			**South coast**		
	immature	**mature**	**mean**	**immature**	**mature**	**mean**	**immature**	**mature**	**mean**	**immature**	**mature**	**mean**
Saturated fatty acids (SFA)												
C 14:0	4.99±1.58	4.89±2.05	4.94±1.75**	1.88±0.96	3.25±1.62	2.51±1.42**	4.19±0.48	4.09±0.36	4.14±0.40	4.06±0.72	3.94±0.52	3.99±0.58
C 16:0	22.62±4.35	18.34±5.71	20.48±5.33	22.08±14.61	18.48±9.58	20.44±12.14	19.25±1.98*	23.85±1.18*	21.73±2.83	18.78±2.44	22.31±3.50	20.83±3.48
C 18:0	13.73±4.27	11.63±0.32	12.68±3.09	12.88±1.58	11.21±1.78	12.12±1.80	8.68±1.33*	6.36±0.93*	7.43±1.62	7.47±1.26	5.90±1.00	6.56±1.33
Other SFA	6.21±2.98	6.60±2.66	6.40±2.70**	3.85±1.08	3.58±0.62	3.72±0.87**	2.19±0.22	2.25±0.45	2.22±0.35**	2.04±0.14	1.93±0.38	1.98±0.30**
Σ SFA	47.55±8.68	41.46±8.25	44.51±8.68	40.68±13.32	36.52±13.50	38.79±12.90	34.32±1.33*	36.55±0.85*	35.52±1.56**	32.36±1.58	34.08±3.41	33.37±2.83**
Monounsaturated fatty acids (MUFA)												
C 16:1n-7	6.61±1.51	6.78±2.84	6.69±2.17**	3.05±0.52	4.11±1.34	3.53±1.08**	2.68±0.34*	2.05±0.42*	2.34±0.49	2.79±0.51	2.63±0.73	2.70±0.63
C 17:1	1.77±0.62	1.15±0.10	1.46±0.53	1.56±0.22*	1.25±0.26*	1.42±0.28	2.53±0.40	2.62±0.55	2.58±0.47**	2.26±0.42	1.90±0.42	2.05±0.44**
C 18:1n-9	11.49±2.85	10.49±6.77	10.99±4.98	8.54±3.46	14.51±3.22	11.26±4.45	6.23±1.33	5.37±0.56	5.76±1.05	5.34±1.32	4.41±1.74	4.80±1.59
C 20:1n9	2.25±0.60	2.72±0.94	2.49±0.79**	3.33±1.08	3.98±0.96	3.34±1.08**	5.50±0.63*	4.48±0.21*	4.95±0.69**	6.11±1.18	7.90±6.51	7.15±4.95**
other MUFA[2]	2.19±0.98	1.78±0.86	1.98±0.90	2.23±0.78	3.04±1.08	2.60±0.97	3.50±0.40*	2.46±0.36*	2.94±0.65**	2.88±0.38	2.13±0.65	2.44±0.66**
ΣMUFA	24.30±3.76	22.92±9.26	23.61±6.78	18.19±3.22*	26.88±4.86*	22.14±5.94	20.45±1.21*	16.99±1.17*	18.59±2.13	19.39±1.45	18.97±7.69	19.15±5.31
Polyunsaturated fatty acids (PUFA)												
C: 20:4n-6[3]	4.34±1.55	5.18±2.62	4.76±2.09**	7.64±3.22	6.06±2.06	6.92±2.75**	9.86±1.71*	7.51±0.96*	8.59±1.78	9.80±1.00	9.18±0.68	9.48±0.87
C 20:5n-3	8.54±4.06	17.45±11.53	13.00±9.46	10.82±9.46	11.00±6.08	10.90±5.02	1.64±0.32	1.47±0.28	1.55±0.30	2.58±2.20*	1.14±0.20*	1.74±1.53
C 22:6n-3	11.43±7.92	8.42±6.23	9.92±6.97**	19.54±8.70	16.28±8.97	18.06±8.54**	1.09±0.30*	0.27±0.22*	0.65±0.49	1.27±0.44*	0.67±0.31*	0.92±0.47
Other PUFA[4]	2.32±0.77	3.15±1.75	2.73±1.36**	1.92±0.30	1.88±0.41	1.91±0.34**	n.d	n.d	n.d	n.d	n.d	n.d
ΣPUFA	26.63±11.34	34.19±16.02	30.41±13.81	39.92±15.68	35.23±16.73	37.79±15.52	12.60±1.64	9.25±1.04	10.79±2.16	13.75±3.26	10.99±0.82	12.14±2.50
Σ FAME (ug/mg PS)	31.35±9.55	33.14±8.30	31.25±8.53**	48.79±11.56	43.40±9.59	46.34±10.56**	63.74±15.67	67.99±9.53	66.03±12.35	58.27±16.11	67.56±15.02	63.69±15.50
Σn-3/Σn-6	2.03±1.46	1.11±0.55	1.57±1.15	2.11±0.83	2.07±0.98	2.09±0.85	3.52±0.67*	5.07±0.76*	4.35±1.06	3.66±0.53	4.01±0.36	3.86±0.52
DHA/EPA	1.31±0.53	0.63±0.37*	0.97±0.56**	1.76±0.34	1.57±0.31	1.68±0.36**	0.66±0.10*	0.18±0.15*	0.40±0.28	0.65±0.03	0.57±0.05	0.60±0.23

[1] – other minor SFA C15:0, C17:0 and C24:0;
[2] – other minor MUFA C18:1n-7, C22:1n-9 and C24:1n-9;

[3] – as the FA C20:3n-3 and the FA C20:4n-6 have the same retention time, and the concentration of the FA C20:4n-6 is dominant in marine products, the concentration presented here is representative of C20:4n-6.
