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

Running head: Stylet size in *Octopus vulgaris* hatchlings

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ABSTRACT

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

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

INTRODUCTION

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

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

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

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

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

MATERIAL AND METHODS

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

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

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

A set of six paralarvae was used to establish the most adequate protocol that could simultaneously permit locate and examine several cross-sections of the paralarval stylet. These were embedded in paraffin and sectioned (in 6 µm width sections) according to three morphological planes: the sagittal, transversal, and frontal planes. Sections were stained with acetic alcian blue solution (n = 3) and Masson's trichrome (n = 3) in order to enhance the fibrous nature of the stylets, by staining fibrin tissue in a solution of acetic alcian blue (adapted from Vecchione, 1991) or light green/blue (Jones, 2002), respectively. It was expected that the staining would improve the identification of the structures inside the mantle. Stained sections were observed under a binocular microscope equipped with transmitted light, at 400 x and 1000 x magnification. All sections where sequentially photographed. Taking into account the results of the experiment above, the two groups of 3 days-old paralarvae (18ºC group and 22ºC group) were subsequently sectioned in the transversal plane in 6 µm sections and stained with the 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 µm), stylet area (SA in μm^2), stylet major radius (SRmax in µm) and nucleus diameter
128 (SDnucleus in µm).

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®) 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 ºC and 19.2 ºC during embryologic development (data source: Seawatch buoy
135 located off Cape Silleiro , 42º 7.80 N, 9º 23.40 W, 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 "18ºC" and
141 "22ºC" 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

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

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

RESULTS

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

The use of Masson trichrome as a stain clearly improved the ability to locate the stylet inside the mantle – funnel retractor muscle insertion in comparison with alcian blue. Using this stain the stylet appeared in most paralarvae sections as green/blue contrasting with the surrounding tissue (Figure 1B). The transversal sectioning plane gave best results to obtain good cross sections of the stylet near the bend were it was possible to locate the stylet primordium. This transversal plane allowed firstly to identify the stylet at the bend level in the mantle-funnel retractor muscle insertion and then to identify the best cross-section where it was possible to detect the hatch check in the stylet and to measure the diameter, perimeter, area and major radius of the stylet (Figure 2). The stylet is anterior-posteriorly oriented in the mantle with the 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 ± 0.46 μm and the mean area measured was 13.00 ± 6.13 μm^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 ± 0.42 μm.

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 ± 0.95 μm and the area measured 27.54 ± 8.62 μm^2. The diameter of
181 the stylet nucleus measured 3.02 ± 0.55 μm. 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 18ºC group
186 and the 22ºC group when comparing both stylet and paralarvae dimensions, although paralarvae
187 from 22ºC group presented larger sizes and also bigger stylets. On the other hand, the wild
188 paralarvae are larger and weight more than the 18ºC 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 18ºC, 22ºC 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 µm 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 µm (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 subantartic 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 18ºC 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 ± 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

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281

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Figures legends:

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

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

Figure 3 – *Octopus vulgaris* juvenile and adult stylet cross-sections showing the central area corresponding in size to the stylet diameter in 3 days-old paralarvae (magnification 630x). SD – diameter of the stylet at hatching; A – stylet cross-section of a juvenile weighing 384 g (SD = 3.5 µm); B – stylet cross-section of a juvenile weighing 700 g (SD = 3.39 µm).

384 Table 1 – *Octopus vulgaris* paralarvae and stylets mean (± Standard deviation)
385 dimensions by group. Different superscripts indicate statistically significant differences
386 between 18ºC group and 22ºC group and between 18ºC 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
18ºC	0.96 ± 0.15^{a}	1.9 ± 0.07^{a}	0.33 ± 0.03^{a}	1.05 ± 0.05^{a}
22ºC	1.09 ± 0.10^{a}	1.95 ± 0.07^{a}	0.32 ± 0.03^{a}	1.13 ± 0.10^{a}
Wild	1.61 ± 0.19^{b}	2.41 ± 0.30^{b}	0.44 ± 0.05^{b}	2.45 ± 0.30^{b}

Stylet

Group	Stylet diameter	Stylet Area	Stylet major radius	Stylet nucleus diameter
18ºC	3.91 ± 1.19^{a}	12.88 ± 7.56^{a}	2.28 ± 0.84^{a}	2.52 ± 0.48^{a}
22ºC	4.06 ± 0.76^{a}	13.11 ± 4.94^{a}	2.43 ± 0.64^{a}	2.82 ± 0.92^{a}
Wild	5.88 ± 0.95^{b}	27.54 ± 8.62^{b}	3.39 ± 0.72^{b}	3.02 ± 0.55^{a}

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