

1 **Stylet (Vestigial Shell) size in *Octopus vulgaris* Cephalopoda) hatchlings used to**  
2 **determine stylet nucleus in adults,**

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4 **Running head:** Stylet size in *Octopus vulgaris* hatchlings

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21 **ABSTRACT**

22 *The estimation of age and growth of cephalopod stocks is a key issue for their sustainable*  
23 *management. Recently, several studies have successfully validated the daily deposition of*  
24 *growth rings in the vestigial shell or stylets of several octopus species. Octopus vulgaris eggs*  
25 *were incubated at two different temperatures, 18°C and 22°C, until hatching to determine stylet*  
26 *size at hatching and assess the effect of temperature in the stylet dimensions. The 3 days-old*  
27 *hatchlings were sectioned transversally and 6 µm sections were stained to enhance the stylet*  
28 *position and visibility. The sections were observed under transmitted light microscopy at 1000x*  
29 *magnification, and the stylets identified as blue/green structures inside of the mantle – funnel*  
30 *retractor muscle. The transversal sections of the whole paralarvae allowed the diameter of the*  
31 *embryonic stylet of an octopus species to be measured for the first time. The mean stylet*  
32 *diameter in three-day old paralarvae is 3.99 µm independently of the thermal conditions.*  
33 *Moreover, significant differences in stylet size between captive and wild paralarvae were*  
34 *observed; the latter showed significantly larger stylets, an indication that they are over three-*  
35 *days old. Our results also evidence that the stylet nucleus is much smaller than previously*  
36 *thought based on measurements in stylets of juveniles and adults.*

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38 **Keywords:** *Octopus vulgaris*, hatchlings, stylets, age.

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## 44 INTRODUCTION

45 The assessment of growth and age provides important input data for many stocks  
46 assessment models and thus is very important for the sustainable management of fisheries  
47 stocks. In cephalopods, considering that the success of recruitment depends almost entirely on  
48 environmental conditions, it is quite important to understand how reproduction, life span and in  
49 particular growth, are affected by those conditions.

50 *Octopus vulgaris*, Cuvier 1797 is an important resource for the artisanal and industrial  
51 fisheries in all of the Atlantic margin of the Iberian peninsula, with annual average landings of  
52 9185 tons in Portugal (INE, 2013) and 4 000 in Galicia (Otero *et al.*, 2005). The life span of *O.*  
53 *vulgaris* was estimated in one to two years (Domain *et al.*, 2000; Katsanevakis & Verriopoulos,  
54 2006). After hatching, the paralarvae go through a short period of no net growth depending of  
55 the yolk reserves consumption to survive (Villanueva & Norman, 2008). Then the paralarvae  
56 grow exponentially until settlement reaching the sub-adult stage. Here, the logarithmic growth  
57 phase starts with a decreasing instantaneous growth rate until the maturation phase is complete  
58 (Mangold, 1983; Villanueva, 1995).

59 Direct ageing methods based on statolith increment analysis were not found to be useful  
60 in incirrate octopods, while approaches using beaks in *O. vulgaris* still need proper validation,  
61 in particular due to erosion by feeding (Perales-Raya *et al.*, 2010; Canali *et al.*, 2011). An  
62 alternative to perform direct age assessments is the use of the vestigial shell or stylet (Sousa  
63 Reis & Fernandes, 2002). Stylets are needle-shaped rods located on the dorso-lateral side of the  
64 mantle, that arose from the reduction of the shell in the Incirrata (Budelmann *et al.*, 1997;  
65 Naef, 1921/1923 in Bizikov, 2004). The growth of stylets progresses from the centre of growth  
66 (stylet primordium) located in the bend through the regular deposition of concentric layers of  
67 semi-transparent chitin (Bizikov, 2004) that can be used to assess age. Stylets have recently  
68 been used successfully to assess age in wild populations of some octopus species (e.g. *O.*