[4] – other minor FA C18:2n-6 and C20:2n-6.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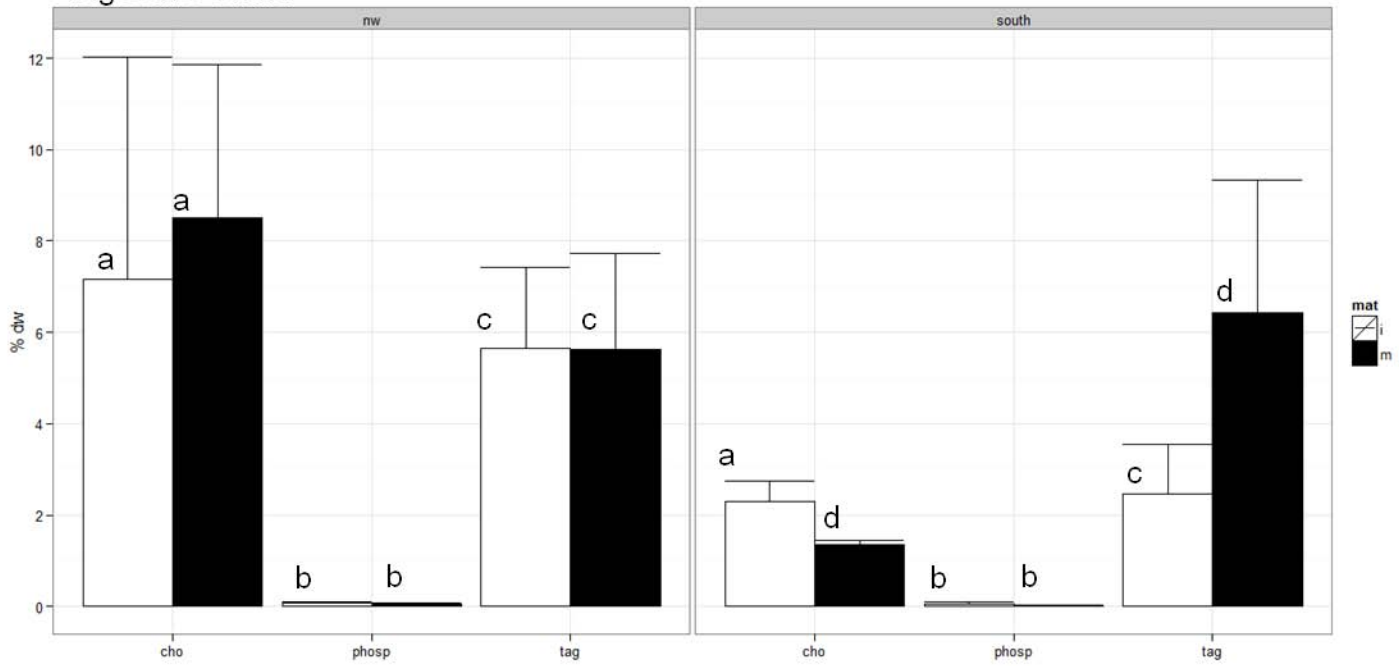
