

1 **Molecular prey identification in wild *Octopus vulgaris* paralarvae**

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9

10 **Abstract**

11 The trophic ecology of *Octopus vulgaris* paralarvae collected in 2008 off the Ría de Vigo, NW Spain
12 (42°12.80'N–9°00.00'W) was approached by both morphological and molecular methods. External
13 digestion of prey and posterior suction of the liquefied contents by wild *O. vulgaris* paralarvae made the
14 morphological identification of gut contents impossible. Thus, a PCR-based method using group specific
15 primers was selected to identify prey consumed by *O. vulgaris* paralarvae in the pelagic realm. The
16 mitochondrial ribosomal 16S gene region was chosen for designing group specific primers, which
17 targeted a broad range of crustaceans and fishes but avoided the amplification of predator DNA. These
18 primers successfully amplified DNA of prey by using a semi-nested PCR-based approach and posterior
19 cloning. Homology search and phylogenetic analysis were then conducted with the 20 different
20 operational taxonomic units (OTUs) obtained to identify the putative organisms ingested. The
21 phylogenetic analysis clustered ingested prey into 12 families of crustaceans (11 belonging to the order
22 Decapoda and 1 to the order Euphausiacea) and two families of fishes (Gobiidae and Carangidae).
23 According to the Czekanowski's Index (CI), the trophic niche breadth of *O. vulgaris* paralarvae is low
24 (CI=0.13), which means that these paralarvae are specialist predators at least during the first weeks of
25 their life cycle. It is the first time that natural prey has been identified in *O. vulgaris* paralarvae collected
26 from the wild and such knowledge may be critical to increasing the survival of *O. vulgaris* hatchlings in
27 captivity, a goal that has been actively pursued since the 1960's by aquaculture researchers.

28 **Key words:** *Octopus vulgaris*, paralarval diet, group specific primers, gut content, PCR, trophic ecology,
29 aquaculture.

30 **Introduction**

31 Dietary analysis in cephalopods is hampered by problems arising from the anatomy, physiology and mode
32 of ingestion (Rodhouse and Nigmatullin 1996) of these organisms. The oesophagus diameter is limited
33 physically as it passes through the brain, so the cephalopod beak bites small pieces of tissue to swallow.
34 Rapid digestion rates in the stomach result in short residence times (two to six hours) making the prey
35 remains visually unidentifiable (Altman and Nixon 1970; Andrews and Tansey 1983; Nixon 1985). The
36 mode of prey ingestion can be internal, by biting with the beak, or external, where salivary enzymes
37 paralyse and digest the flesh followed by the ingestion of the liquefied content (Nixon 1984; Guerra and
38 Nixon 1987). These specialised feeding strategies largely avoid the ingestion of hard skeletal material and
39 tend to bias data on both prey species and size when morphological analysis are used (Nixon 1985).

40 Cephalopods are known to be highly versatile predators with opportunistic predation behaviours
41 (reviewed in Rodhouse and Nigmatullin 1996). While numerous works have focused on the trophic role
42 of adults (Nixon 1987; Boyle et al. 1996; Raserio et al. 1996; Rodhouse and Nigmatullin 1996), the
43 knowledge of diet in wild paralarvae is scarce due to the small size of this life history stage. The few
44 attempts made to clarify the diet showed that paralarvae are mainly generalist feeders preying primarily
45 on crustaceans, as observed by visual analysis by Passarella and Hopkins (1991) and Vecchione (1991).
46 Further visual analysis made by Vidal and Haimovici (1998) showed that 11.4% of ommastrephid squid
47 paralarvae contained copepod appendages. Additionally, Venter et al. (1999) developed an immunoassay
48 that detected copepods, euphausiids and polychaetes in the gut of six *Loligo reynaudii* paralarvae.

49 While some squid and cuttlefish paralarvae preying on pelagic crustaceans ingest exoskeleton pieces, thus
50 allowing morphological analysis (Vecchione 1991; Passarella and Hopkins 1991; Vidal and Haimovici
51 1998); the external digestion exhibited in octopod paralarvae hatchlings rejects the entire crustacean
52 zoeae exoskeleton therefore preventing morphological analysis of the dietary items (Hernández-García et
53 al. 2000). Occasionally, the presence of thoracic appendages has been observed in the stomach of
54 *Octopus vulgaris* hatchlings fed on *Artemia* under laboratory conditions, because *Artemia* has a thinner
55 exoskeleton than other crustacean zoeae (Iglesias et al. 2006).

56 *Octopus vulgaris* is a generalist predator as both a juvenile and an adult, feeding upon a variety of
57 organisms mainly within the class Crustacea, but also Gastropoda, Lamellibranchiata, Osteichthyes,
58 Ophiuroidea, Polychaeta and Cephalopoda (Nigmatullin and Ostapenko 1976; Guerra 1978; Smale and
59 Buchan 1981; Nixon 1987; Mather 1991). The industrial rearing of this octopus species has been
60 hampered by the high mortality during the pelagic stage, despite the broad range of experimental diets

61 assayed throughout the past sixty years (reviewed in Iglesias et al. 2007). Although some authors have
62 hypothesised that *O. vulgaris* prey upon crustaceans during the planktonic stage (Mangold and Boletzky
63 1973; Nixon 1985; Rodhouse and Nigmatullin 1996; Villanueva and Norman 2008), the feeding habits of
64 wild *O. vulgaris* paralarvae are still unknown.