69 *pallidus*, Leporati *et al.*, 2008; *O. cyanea*, Herwig *et al.*, 2012). The fast degradation of the  
70 structure upon contact with air and the abrasive techniques used to expose the growth structures  
71 are major concerns to the standardization of the techniques and their regular implementation.  
72 Nevertheless, new preparation methods are being developed, which appear to produce good  
73 quality stylet sections (Barratt & Allcock, 2010) and consequently the age determination by  
74 stylet increment analysis (SIA) is potentially an effective tool for the age determination in *O.*  
75 *vulgaris*, as was first advanced by Sousa-Reis & Fernandes (2002). It is also worth noting that  
76 the daily deposition of growth increments in the stylets of adults of this species was validated by  
77 Hermosilla *et al.* (2010). However, the validation of the daily deposition of growth increments  
78 in adults do not validate the same periodicity in the increments deposition in earlier life stages  
79 and neither identifies the deposition of first growth increment in paralarvae, essential criteria  
80 for a rigorous age validation of the SIA in each species (Campana, 2001). The difficulties and  
81 potential inaccuracies associated with determining the age of merobenthic octopuses (such as *O.*  
82 *vulgaris*) using SIA and the importance of validating age at first increment formation are  
83 discussed in Doubleday *et al.* (2011).

84 The present study aimed firstly to develop a technique to rapidly locate the stylets in the  
85 muscle of paralarvae, and secondly to determine the stylet size at hatching in newly hatched *O.*  
86 *vulgaris* paralarvae as a tool to define the starting point for age determination in stylets of later  
87 stages. Additionally, the stylets of three-day old paralarvae were compared to unknown age  
88 paralarvae captured in the wild to determine if the stylet nuclear area is conservative between  
89 paralarvae of different sizes and ages and between animals incubated at different temperatures.

## 90 MATERIAL AND METHODS

91 The captivity paralarvae used in this study were collected opportunistically from  
92 experiments on ocean warming effects on *O. vulgaris* earlier life stages, conducted at Guia  
93 Marine Laboratory (more details about the rearing conditions are described in Repolho *et al.*,

94 2014). These paralarvae hatched from eggs clutches collected in the beginning of the  
95 embryogenesis (Stage I: Naef, 1965) from traps of local fisherman between October 2010 and  
96 November 2011 in Cascais, Portugal. After collection, eggs were transferred to the aquaculture  
97 systems in Guia Marine Laboratory, Cascais. Here, the eggs were reared at different water  
98 temperatures including 18°C and 22°C until hatch 39 to 25 days respectively, after eggs  
99 incubation. After hatch, the paralarvae were kept at the same temperatures for three days  
100 without food and then 10 paralarvae from each temperature were sacrificed for this study. The 3  
101 days-old paralarvae were chosen to ensure some growth past the hatch check and the  
102 observation of increments if already formed. All paralarvae were preserved in 70% ethanol.

103         The paralarvae were measured under transmitted light binocular microscopy at 30 x  
104 magnification. Measurements were taken as follows: total length (TL in mm), mantle ventral  
105 length (ML in mm), eye diameter (D-eye in mm) and total weight (W in mg). Before weighing,  
106 the paralarvae were dried with filter paper.

107         A set of six paralarvae was used to establish the most adequate protocol that could  
108 simultaneously permit locate and examine several cross-sections of the paralarval stylet. These  
109 were embedded in paraffin and sectioned (in 6 µm width sections) according to three  
110 morphological planes: the sagittal, transversal, and frontal planes. Sections were stained with  
111 acetic alcian blue solution (n = 3) and Masson's trichrome (n = 3) in order to enhance the  
112 fibrous nature of the stylets, by staining fibrin tissue in a solution of acetic alcian blue (adapted  
113 from Vecchione, 1991) or light green/blue (Jones, 2002), respectively. It was expected that the  
114 staining would improve the identification of the structures inside the mantle. Stained sections  
115 were observed under a binocular microscope equipped with transmitted light, at 400 x and 1000  
116 x magnification. All sections were sequentially photographed. Taking into account the results  
117 of the experiment above, the two groups of 3 days-old paralarvae (18°C group and 22°C group)  
118 were subsequently sectioned in the transversal plane in 6 µm sections and stained with the  
119 Masson Trichrome method. All sections were observed under transmitted light at a

120 magnification of 400 x and 1000 x and photographed.