Long W

Digestive Gland



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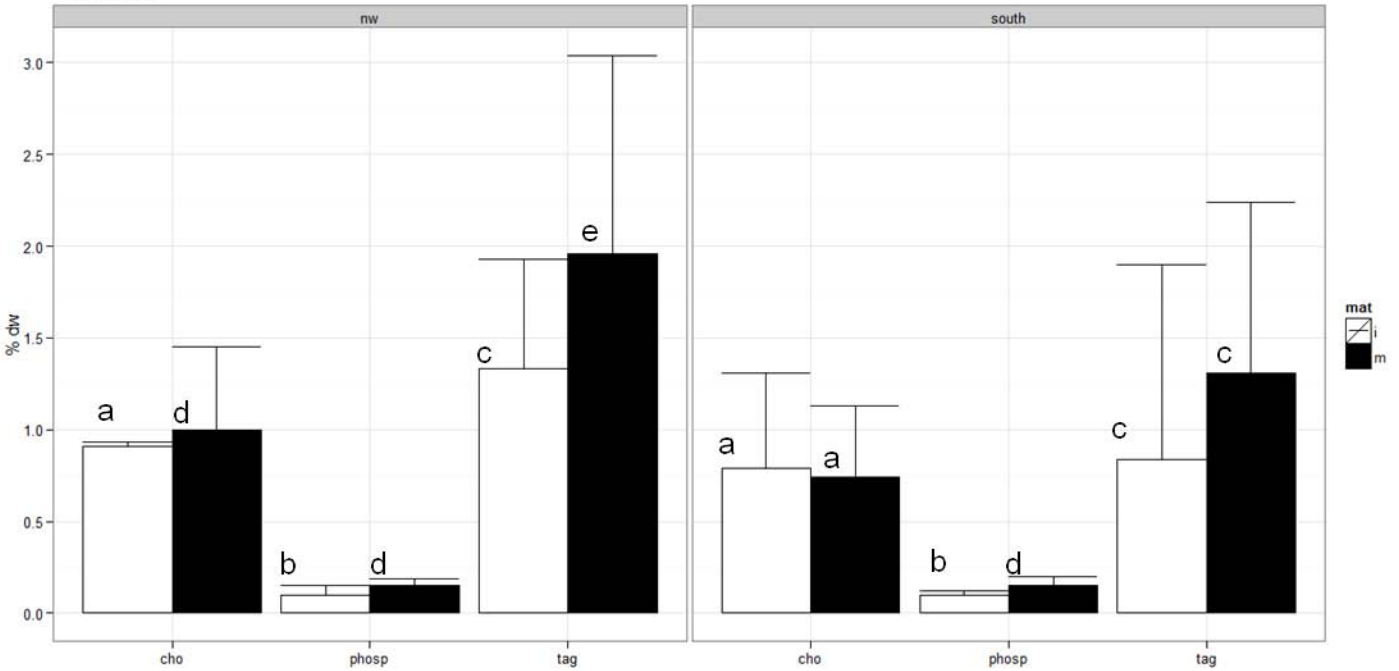