65 The trophic ecology of *Octopus vulgaris* paralarvae was tackled using both morphological and molecular
66 methods, which have been shown to provide a comprehensive understanding of both invertebrate and
67 vertebrate diets (Casper et al. 2007; Deagle et al. 2007, 2010; Braley et al. 2010). Given that *Artemia* was
68 successfully detected in a single *O. vulgaris* paralarvae reared in laboratory by using species specific
69 primers (Roura et al. 2010), the next step involved developing a molecular technique to detect the natural
70 prey of wild paralarvae. This approach requires *a priori* knowledge of the fauna that coexist with
71 paralarvae in the zooplankton. Hence, ten surveys were undertaken in the Ría de Vigo, a region of coastal
72 upwelling off NW Spain (Otero et al. 2009), to obtain wild paralarvae as well as relative abundances of
73 the different zooplankton species present in the area. Due to the enormous variety of suitable prey species
74 in the zooplankton community; neither the species specific primer approach (King et al. 2008) nor the
75 serological methods (Boyle et al. 1986, Venter et al. 1999) would be practical to identify prey. Therefore,
76 we developed a technique to amplify small, multi-copy DNA fragments with universal primers for the
77 16S rRNA gene (Simon et al. 1994) in conjunction with group specific primers, designed within this
78 gene, that anneal to short target templates of potential prey items (Deagle et al. 2005, 2007, 2009, 2010;
79 Braley et al. 2010). The group specific primers were designed to amplify a wide range of crustaceans and
80 fishes, likely the most suitable prey of wild *O. vulgaris* paralarvae, based upon reports that the feeding
81 habits of cephalopods shift from crustacean feeders during early stages (Vecchione 1991; Vidal and
82 Haimovici 1998; Venter et al. 1999) towards piscivory in juvenile and adult stages (Passarella and
83 Hopkins 1991; Rasero et al. 1996).

84 The aim of this work was to identify natural prey of *Octopus vulgaris* paralarvae collected in the wild,
85 using both morphological and molecular methods. Additionally, trophic selectivity of the paralarvae was
86 addressed by comparing the composition of the zooplankton community they inhabit with the prey
87 detected, under the assumption that cephalopod paralarvae are generalist predators. This molecular
88 method is also immediately transferable to other oceanographic predator/prey scenarios as well as to other
89 dietary studies on cephalopod paralarvae.

90

91 **Methods**

92 *Sample collection, morphologic analysis and DNA extraction*

93 Ten surveys to collect zooplankton and hydrographical data were undertaken at night during July and
94 September-October 2008 in the Ría de Vigo, NW Spain (42°12.80'N–09°00.00'W) onboard RV
95 “Mytilus”. Biological sampling consisted of four transects as in González et al. (2005); three located
96 outside the Cies Islands and one inside the Ría de Vigo (T2, T3, T4 and T5) parallel to the coast
97 following an onshore-offshore depth gradient with an average depth of 26, 68, 85 and 110m, respectively.

98 On each transect two double oblique trawls were deployed, one at the surface and one near the bottom,
99 using a 75-cm diameter bongo net equipped with 375- μ m mesh and a current meter. Zooplankton samples
100 were fixed onboard with 96% ethanol and stored at -20°C. In the laboratory, cephalopod paralarvae were
101 separated and classified according to Sweeney et al. (1992) and our own reference collections.
102 Zooplankton composition and abundance was estimated by Roura et al. (unpublished).

103 Morphological analyses of the gut contents were carried out from two batches of eighteen randomly
104 selected *Octopus vulgaris* paralarvae, following two different procedures. In the first batch, the digestive
105 tracts were removed and gut contents were distributed in water on a microscope slide and then examined
106 under an inverted microscope at 100x to 400x magnification (Nikon Eclipse TS100) as in Passarella and
107 Hopkins (1991). The second batch was prepared for routine histological analysis by staining with
108 haematoxylin-eosin and examined under a microscope at 100x to 400x magnification (Nikon Eclipse 80i).

109 Genetic analysis was carried out with eighteen *O. vulgaris* paralarvae randomly sorted that were
110 preserved in 70% ethanol at -20°C. To avoid potential contaminants from the body surface before DNA
111 extraction, individual paralarvae were washed with sterile distilled water, which was recovered and used
112 as a negative control (Suzuki et al. 2006). Paralarvae were then dissected and their digestive system was
113 removed and placed into DNA-free tubes. All dissections were performed in a UV-sterilized laminar flow
114 hood with flame-sterilized dissection tools to avoid contamination. Gut and content DNA was extracted
115 with a QIAamp DNA Micro Kit (QIAGEN), using RNA carrier in buffer AL. All steps followed
116 manufacturer’s instructions, with the exception of the 56°C digestion step which was done overnight and
117 the final elution step was done in two steps using 15 μ l buffer AE in each elution.

118 *Group specific primer design*

119 Group specific primers were designed by obtaining 16S rRNA sequences from GenBank (Benson et al.
120 2002) corresponding to 30 taxonomically diverse crustaceans, 3 fishes, 2 echinoderms and 2 cephalopods

121 (one of them *Octopus vulgaris*) which are known to be present in the NE Atlantic Ocean (Table 5,
122 supplementary material). These sequences were then aligned with MAFFT (Kato et al. 2002). The
123 software AMPLICON (Jarman 2004) was used to identify conserved regions within the target group of
124 potential prey species, but with nucleotide mismatches at the 3' end of the *O. vulgaris* forward primer
125 sequence to prevent its amplification (Deagle et al. 2007). Group specific primer specificity was tested by
126 PCR using a gradient between 49°C and 60°C on known template DNA from across the Crustacea (the
127 euphausiacid *Nyctiphanes couchii*, the crab *Necora puber*, the squat lobster *Galathea strigosa*, the hermit
128 crab *Anapagurus laevis*, the prawn *Palaemon longirostris*, the mysid *Leptomysis gracilis* and the copepod
129 *Calanus helgolandicus*), Chaetognata (*Sagitta elegans*) and *O. vulgaris*.

130 *Genetic database of planktonic organisms from the Ría de Vigo*

131 To ensure the correct identification of sequences obtained from the gut of *Octopus vulgaris* paralarvae,
132 mtDNA16S sequences were obtained from 25 species of crustaceans collected in the zooplankton
133 sampling done in the Ría de Vigo (Table 2). One individual of each species was visually identified,
134 washed with distilled water to remove surface contaminants and DNA was extracted with the QIAamp
135 DNA Micro Kit (QIAGEN), eluting the DNA in ultrapure water.