121 | The selected transversal sections of the stylet (the best transversal section closer to the  
122 stylet bend), were used to identify the embryonic primordium or nucleus of the stylet. The  
123 nucleus was limited by a discontinuity in the ageing structure which appeared as a high-contrast  
124 micro-increment with a deeply darker zone under transmitted light, or an abrupt change in the  
125 micro-structural growth pattern (Panfili *et al.*, 2002). Stylet measurements were taken under  
126 1000x magnification from the selected cross-section of the stylet, as follows: stylet diameter  
127 (SD in  $\mu\text{m}$ ), stylet area (SA in  $\mu\text{m}^2$ ), stylet major radius (SRmax in  $\mu\text{m}$ ) and nucleus diameter  
128 (SDnucleus in  $\mu\text{m}$ ).

129 | Additionally, wild paralarvae (n=9) of unknown age were collected in July and  
130 September 2010 in the Ría de Vigo (Southwest Galicia, Spain) during mesozooplankton  
131 surveys. These paralarvae were collected in depth and surface strata using a multitrawl  
132 (MultiNet<sup>®</sup>) sampler (0.71 × 0.71 m opening frame, see Roura (2013) for details). Local Sea  
133 Surface Temperature recorders indicate that these paralarvae mean surface temperatures  
134 between 16.5 °C and 19.2 °C during embryologic development (data source: Seawatch buoy  
135 located off Cape Silleiro , 42° 7.80 N, 9° 23.40 W, [www.puertos.es](http://www.puertos.es)). The wild paralarvae were  
136 stored in 70 % ethanol and measured similarly to the captive paralarvae. These were then  
137 transversally sectioned accordingly and stained with Hemotoxylin & Eosin. Selected cross-  
138 sections were measured following the same procedure defined for the 3 days-old paralarvae.

139 | To assess the effect of temperature on paralarva and stylet sizes, measurements data  
140 were grouped according to the incubation temperature and sampling source, as “18°C” and  
141 “22°C” groups for the 3 days-old paralarvae and “wild” group for the paralarvae collected  
142 during the mesozooplankton surveys. Prior to the statistical analysis, the assumptions of sample  
143 normality and homogeneity for paralarvae and stylet dimensions were assessed by group with  
144 Shapiro-Wilk’s and Bartlett’s tests, respectively. A non-parametric Kruskal-Wallis test was

145 used to identify differences in mean measurements between groups. The Spearman correlation  
146 index was used to identify cases of colinearity between the measurements, as well as to identify  
147 strong correlations between the size of the paralarva and measurements of the respective stylet.

148 Additionally, the Kruskal-Wallis test was used to compare the mean SD of 3days-old  
149 with the mean diameter of the nucleus identified in stylet cross-sections of *O. vulgaris* juveniles  
150 (n=13) captured in the Portuguese northeast coast. The sampling design and methodology  
151 applied to prepare and measure juvenile stylets cross-section are described in Lourenço, 2014).

## 152 RESULTS

153 As in adults, the stylets of *O. vulgaris* paralarvae were located in the insertion of the  
154 funnel retractor muscles, in the posterior region of the mantle. In relation to adults, these  
155 structures were situated more dorsally and mid region of the mantle (Figure 1A). Having in  
156 mind that some degree of body shrinkage can occur due to the preservation method (up to 20%  
157 with ethanol 70% accordingly with Goto, 2005), in the paralarva, the stylet bend (where the  
158 primordium of the structure is located) was found to lie between 100  $\mu\text{m}$  and 200  $\mu\text{m}$  from the  
159 tip of the mantle in paralarvae measuring between 0.57 mm and 3 mm of dorsal mantle length.

160 The use of Masson trichrome as a stain clearly improved the ability to locate the stylet  
161 inside the mantle – funnel retractor muscle insertion in comparison with alcian blue. Using this  
162 stain the stylet appeared in most paralarvae sections as green/blue contrasting with the  
163 surrounding tissue (Figure 1B). The transversal sectioning plane gave best results to obtain good  
164 cross sections of the stylet near the bend where it was possible to locate the stylet primordium.  
165 This transversal plane allowed firstly to identify the stylet at the bend level in the mantle-funnel  
166 retractor muscle insertion and then to identify the best cross-section where it was possible to  
167 detect the hatch check in the stylet and to measure the diameter, perimeter, area and major  
168 radius of the stylet (Figure 2). The stylet is anterior-posteriorly oriented in the mantle with the  
169 anterior branch (or rostrum) inserted deep inside the mantle muscle, the bend was located inside

170 the mantle – funnel retractor muscle insertion, and the post-rostral branch positioned more  
171 superficially along the interior wall of the mantle (Figure 2).