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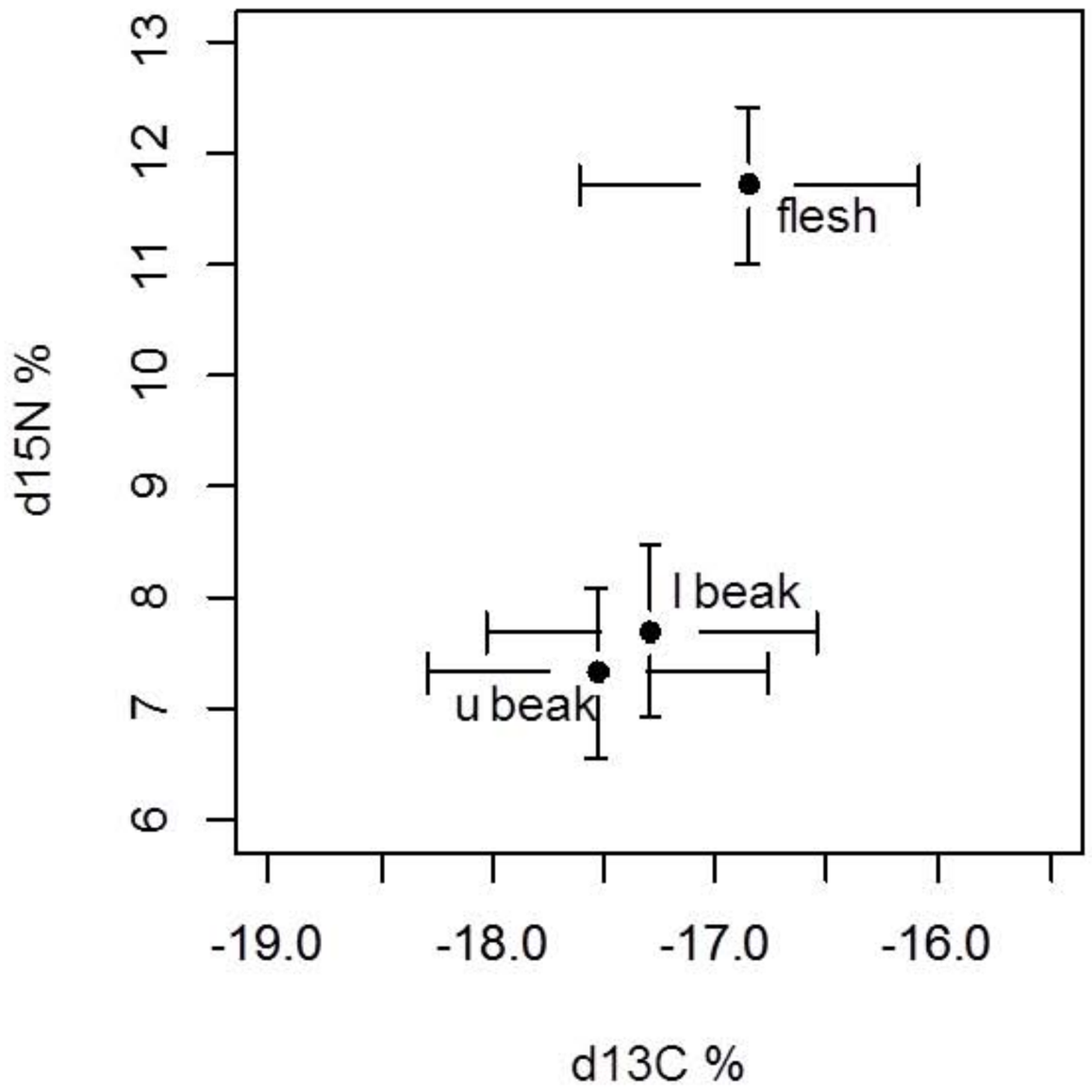