136 Due to difficulties amplifying crustacean 16S rRNA, PCR products were generated with different
137 combinations of the universal primers 16Sar-16Sbr (Simon et al. 1994) and the designed group specific
138 primers 16Scruf-16Scrur (Table 2). Copepod specific primers 16Sca and 16Scb (Braga et al. 1999) were
139 needed to amplify a region that is nested in the 16S rRNA universal fragment and encompasses the
140 sequence amplified with the designed group specific primers. Cycling conditions for the primers 16Sar-
141 16Scrur and 16Scruf-16Sbr, consisted of an initial denaturation at 94°C for 2 min followed by 39 cycles
142 of: denaturation at 94°C for 30 s, annealing at 57°C for 35 s, extension at 72°C for 40 s and a final step of
143 7 min at 72°C. Cycling conditions for copepod primers 16Sca-16Scb consisted of an initial denaturation
144 at 94°C for 2 min followed by 38 cycles of: denaturation at 94°C for 60 s, annealing at 50°C for 60 s,
145 extension at 72°C for 60 s and a final step of 7 min at 72°C.

146 All reactions were carried out in 25 µL, containing 10-100 ng of template 2.5 µL 10X PCR reaction
147 buffer, 0.5 µL dNTPs, 0.75 µL each primer and 0.025 U µL⁻¹ Taq polymerase (Roche). PCR
148 amplifications were carried out in a TGradient thermocycler (Biometra). Aerosol resistant pipette tips
149 were used to set up all PCR reactions. Negative controls, extraction controls and distilled water were
150 included for each set of PCR amplifications. An aliquot of 1.5 µL from each PCR reaction was quantified

151 using Nanodrop 2000 spectrophotometer (Thermo Scientific), then electrophoresed on 1.75% agarose gel,
152 stained with RedSafe™ (iNtRON biotechnology) and scanned in a GelDoc XR documentation system
153 (Bio-Rad Laboratories).

154 PCR products were purified with Exo-SAP (USB, Affymetrix) and sequencing reactions were carried out
155 with an automated DNA sequencer (Applied Biosystems 3130), using the BigDyeTerminator V3.1 Cycle
156 Sequencing Kit (Applied Biosystems) with forward primers. Chromatograms were examined using
157 BioEdit Sequence Alignment Editor version 7.0.9 (Ibis Biosciences). All sequences were assessed for
158 similarity using BLAST (Basic Local Alignment Search Tool) and were submitted to GenBank
159 (Accession numbers in Table 2)

160 *Identification of prey: semi nested PCR and cloning.*

161 Two sets of semi-nested PCR amplifications were performed independently on the extracted DNA from
162 the digestive tract of each *Octopus vulgaris* paralarvae (Fig. 1). In both sets, the first PCR was carried out
163 with the universal primer 16Sar plus a reverse group specific primer (16Scruf for crustaceans/fishes and
164 16Scb for copepods) to increase the copies of prey DNA. The second PCR was carried out using 1 µL of
165 the first PCR as a template, with forward and reverse group specific primers for crustaceans/fishes and
166 copepods to amplify only prey DNA.

167 Cycling conditions for the primers 16Scruf-16Scruf consisted of an initial denaturation at 94°C for 2 min
168 followed by 33 cycles of: denaturation at 94°C for 30 s, annealing at 57°C for 35 s, extension at 72°C for
169 40 s and a final step of 7 min at 72°C. Cycling conditions for primers 16Sar-16Scb and subsequent 16Sca-
170 16Scb as described above.

171 All reactions were carried out in 25 µL, containing 50 ng of template the first PCR and the semi nested
172 with 1 µL from the product of the first PCR 2.5 µL 10X PCR reaction buffer, 0.5 µL dNTPs, 0.3 µL
173 MgCl₂, 0.5 µL each primer and 0.05 U µL⁻¹ Taq polymerase (Roche).

174 Semi-nested PCR products from the digestive tract of the *Octopus vulgaris* paralarvae obtained with
175 group specific primers (16Scruf-16Scruf) and copepod-specific primers (16Sca-16Scb) were ligated to a
176 pCR 4-TOPO plasmid vector for 15 min at room temperature and cloned using TOPO TA Cloning kit
177 (Invitrogen) with One Shot TOP10 chemically competent cells following the manufacturer's protocol.
178 Plasmids were extracted from 10 colonies, when possible, with the Quick Plasmid Miniprep Kit
179 (Invitrogen). Insert size was checked by PCR with universal vector specific T7 and T3 primers and

180 visualised by gel electrophoresis. Sequencing was carried out on 200 ng of plasmid DNA using primer
181 T7.

182 Sequences recovered from clone libraries were edited and were considered to be part of the same
183 “operational taxonomic unit” (OTU) if there was less than 1% sequence divergence, allowing for intra-
184 specific variation and *Taq* polymerase errors (Braley et al. 2010). OTUs were compared to sequences
185 found in GenBank using the BLAST algorithm. A phylogenetic tree was constructed to assign unknown
186 sequences to the highest taxonomic level and to verify the OTU identifications. The tree contained all
187 OTUs obtained from *Octopus vulgaris* with primers 16Scruf-16Scrur, together with the five closest
188 matches of each OTU that were downloaded from GenBank. These sequences were aligned using
189 MAFFT v5.7 (Katoh et al. 2002) with default settings. A substitution model was selected under the
190 Akaike information criterion corrected for short sequences (AICc, Akaike 1974) as implemented in
191 jModeltest (Posada 2008). The HKY + γ (Hasegawa et al. 1985) model was chosen to infer the
192 evolutionary history by using the Maximum Likelihood (ML) method. The analysis involved 79
193 nucleotide sequences with a total of 164 positions in the final dataset. Bootstrap probabilities with 1000
194 replications were calculated to assess reliability on each node of the ML tree. Evolutionary analyses were
195 conducted in MEGA5 (Tamura et al. 2011). If sequence similarity displayed in the BLAST was <98%,
196 identification for the OTUs was restricted to the highest taxonomic lineage supported by bootstrap
197 probabilities higher than 70% in the consensus tree.

198 Trophic niche breadth was calculated using Czekanowski's Index (CI) with the formula:

$$199 \text{ CI} = 1 - 0.5 \sum_i |p_i - q_i|$$

200 where p_i is the proportion of resource item i out of all items eaten by the paralarvae, and q_i is the
201 proportion of item i in the zooplankton available to the paralarvae (Feinsinger et al. 1981). Values for CI
202 range from 1 for the broadest possible niche (a population uses resources in proportion to their
203 availability) to $[\min q_i]$ for the narrowest possible niche (a population is specialized exclusively on the
204 rarest resource).