172 In the 3 days-old paralarvae, the mean diameter of the stylet (measured between the most  
173 distant points) was  $3.99 \pm 0.46 \mu\text{m}$  and the mean area measured was  $13.00 \pm 6.13 \mu\text{m}^2$ . In those  
174 stylets, the nucleus was only identifiable in the cross-sections near the bend. It was identified as  
175 a distinctively darker area circumscribed by one highly-contrasted micro-increment (with a  
176 deeply darker zone), and within which first order growth rings are not observed. The mean  
177 diameter of the nucleus was  $2.71 \pm 0.42 \mu\text{m}$ .

178 The nuclear area previously defined in the captive paralarvae was easily identified in the  
179 nine stylets of wild paralarvae by its micro-structure. In the wild paralarvae group, the diameter  
180 of the stylet measured  $5.88 \pm 0.95 \mu\text{m}$  and the area measured  $27.54 \pm 8.62 \mu\text{m}^2$ . The diameter of  
181 the stylet nucleus measured  $3.02 \pm 0.55 \mu\text{m}$ . And, it was only possible to identify the deposition  
182 of one growth increment in the post-nuclear area (Figure 1C) of the stylets of two wild  
183 paralarvae.

184 Table 1 shows the mean values obtained for each of the paralarvae and stylet dimensions  
185 studied by group. The results show that there is no statistical difference between the 18°C group  
186 and the 22°C group when comparing both stylet and paralarvae dimensions, although paralarvae  
187 from 22°C group presented larger sizes and also bigger stylets. On the other hand, the wild  
188 paralarvae are larger and weight more than the 18°C group with the stylet being also bigger in  
189 the wild paralarvae, with exception to the stylet nucleus diameter that did not show between a  
190 18°C, 22°C and wild group (Table 1).

191 The stylet area (SA) and SD (collinear with SA) correlates positively with the SRmax  
192 (SA vs SRmax:  $r_s = 0.63$ , p-value < 0.001). SDnucleus do not correlates with neither of the other  
193 stylet dimensions (SDnucleus vs SA,  $r_s = 0.13$ , p-value > 0.05; SDnucleus vs SRmax,  $r_s = 0.22$ ,  
194 p-value > 0.05). The Spearman index determined for the correlation between the paralarvae



195 dimensions and the stylet size show that SA (colinear with SD) and SRmax correlate positively  
196 with the D\_eye and with W (SA vs D\_eye:  $r_s = 0.60$ , p-value = 0.001; SA vs W:  $r_s = 0.55$ , p-  
197 value = 0.002; SRmax vs D\_eye:  $r_s = 0.60$ , p-value = 0.001; SRmax vs W:  $r_s = 0.58$ , p-value =  
198 0.001), while the Srnucleus did not show any significant correlation with none of the paralarvae  
199 dimensions.

200           The mean SD determined in the 3 days-old paralarvae is statistically identical to the  
201 diameter of the nucleus identified and measured in the juveniles stylet cross sections ( $k = 235$ ,  
202 p-value > 0.05).

203

## 204 DISCUSSION

205           To our knowledge, this is the first time that the stylet was identified in pelagic paralarvae  
206 of a merobenthic octopus, proving its formation in an earlier embryonic stage. In the adults of  
207 *O. vulgaris*, the stylet is a recognizable structure in the dorso-anterior region of the mantle,  
208 easily extracted by dissection. However, in newly hatched individuals, the body size and the  
209 fragile structure of non-mineralized chitin of the stylet make it particularly difficult to collect  
210 the stylets by dissection. Several methods to isolate and collect the stylet from the body of the  
211 larvae were tried, including staining the paralarva body with an acetic alcian blue solution, in an  
212 adaptation of the method used by Vecchione (1991) to identify stomach contents in squids.  
213 According to that author, the alcian blue efficiently stains eye crystalline lenses and  
214 funnel/mantle-locking cartilages in squid paralarvae. We observed that, although the alcian blue  
215 successfully stained the eye lenses of *O. vulgaris* paralarvae, the staining achieved for the  
216 stylets was not effective and resulted in unclear structures.