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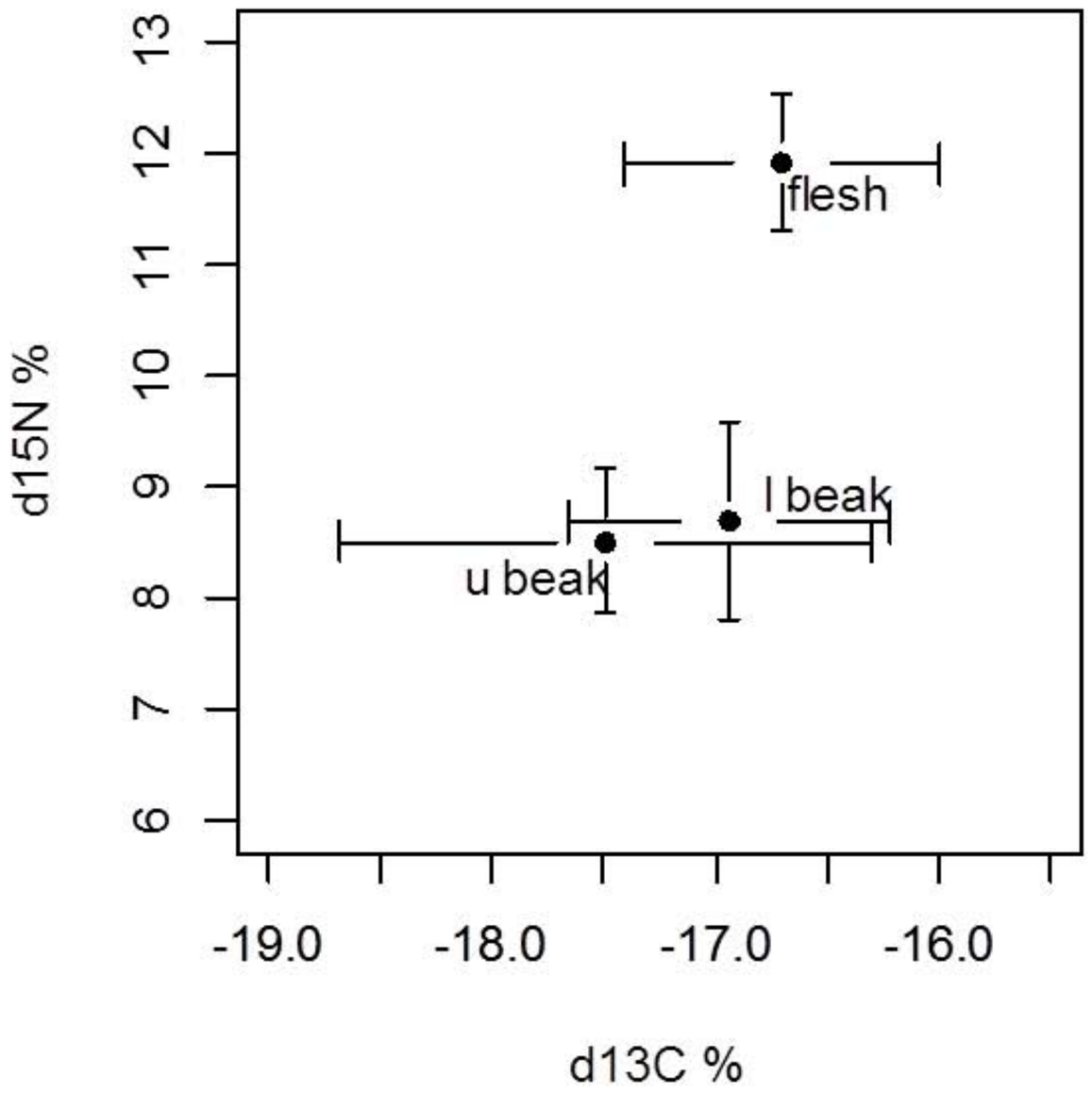
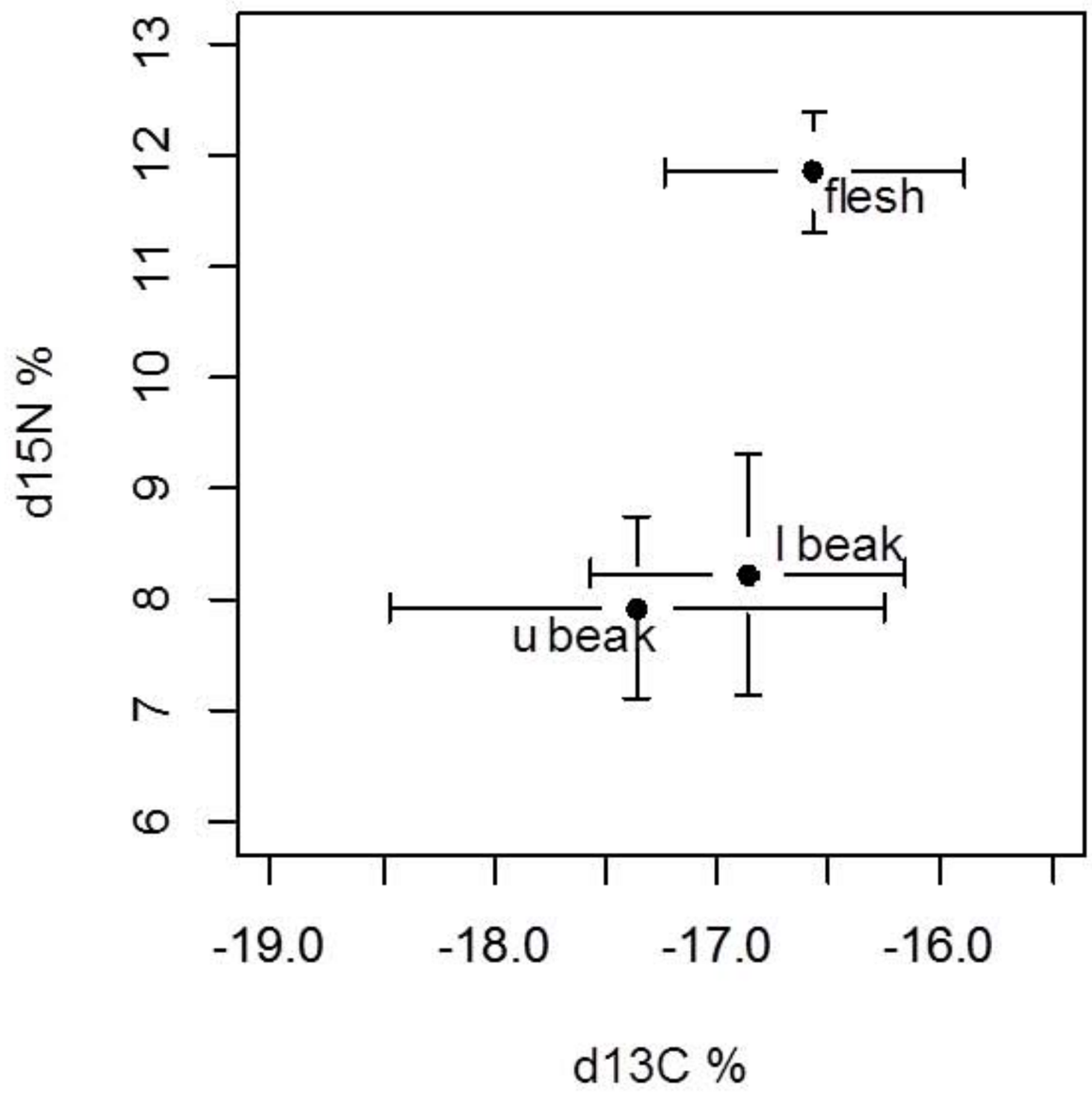