205

206 **Results**

207 *Octopus vulgaris* paralarvae and morphological analysis of the digestive tracts

208 All specimens used for morphological and genetic analysis were early hatchlings of less than 10 days
209 according to the size (1.28-2.05 mm dorsal mantle length) and that each paralarva had 3 suckers per arm

210 (Villanueva 1995). Visual identification of the gut contents was inconclusive, because no solid remains
211 were found. Histological sections made to the digestive tract also revealed empty digestive tracts (Fig. 2a)
212 with the exception of two stomachs which were filled with liquefied material that was impossible to
213 identify (Fig. 2b).

214 *Group specific primers and genetic database*

215 PCR tests using the designed group specific primers yielded a target band of the expected fragment size in
216 all the crustaceans and chaetognat tested. However, copepods yielded only faint bands that did not
217 correspond to copepod DNA when sequenced, so we decided to use the copepod specific primers (Braga
218 et al. 1999) in conjunction with the designed group specific primers for dietary analysis and for
219 submissions to the genetic database. No PCR products were obtained at any annealing temperature when
220 *Octopus vulgaris* DNA was used as template. All sequences obtained from the zooplankton collected
221 from the Ría de Vigo were submitted to GenBank (Accession numbers in Table 2).

222 *Identification of preys in paralarvae by cloning*

223 All octopus digestive tracts yielded amplifiable DNA when PCR was performed with the designed group
224 specific primers 16Scruf-16Scrur. Although we intended to sequence 10 colonies per larvae, some
225 samples did not yield the minimum number of colonies (Table 3). Overall, a total of 122 clones were
226 sequenced, and 115 readable sequences were obtained. All sequences corresponded to prey species, with
227 114 clones corresponding to the semi-nested PCR band (16Scruf-16Scrur) and 1 clone corresponding to
228 the first PCR (16Sar-16Scruf) identified as *Trachurus trachurus* (OTU 19, Table 3).

229 Cloning of the amplicons obtained with copepod specific primers 16Sca-16Scb in *Octopus vulgaris* gut
230 contents resulted in 135 colonies, but all the sequences obtained from 125 readable clones corresponded
231 to *O. vulgaris* except one that amplified the DNA of *Anapagurus laevis* (OTU 13, Table 3).

232 Prey detected consisted of 20 different OTUs with between 1 and 5 different OTUs per paralarvae (Table
233 3). Eight OTUs were assigned to species with 78 clones displaying 100% similarity, and 1 clone
234 displaying 98% similarity to sequences from GenBank. Six OTUs showed similarities higher than 90%
235 (13 clones), three were assigned to genus (94-95%), two to a subfamily (Gobiinae, 93 and 92%) and the
236 last one to a family (Goneplacidae, 90%). The remaining four OTUs, corresponding to 22 clones,
237 displayed between 76-81% similarities and were assigned to the familial level on the basis of their
238 supported topographical position on the bootstrap consensus tree (Table 3, Fig. 3).

239 Summarizing, prey detected in *Octopus vulgaris* consisted mainly of crustaceans which accounted for
240 97.4% of the clones detected and the remaining 2.6% corresponded to fishes (Table 4). Three taxa
241 accounted for 95% of the clones; prawns (37.1%), crabs (37.1%) and krill (19.8%). When considering the
242 importance of these groups in the diet of *O. vulgaris*, it is remarkable that prawns and crabs are the most
243 common prey species, detected in 14 and 12 paralarvae out of 18 respectively (Table 4). In spite of the
244 high number of krill clones, these corresponded to only three paralarvae. The rest of the taxa were
245 detected in only three paralarvae, or in just one in the case of the Thalassinidae. According to the CI the
246 trophic niche breadth is low (0.13) indicating that *O. vulgaris* paralarvae are specialist predators. All
247 OTUs were submitted to GenBank, accession numbers in Table 3.

248

249 **Discussion**

250 This is the first time that prey items have been identified in *Octopus vulgaris* paralarvae collected in the
251 wild. This was approached by using two morphological techniques; visual analysis of the digestive tracts
252 and histological sections, as well as one molecular technique using group specific primers. Although the
253 combined approach of morphological and molecular methods has been documented as a more
254 comprehensive way to understand the diet of both vertebrates and invertebrates (Casper et al. 2007;
255 Deagle et al. 2007, 2010; Braley et al. 2010), only the molecular method succeeded identifying prey in *O.*
256 *vulgaris* paralarvae. The small size of the paralarvae, the limitation of the oesophagus diameter, the high
257 digestion rates, and the external digestion (Nixon 1985; Parra et al. 2000; Hernández-García et al. 2000),
258 made it impossible to carry out morphological analyses of prey in *O. vulgaris* paralarvae during their first
259 days of life in the pelagic realm.

260 The advantage of molecular methods is that when morphological methods were ineffective, i.e. digestive
261 tract is empty or filled with unidentifiable remains, prey cells with sufficient DNA to be detected by PCR
262 are able to be recovered (King et al. 2008). The main obstacle in employing molecular techniques in small
263 animals is distinguishing prey DNA among the overall volume of host DNA (Symondson 2002). To
264 overcome this obstacle we designed group specific primers within the 16S rRNA region for crustaceans
265 and fishes, which selectively avoided amplification of *Octopus vulgaris* DNA. Other studies previously
266 used this region of the 16S rRNA to design group specific primers for dietary purposes (Deagle et al.
267 2005, 2007, 2009; Braley et al. 2010). Braley et al. (2010) designed a reverse group specific primer for
268 crustaceans used in conjunction with the universal 16Sar, but only 11 of 184 PCR attempts produced

269 successful amplifications of krill and shrimp. In contrast, the group specific primers designed in this study
270 effectively amplified DNA, both alone and in conjunction with the universal 16Sar-16Sbr, from a wide
271 range of crustacean taxa: cladocerans, crabs, prawns, thalassinids, krill, hermit crabs, porcellanids,
272 carideans (Palaemonidae, Crangonidae and Alpheidae), mysids as well as fishes.