217           To overcome this difficulty and considering the fragile nature of newly-hatched  
218 paralarvae with the beaks and radula still under-developed, we chose to adopt a histological  
219 approach to obtain and observe cross-sections of the stylets. Nevertheless, other challenges arise

220 with this approach. The stylets of paralarvae are, as in adults, needle-shaped rods with an  
221 irregular shape, presenting a middle bended region with concave and convex arms in insertion  
222 area of the mantle-funnel retractor muscles. Both sagittal and transversal cutting planes result in  
223 good cross sections of the stylet, but only the transversal plane allowed a greater number of  
224 sections in the vicinity of the primordium. Additionally, this sectioning plane allowed the  
225 definition of a methodology to identify the bend and the closest cross-section in which it is  
226 possible to identify the nucleus and to measure the structure in a replicable manner.

227         The nucleus (primordium) is visible in the nearest cross-section to the stylet bend, with a  
228 mean diameter of 2.71  $\mu\text{m}$  independently of the developmental temperature, indicating that the  
229 stylet primordium size is and independent of both biological and environmental factors,  
230 suggesting that the nuclear region (corresponding to stylet size at hatching) can be used as a  
231 reference point to determine age and growth and related measurements.

232         Under a magnification of 1000x, the stylet does not have visible growth rings in the  
233 majority of the sections. Here the size limitation factor must be considered and in only two  
234 stylets of the wild paralarvae group post-nuclear growth increments were visible. Although  
235 stylets smooth core regions seem to be particularly common in holobenthic octopus as *O.*  
236 *pallidus* (Doubleday *et al.*, 2006) and other merobenthic octopus as *Macroctopus maorum*  
237 (Doubleday *et al.*, 2011) one should hypothesize that the absence of visible growth increments  
238 near the nucleus may reflect an inadequate resolution power of light microscopy to resolve  
239 distances of less than 1  $\mu\text{m}$  (Campana, 1992; Doubleday *et al.*, 2011) rather than an actual  
240 feature of the structure. The use of scanning electronic microscopy associated with crio-  
241 sectioning of the paralarvae could be useful tools to improve the analysis of the stylet.         In  
242 *O. vulgaris* a merobenthic species, both stylet diameter and nuclear region of paralarvae are  
243 considerably smaller than in *O. pallidus*, a holobenthic species and particularly identical to  
244 stylet sizes and characteristics described by Doubleday *et al.* (2011) for *Macroctopus maorum*, a  
245 merobenthic octopus living in the temperate and the subantarctic waters in Australian coastal

246 waters. In comparison with *O. pallidus*, the *O. vulgaris* paralarvae are small and pelagic until  
247 settlement 30 to 60 days after hatching (Villanueva, 1995; Villanueva & Norman, 2008), while  
248 *O. pallidus* paralarvae are larger in relation to adult's size and already benthic at hatch. This  
249 results in two orders of magnitude difference in weight (2 mg weight for *O. vulgaris* hatchlings  
250 and 0.10 g to 0.54 g for *O. Pallidus*, Semmens *et al.* 2011) at hatching and fully accounts for  
251 size differences between stylet diameter and nuclear area. Such differences illustrate the  
252 importance of investigating and validating growth structures and check marks in the stylets of  
253 each species.

254           We were not able to determine the age of the nine paralarvae captured in the Cies  
255 Islands. Considering the temperature conditions recorded, we can hypothesise that they  
256 developed under a temperature close to the 18°C group. Comparing both groups, the wild  
257 paralarvae were in all cases larger in size, weight and eye diameter than the ones hatched in  
258 captivity, indicating that they may be over 3-days old (Villanueva, 1995), and even though the  
259 nucleus has the same diameter for both groups, the larger stylet area in the wild paralarvae  
260 indicates that some material have been deposited in the stylet while they grow.