immature



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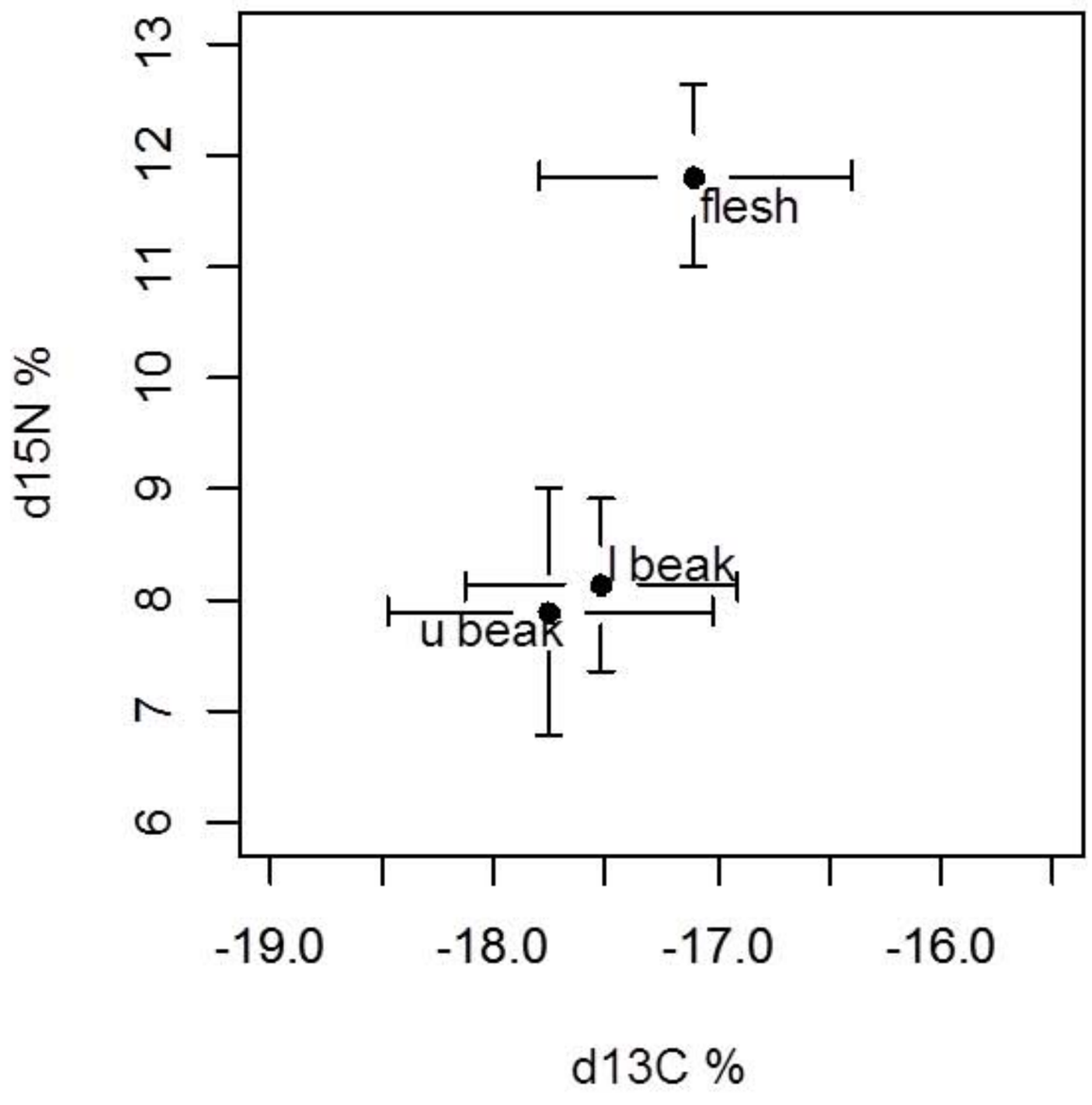