273 The unexpected failure to amplify copepod DNA is a potential consequence of using group specific
274 primers (Jarman et al. 2004; Deagle et al. 2005, 2007; Braley et al. 2010), which have been designed to
275 exclude from amplification *Octopus vulgaris* DNA. For this reason PCR had to be run with the copepod
276 specific primers 16Sca -16Scb (Braga et al. 1999) both in copepods and octopus paralarvae. These
277 primers effectively amplified copepod DNA for the genetic library (Table 2), however failed to amplify
278 copepod DNA from the digestive tract of *O. vulgaris* paralarvae. This suggests that early hatchlings of *O.*
279 *vulgaris* do not eat copepods, despite their presence as one of the main zooplankton taxa (table 4) and
280 being the most common prey in previous studies undertaken with other cephalopod paralarvae (Passarella
281 and Hopkins 1991; Vecchione 1991; Vidal and Haimovici 1998; Venter et al. 1999). Nonetheless, the
282 erratic movements and the extremely fast escape responses that copepods display (Yen and Fields 1992)
283 potentially pose a challenge for the early *O. vulgaris* hatchlings when compared with the predictable
284 swimming behaviour of crab and prawn zoeae or krill calyptopis. Indeed, Chen et al. (1996) found in
285 *Loligo opalescens* paralarvae that copepod capture is a skill acquired in an experience-dependent manner
286 during the post-hatchling stage.

287 In the current study, seven OTUs (29 clones) could not be identified to species or genus because no
288 similar sequences were present in GenBank. Phylogenetic relatedness was used to assign the unidentified
289 sequences to the highest taxonomic lineage based on the bootstrap values of the consensus tree nodes.
290 This reflects the difficulty when working with the diet of an expected generalist predator, due to the
291 limited sequence information available to target the large diversity of potential prey taxa (Blankenship
292 and Yayanos 2005; Suzuki et al. 2006, 2008). A prerequisite for resolving the diet of any predator living
293 in such a complex environment is the extensive characterization of the system (Sheppard et al. 2005; King
294 et al. 2008). In this work, five sequences that were submitted to GenBank from zooplankton species found
295 in the Ría de Vigo, were detected in the gut of the paralarvae, which highlights the importance of an
296 appropriate genetic database to obtain the highest level of identification and to reduce the uncertainty of
297 any species identification.

298 While previous work on cephalopod paralarvae diet found that paralarvae are generalist predators, prey
299 species detected in early hatchlings of *Octopus vulgaris* suggest that they are actually specialist predators
300 according to the CI obtained (0.13). Among the crustaceans, the group that primarily contribute to the
301 total abundance of zooplankton in the Ría de Vigo are krill, or Euphausiacea, which were only detected in
302 three paralarvae (Table 4). By contrast, all the paralarvae analysed ate some Decapoda, which include
303 Brachyura (crabs), Caridea (shrimps), Anomura (hermit crabs) and Thalassinidea (mud shrimps), despite
304 their much smaller contribution to the total abundance of zooplankton which was less than 4.26% (Table
305 4). In fact, the trophic selection is quite evident for carideans, which were the most abundant prey present
306 in 14 out of 18 *O. vulgaris* paralarvae, but whose contribution to the total zooplankton abundance was
307 only 0.28%.

308 The specialist trophic strategy during the first days in the pelagic ecosystem could be a consequence of a
309 lack of skills to capture fast moving and more abundant prey, as proved in paralarvae of *Loligo*
310 *opalescens* (Chen et al. 1996). As it occurs in the former species, an ontogenic switch into a generalist
311 predation strategy would be expected as the *Octopus vulgaris* paralarvae grow and gain experience, but
312 further research is needed to test this hypothesis. On the other hand, if paralarvae were truly specialists
313 throughout the planktonic phase, this might explain the high mortality of *O. vulgaris* hatchlings both
314 under culture and in the wild, due to prolonged starvation periods (Vecchione 1991).

315 In conclusion, up to 20 prey species have been detected in *Octopus vulgaris* paralarvae obtained from the
316 wild with a PCR-based method. This is the first successful attempt to unravel the complex trophic
317 interactions that occur in the pelagic ecosystem for *O. vulgaris* paralarvae. Based on the prey species
318 detected and their relative abundances in the zooplankton, *O. vulgaris* paralarvae can be considered
319 specialist predators during their first days of life in the pelagic ecosystem. Such knowledge can be critical
320 to solving the primary problems associated with the integral culture of this species, which is the low
321 survival of the paralarvae likely due to inadequacy of food supplied (Iglesias et al. 2007). Further effort
322 will progress in this direction to enhance the knowledge of this species during its planktonic phase.

323

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333

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- 451

452

453 **Fig.1** Diagram of the two semi nested PCR undertaken on each paralarvae, showing the prey targeted and
454 the primers used on each PCR

455

456 **Fig.2** Histological sections of *Octopus vulgaris* paralarvae stained with haematoxylin-eosin showing (a)
457 an empty stomach and (b) a stomach filled with undefined material (*) impossible to recognise.

458 Abbreviations, br: brain; di gl: digestive gland; oe: oesophagus; ra: radula; st: stomach; su: sucker. Scale
459 bars 100 nm.

460

461 **Fig.3** Maximum Likelihood tree for affiliating 18 operational taxonomic units (OTUs) obtained from the
462 digestive tract *Octopus vulgaris* paralarvae. OTUs obtained from the digestive tract are shown in bold.