261           The diameter of the stylet in the 3 days-old paralarvae is close to 5 µm. Comparing our  
262 observations between *O. vulgaris* paralarva and juvenile stylet cross-sections it is possible to  
263 observe correspondences of the nuclear area among the two life stages (Figure 3). In fact, the  
264 absence of statistical differences between the SD of 3 days-old paralarvae stylets with the  
265 diameter of the nucleus (mean nuclear diameter  $5.80 \pm 2.21$  µm, see Lourenço, 2014) identified  
266 in the juveniles cross-sections, give us security to use the stylet diameter in post-hatch  
267 paralarvae nuclear area to validate the limit of the nucleus in the juvenile stylet cross sections as  
268 the first post-hatch increment. Nevertheless, more studies on the stylet structure are needed to  
269 understand how the structure grows in both girth and length at this pre-settlement stage.

270

271 ACKNOWLEDGMENTS

272 The first author is grateful to José António Durán for teaching and support with histological  
273 methodologies. The authors want to express their acknowledgements to the two anonymous  
274 referees which comments largely improve this manuscript.

275 FINANCIAL SUPPORT

276 This study was supported by the projects CAIBEX (CTM2007-66108-C02-01) and LARECO  
277 (CTM2011-25929). The MultiNet<sup>®</sup> sampler used to collect wild paralarvae was funded by  
278 FEDER funds. The Portuguese Foundation for Science and Technology (FCT) supported this  
279 study through a PhD grant to Sílvia Lourenço (grant number SFRH/BD/44182/2008) and the  
280 project grant PTDC/BIA-BEC/103266/2008 to Rui Rosa.

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282 REFERENCES

- 283 **Barratt, I. M and Allcock, A.L.** (2010) Ageing octopods from stylets: development of a  
284 technique for permanent preparations. *ICES Journal of Marine Science* 67, 1452-1457.
- 285 **Bizikov, V. A.** (2004) The Shell in Vampyropoda (Cephalopoda): Morphology, functional role  
286 and evolution. *Ruthenica*, suplement 3. 87 pp.
- 287 **Budelmann, B. U., Schipp, R. and Boletzky, v S.** (1997) Cephalopoda. In: Harrison, F. W.,  
288 Kohn A. J. (Ed) *Microscopic Anatomy of Invertebrates*. Vol 6A: Mollusca II. 119 - 414.
- 289 **Campana, S. E.** (1992) Measurement and Interpretation of the Microstructure of Fish Otoliths.  
290 In: Stevenson, D., and Campana, S.E. (Eds). *Otolith Microstructure Examination and*  
291 *Analysis. Canadian Special Publication of Fisheries and Aquatic Sciences* 117, 59 - 72.
- 292 **Campana, S. E.** (2001) Accuracy, precision and quality control in age determination, including

293 a review of the use and abuse of age validation methods. *Journal of Fish Biology* 59:  
294 197–242.

295 **Canali, E., Ponte, G., Belcari, P., Rocha, F. and Fiorito, G.** (2011) Evaluating age in *Octopus*  
296 *vulgaris*: estimation, validation and seasonal differences. *Marine Ecology Progress*  
297 *Series* 441, 141–149.

298 **Domain, F., Jouffre, D. and Caverivière, A.** (2000) Growth of *Octopus vulgaris* from tagging  
299 in Senegalese waters. *Journal of Marine Biological Association UK* 80, 699–705.

300 **Doubleday, Z., Semmens, J.M., Pecl, G. and Jackson, G.** (2006) Assessing the validity of  
301 stylets as ageing tools in *Octopus pallidus*. *Journal of Experimental Marine Biology*  
302 *and Ecology* 338, 35–42.

303 **Doubleday, Z. A., White, J., Pecl, G. T. and Semmens, J. M.** (2011) Age determination in  
304 merobenthic octopuses using stylet increment analysis: assessing future challenges  
305 using *Macroctopus maorum* as a model. *ICES Journal of Marine Science* 68: 2059–  
306 2063.

307 **Goto, T.** (2005) Examination of different preservative for *Todarodes pacificus* paralarvae fixed  
308 with borax-buffered formalin-seawater solution. Phuket Marine Biological Center  
309 Research Bulletin 6, 213-219.