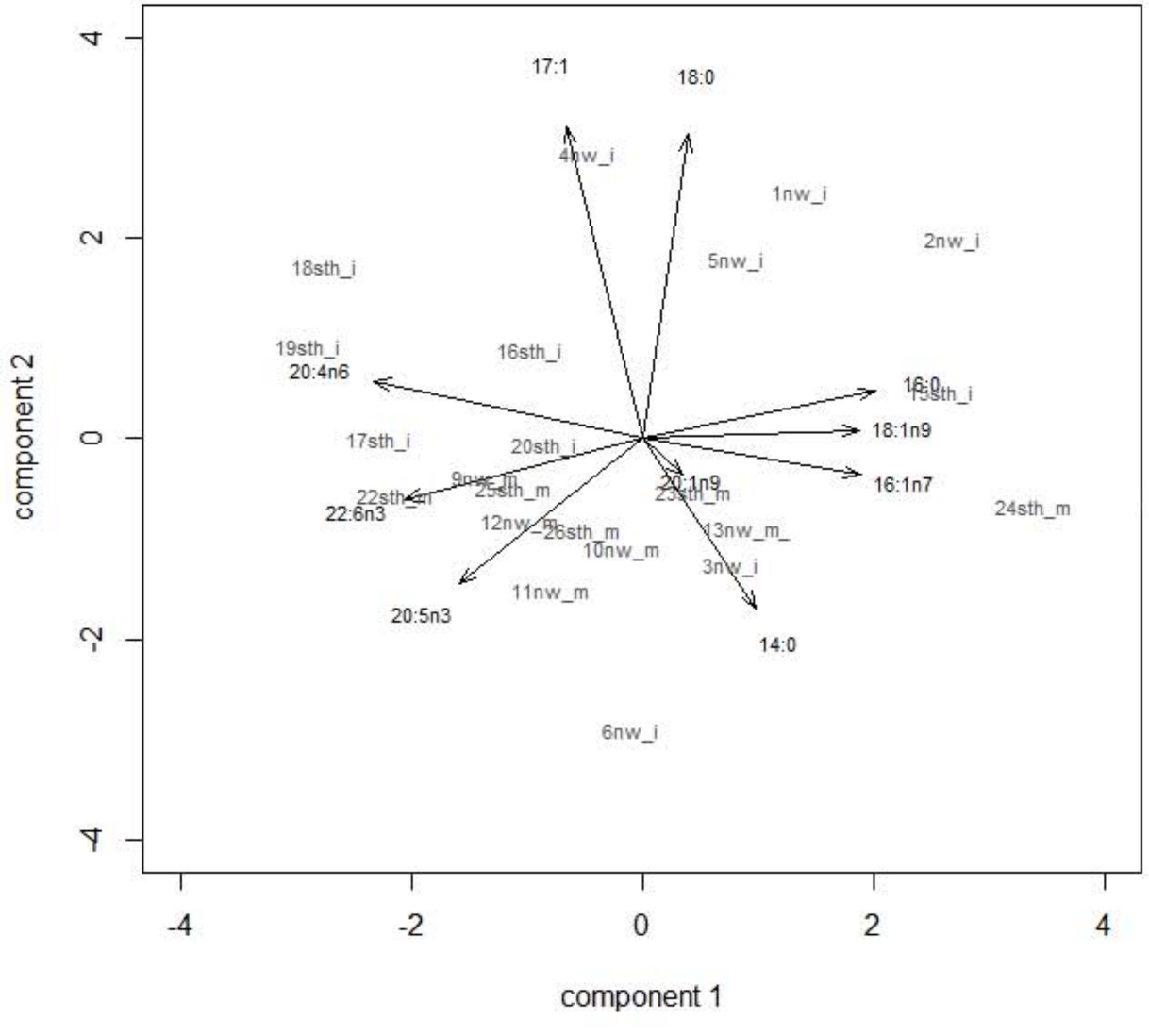


Digestive Gland



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