463 Eukaryote rRNA sequences obtained by the BLAST searches are in italics with accession numbers. Only
464 bootstrap probabilities higher than 60 after 1000 replications are shown in the branches

465

Figure 1
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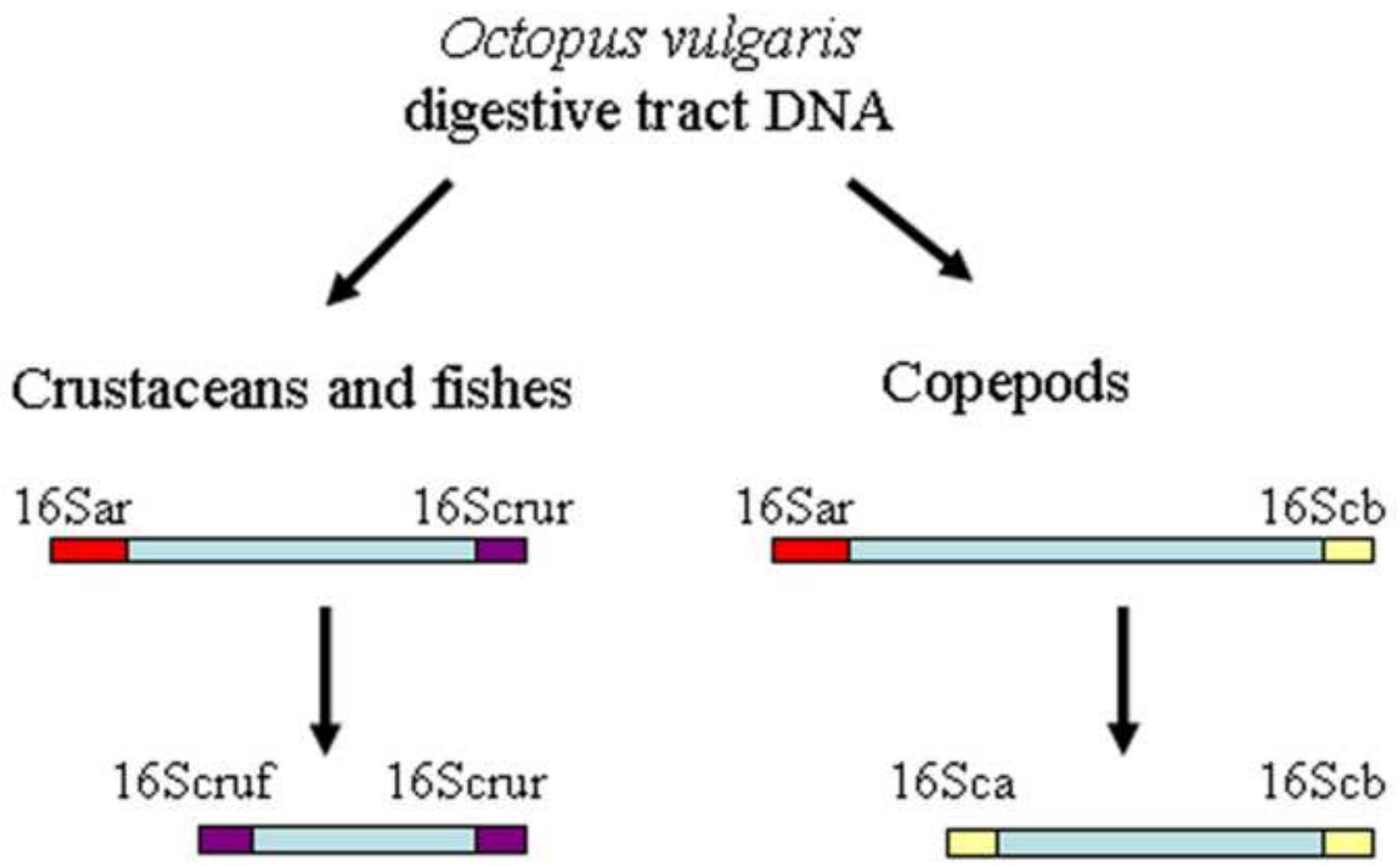


Figure 2
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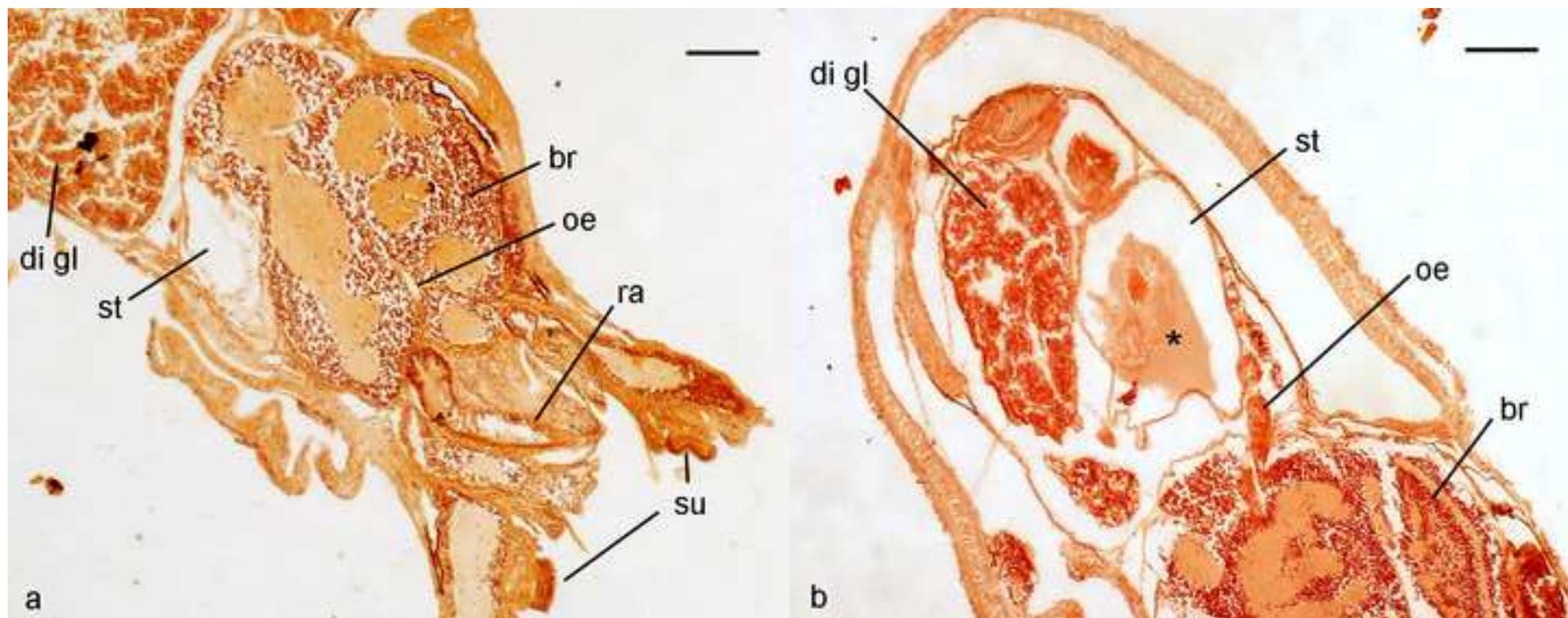