310 **Hermosilla, C. A., Rocha, F., Fiorito, G., González, A.F. and Guerra, A.** (2010) Age  
311 validation in common octopus *Octopus vulgaris* using stylet increment analysis. *ICES*  
312 *Journal of Marine Science* 67, 1458-1463.

313 **Herwig, J.N., Depczynski, M., Roberts, J.D., Semmens, J.M., Gagliano, M. & Heyward, A.**  
314 **J.** (2012) Using age-based life history data to investigate the life cycle and vulnerability  
315 of *Octopus cyanea* *PLoS ONE* 7(8):e43679.

- 316 **INE:** (2013). Statistics Portugal. [www.ine.pt](http://www.ine.pt) (accessed in 31st August 2013)
- 317 **Jones, L.** ( 2002) Connective tissues and stains. In: Bancroft, J. D. and Gamble, M. (eds)  
318 Theory and practice of histological techniques. pp. 139–162.
- 319 **Katsanevakis, S. and Verriopoulos, G.** (2006) Seasonal population dynamics of *Octopus*  
320 *vulgaris* in the eastern Mediterranean. *ICES Journal of Marine Science* 63, 151–160.
- 321 **Leporati, S. C., Semmens, J. M. and Pecl, G.** (2008) Determining the age and growth of wild  
322 octopus using stylet increment analysis. *Marine Ecology Progress Series* 367, 213–222.
- 323 **Lourenço, S.** (2014) *Ecology of the common octopus Octopus vulgaris (Cuvier, 1797) in the*  
324 *Atlantic Iberian Coast: life cycle strategies under different oceanographic regimes.*  
325 PhD Thesis, Faculdade de Ciencias da Universidade de Lisboa. Lisboa. Portugal.
- 326 **Mangold, K.** (1983) *Octopus vulgaris*. In: Boyle, P. (ed) Cephalopod Life cycle: Species  
327 accounts. Vol 1. Academic press, pp. 335-363.
- 328 **Otero, J., Rocha, F., González, A.F., Garcia, J. and Guerra, A.** (2005) Modeling artisanal  
329 coastal fisheries of Galicia (NW Spain) based on data obtained from fishers: the case of  
330 *Octopus vulgaris*. *Scientia Marina* 69, 577–585.
- 331 **Panfili, J., de Pontual, H., Toradec, H. and Wright, P. J.** (2002). Manual of fish  
332 sclerochronology. Brest, France: IFREMER - IRD.
- 333 **Perales-Raya, C., Bartolomé, A., García-Santamaría, M.T., Pascual-Alayón, P. and**  
334 **Almansa, E.** ( 2010) Age estimation obtained from analysis of octopus (*Octopus*  
335 *vulgaris* Cuvier, 1797) beaks: Improvements and comparisons. *Fisheries Research* **106**:  
336 171–176.
- 337 **Repolho, T., Baptista, M., Pimentel, M. S., Dionisio, G., Trübenbach, K., Lopes, V. M.,**

338 **Lopes, A.R., Calado, R., Diniz, M. and Rosa, R.** (2014). Developmental and  
339 physiological challenges of octopus (*Octopus vulgaris*) early life stages under ocean  
340 warming. *Journal of Comparative Physiology B* 184, 55-64.

341 **Roura, A.** (2013) *Ecology of planktonic cephalopod paralarvae in coastal upwelling systems.*  
342 PhD Thesis, Universidade de Vigo, Vigo, Spain.

343 **Semmens, J., Doubleday, Z., Hoyle, K. and Pecl, G.** (2011) A multilevel approach to  
344 examining cephalopod growth using *Octopus pallidus* as a model. *The Journal of*  
345 *Experimental Biology* 214, 2799 - 2807.

346 **Sousa Reis C. and Fernandes, R.** (2002) Growth observations on *Octopus vulgaris* Cuvier,  
347 1797 from the Portuguese waters: Growth lines in the vestigial shell as possible tools  
348 for age determination. *Bulletin of Marine Science* 71, 1099-1103.

349 **Vecchione, M.** (1991) A method for examining the structure and contents of digestive tract in  
350 paralarval squids. *Bulletin of Marine. Science* 49, 300–308.