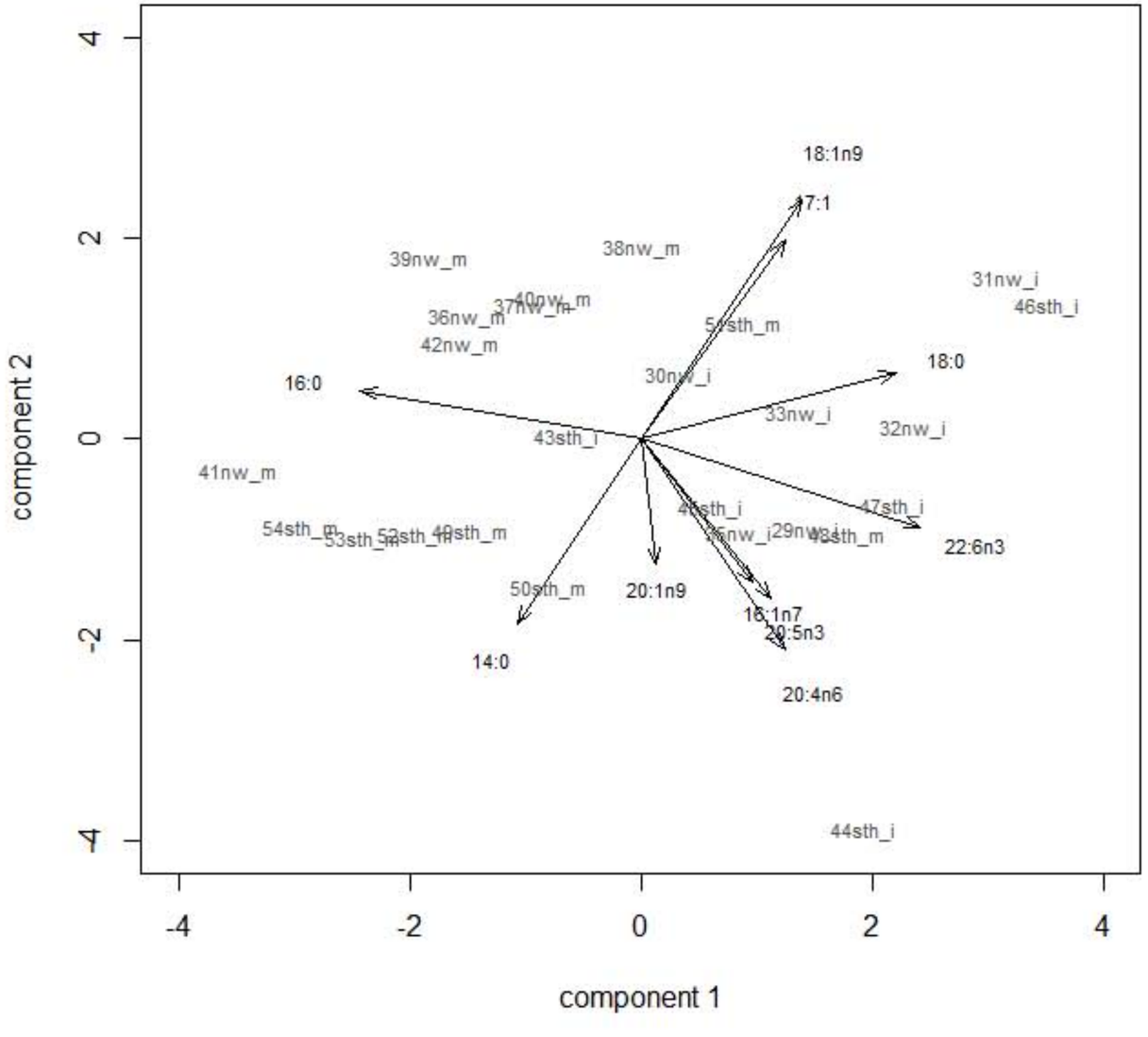

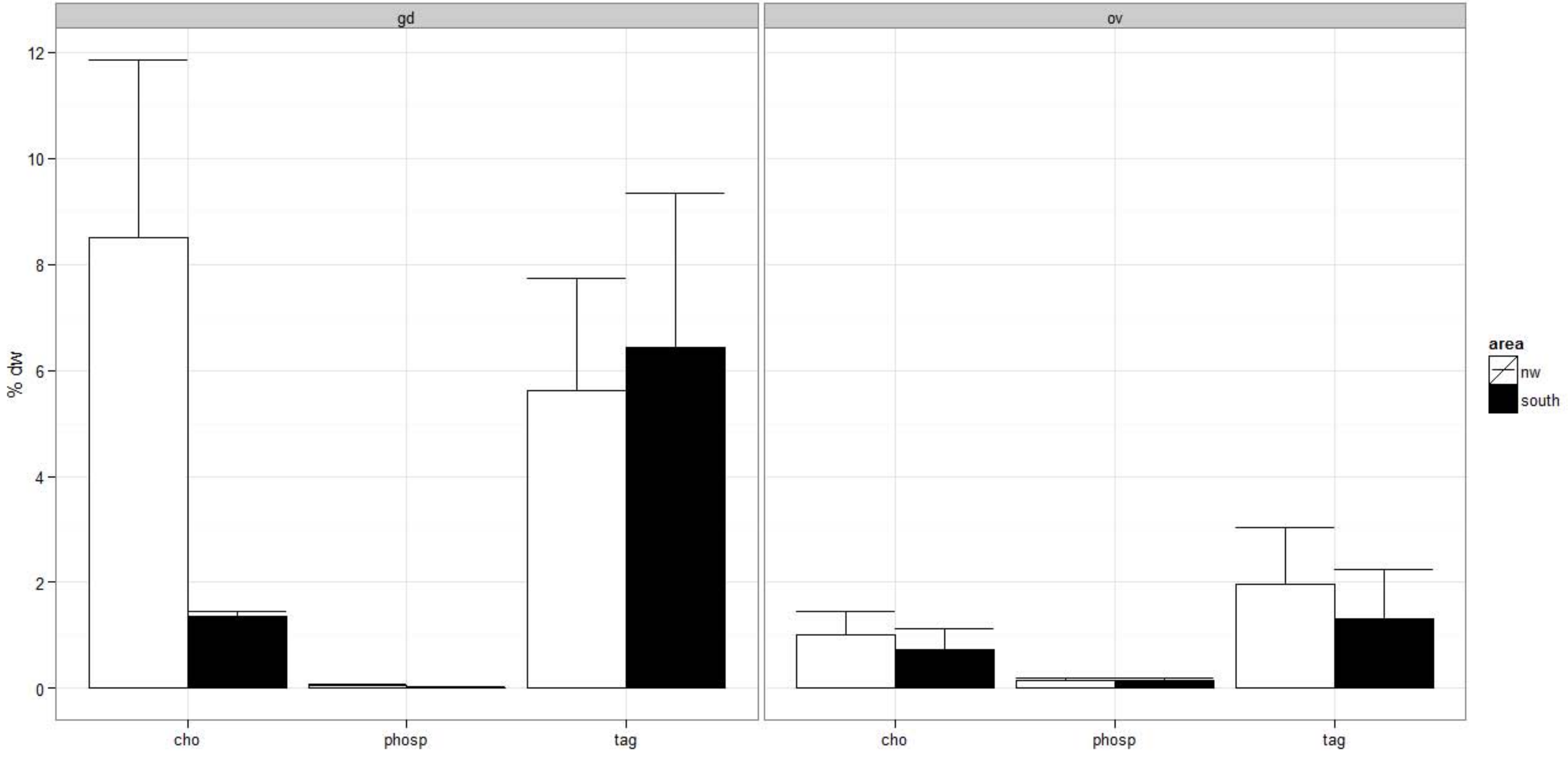
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