Figure 3
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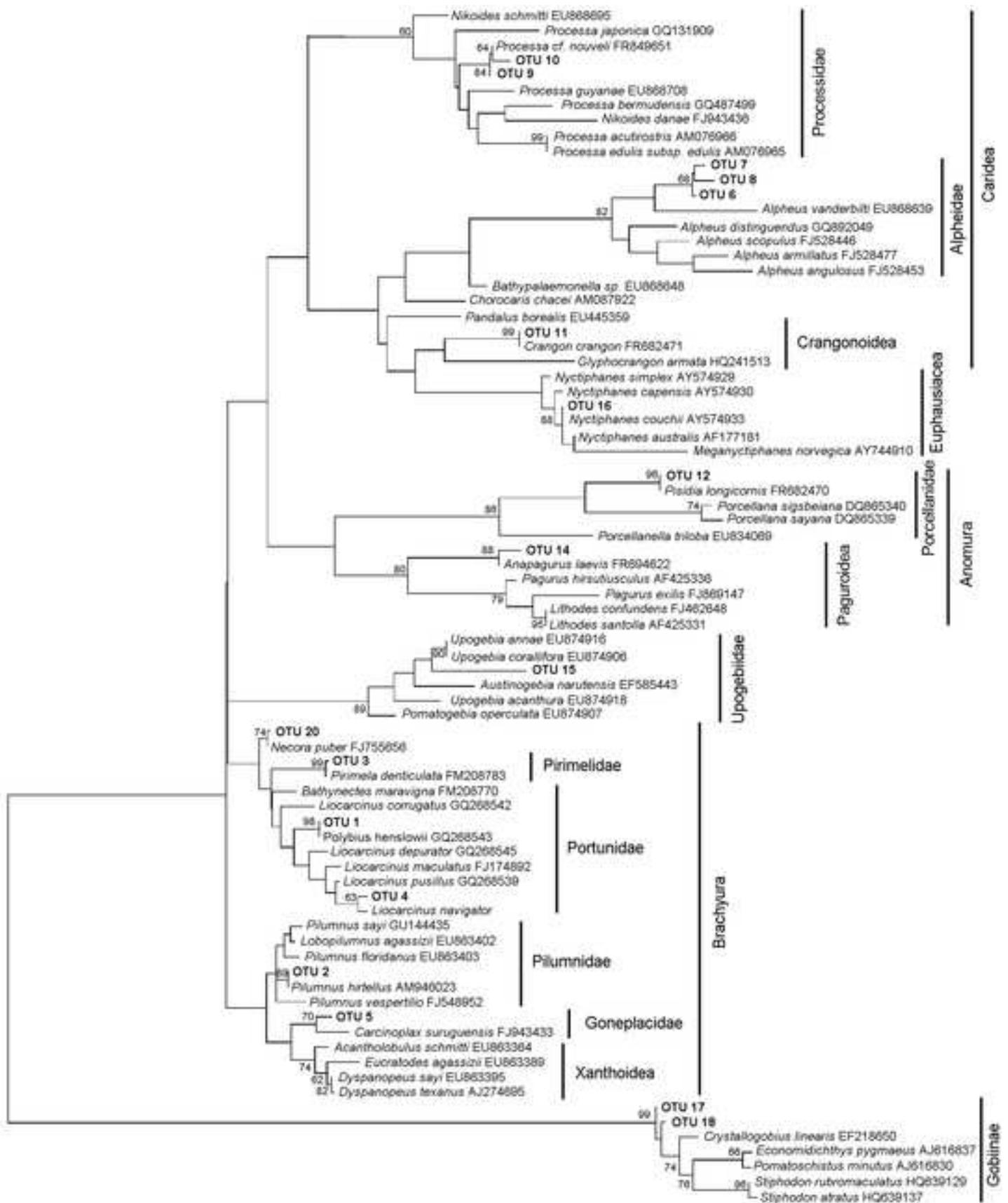


Table 1. Primers used in the current study showing the sequence of forward and reverse primers, the annealing temperature of each primer and the sizes of the amplified PCR products.

Target taxon	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing Temperature	Product size (bp)
Universal	16Sar CGCCTGTTTATCAAAAACAT	16Sbr CCGGTCTGAACTCAGATCACGT	50 °C	550-620
Eucarida	16Scruf GACGATAAGACCCTATAA	16Scrur CGCTGTTATCCCTAAAGTAA	57 °C	194-204
Copepod	16Sca TGTTAAGGTAGCATAGTAAT	16Scb ATTCAACATCGAGGTCACAA	50 °C	356-387

Table 2. List of species sequenced to create a 16S rRNA library of zooplankton present in the Ría de Vigo including GenBank Accession numbers, size of PCR amplicons in base pairs and PCR primers used to amplify each species.