351 **Villanueva, R.** (1995) Experimental rearing and growth of planktonic *Octopus vulgaris* from  
352 hatching to settlement. *Canadian Journal of Fisheries and Aquatic Science* 52, 2639–  
353 2650.

354 **Villanueva, R. and Norman, M. D.** (2008) Biology of the planktonic stages of benthic  
355 octopuses. *Oceanography and Marine Biology - An Annual Review* 46, 105–202.

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361 **Figures legends:**

362 Figure 1 – Transversal section (A) of an *Octopus vulgaris* paralarva (magnification: 40x). The  
363 stylets are well inserted in the antero-dorsal region of the mantle. Detail of a cross-section of an  
364 *Octopus vulgaris* stylet (B and C, magnification: 400x) obtained through a transversal section of  
365 the paralarva. am – adductor muscle; dgl – digestive gland; dmc – dorsal mantle cavity;; mn –  
366 mantle; rfm – funnel retractor muscle; sto – stomach; sty – stylet; vmc – ventral mantle cavity  
367 (after Bizikov, 2004).

368 Figure 2 – Sequence of transversal sections (magnification: 400x) of a one day-old *Octopus*  
369 *vulgaris* paralarva indicating the sequential position of the stylet in the insertion between the  
370 mantle and retractor funnel muscle. Scale bar indicates 20  $\mu\text{m}$ . drm – dermis; dgl – digestive  
371 gland; gl – gills; mc – mantle cavity; rfm – retractor funnel muscle; sty – stylets (after Bizikov,  
372 2004).

373 Figure 3 – *Octopus vulgaris* juvenile and adult stylet cross-sections showing the central area  
374 corresponding in size to the stylet diameter in 3 days-old paralarvae (magnification 630x). SD –  
375 diameter of the stylet at hatching; A – stylet cross-section of a juvenile weighing 384 g (SD =  
376 3.5  $\mu\text{m}$ ); B – stylet cross-section of a juvenile weighing 700 g (SD = 3.39  $\mu\text{m}$ ).

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384 Table 1 – *Octopus vulgaris* paralarvae and stylets mean ( $\pm$  Standard deviation)  
 385 dimensions by group. Different superscripts indicate statistically significant differences  
 386 between 18°C group and 22°C group and between 18°C and Wild group tested by  
 387 Kruskal-Wallis test with significance level of p-value < 0.05.

Paralarva				
Group	Mantle length	Total length	Eye diameter	Weight
18°C	0.96 $\pm$ 0.15 <sup>a</sup>	1.9 $\pm$ 0.07 <sup>a</sup>	0.33 $\pm$ 0.03 <sup>a</sup>	1.05 $\pm$ 0.05 <sup>a</sup>
22°C	1.09 $\pm$ 0.10 <sup>a</sup>	1.95 $\pm$ 0.07 <sup>a</sup>	0.32 $\pm$ 0.03 <sup>a</sup>	1.13 $\pm$ 0.10 <sup>a</sup>
Wild	1.61 $\pm$ 0.19 <sup>b</sup>	2.41 $\pm$ 0.30 <sup>b</sup>	0.44 $\pm$ 0.05 <sup>b</sup>	2.45 $\pm$ 0.30 <sup>b</sup>

  

Stylet				
Group	Stylet diameter	Stylet Area	Stylet major radius	Stylet nucleus diameter
18°C	3.91 $\pm$ 1.19 <sup>a</sup>	12.88 $\pm$ 7.56 <sup>a</sup>	2.28 $\pm$ 0.84 <sup>a</sup>	2.52 $\pm$ 0.48 <sup>a</sup>
22°C	4.06 $\pm$ 0.76 <sup>a</sup>	13.11 $\pm$ 4.94 <sup>a</sup>	2.43 $\pm$ 0.64 <sup>a</sup>	2.82 $\pm$ 0.92 <sup>a</sup>
Wild	5.88 $\pm$ 0.95 <sup>b</sup>	27.54 $\pm$ 8.62 <sup>b</sup>	3.39 $\pm$ 0.72 <sup>b</sup>	3.02 $\pm$ 0.55 <sup>a</sup>

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