Accession number	Species	Taxon	Length (bp)	Primer set	Homology (%)
FR851238	<i>Jaxea nocturna</i>	Thalassinidae	361	16Sar-16Scrur	99
FR851240	<i>Callianasa subterranea</i>	Thalassinidae	365	16Sar-16Scrur	99
FR851239	<i>Podon intermedius</i>	Cladocera	357	16Sar-16Scrur	99
FR682469	<i>Nyctiphanes couchii</i>	Euphausiacea	356	16Sar-16Scrur	99
FR849634	<i>Galathea strigosa</i>	Galatheidae	338	16Sar-16Scrur	
FR682470	<i>Pisidia longicornis</i>	Porcellanidae	380	16Sar-16Scrur	
FR849633	<i>Solenocera membranacea</i>	Penaeidae	367	16Sar-16Scrur	
FR682471	<i>Crangon crangon</i>	Crangonidae	371	16Sar-16Scrur	
FR694622	<i>Anapagurus laevis</i>	Paguridae	363	16Sar-16Scrur	
FR849637	<i>Cestopagurus timidus</i>	Paguridae	276	16Scruf-16Sbr	
FR849651	<i>Processa cf. nouveli</i>	Processidae	170	16scruf-16Scrur	
FR849636	<i>Leptomysis gracilis</i>	Mysidacea	198	16Scruf-16Sbr	
FR849648	<i>Calanus helgolandicus</i>	Copepoda	349	16Sca-16Scb	99
FR849642	<i>Calanoides carinatus</i>	Copepoda	346	16Sca-16Scb	
FR849638	<i>Mesocalanus tenuicornis</i>	Copepoda	341	16Sca-16Scb	
FR849639	<i>Paraeuchaeta hebes</i>	Copepoda	340	16Sca-16Scb	
FR849643	<i>Paracalanus parvus</i>	Copepoda	365	16Sca-16Scb	
FR849645	<i>Pseudocalanus elongatus</i>	Copepoda	275	16Sca-16Scb	
FR849646	<i>Metridia lucens</i>	Copepoda	372	16Sca-16Scb	99
FR849641	<i>Pleuromamma gracilis</i>	Copepoda	329	16Sca-16Scb	
FR849650	<i>Diaxis pygmaea</i>	Copepoda	206	16Sar-16Scb	
FR849649	<i>Acartia clausii</i>	Copepoda	323	16Sca-16Scb	96
FR849634	<i>Clausocalanus sp.</i>	Copepoda	284	16Sca-16Scb	
FR849640	<i>Oithona sp.</i>	Copepoda	397	16Sca-16Scb	
FR849647	<i>Candacia armata</i>	Copepoda	350	16Sca-16Scb	

Table 3. Prey DNA (OTUs 1-20) detected in the eighteen *Octopus vulgaris* paralarvae (Oc1 to Oc18) by cloning the PCR products obtained with group specific primers (16Scruf-16Scrur), including closest matches, their GenBank Accession numbers and percentages of similarity obtained from BLAST.

OTU*	Taxon	Species	Ac. number	Ac. (%)	Oc																	
					1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
OTU 1	Brachyura	<i>Polybius henslowii</i>	DQ388059	100				6					2	1	1							
OTU 2	Brachyura	<i>Pilumnus hirtellus</i>	AM946023	100					3		3		2								1	8
OTU 3	Brachyura	<i>Pirimela denticulata</i>	FM208783	100		3																
OTU 20	Brachyura	<i>Necora puber</i>	FJ755656	100																	4	
OTU 4	Brachyura	<i>Liocarcinus sp.</i>	GQ268541	95													4					
OTU 5	Brachyura	Goneplacidae	FJ943433	90	5																	
OTU 6	Caridea	Alpheidae 1	FJ528488	80		2						2					3	1				
OTU 7	Caridea	Alpheidae 2	DQ682879	79	1		3							1								
OTU 8	Caridea	Alpheidae 3	DQ682895	76					1			1	1				3	2				
OTU 9	Caridea	<i>Processa nouveli</i>	FR849651	100			1			1			1	3	1	3	9				1	
OTU 10	Caridea	<i>Processa sp.</i>	FR849651	94									1									
OTU 11	Caridea	<i>Crangon crangon</i>	FR682471	100					1													
OTU 12	Anomura	<i>Pisidia longicornis</i>	FR682470	98																	1	
OTU 13 ^a	Anomura	<i>Anapagurus laevis</i>	FR694622	98		1																
OTU 14	Anomura	<i>Anapagurus sp.</i>	FR684622	94										1								
OTU 15	Thalassinidea	Upogebiidae	EU874916	81			1															
OTU 16	Euphausiacea	<i>Nyctiphanes couchii</i>	AY574933	100											9	7				7		
OTU 17	Teleostei	Gobiinae	EF218650	93													1					
OTU 18	Teleostei	Gobiinae	EF218650	92																		1
OTU 19 ^b	Teleostei	<i>Trachurus trachurus</i>	AB096007	99																		1
		<i>Trachurus japonicus</i>	AP003092	99																		1

*Each Operational Taxonomic Unit (OTU) has been submitted to GenBank, accession numbers: FR849614-849632 and HE586322. ^a Obtained with primers 16Sca-16Scb. ^b Obtained with primers 16Sar-16Scrur

Table 4. Composition of the zooplankton community during the study expressed as the percentage of each taxon to the total abundance and the diet in *Octopus vulgaris* paralarvae by the number and percentage of clones corresponding to a given taxon and the number of paralarvae where those taxa were detected.

Phyla	Taxon	Wild Zooplankton Abundance (%)	Clones detected and percentage (%)	Number of paralarvae
Crustacea	Euphausiacea	27.8765	23 (19.8)	3
Echinodermata	Ofiuroidea	20.3526		
Crustacea	Copepoda	19.0708		
Chordata	Thaliacea	15.2601		
Crustacea	Cirripeda	3.9272		
Chaetognatha	Sagittidae	2.7184		
Crustacea	Cladocera	2.2304		
Crustacea	Anomura	2.1644	3 (2.6)	3
Crustacea	Brachyura	1.8174	43 (37.1)	12
Cnidaria	Cnidaria	1.5349		
Echinodermata	Equinoidea	1.2949		
Mollusca	Gastropoda	0.8575		
Crustacea	Caridea	0.2777	43 (37.1)	14
Chordata	Teleostei	0.2518	3 (2.6)	3
Crustacea	Misidacea	0.2352		
Crustacea	Amphipoda	0.0297		
Platemintha	Turbellaria	0.0215		
Annelida	Polychaeta	0.0203		
Mollusca	Bivalvia	0.0144		
Briozoa	Ciphonaute	0.0126		
Crustacea	Cumacea	0.0088		
Crustacea	Thalassinoidea	0.0084	1 (0.9)	1
Crustacea	Stomatopoda	0.0068		
Crustacea	Dendrobranchiata	0.0030		
Crustacea	Isopoda	0.0018		
Mollusca	Cephalopoda	0.0016		
Cephalochordata	Branchiostomidae	0.0009		
Crustacea	Ostracoda	0.0007		

Table 5

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