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1 **The embryonic phase and its implication in the hatchling size and**  
2 **condition of Atlantic bobtail squid *Sepiola atlantica***

3

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5

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14

15 **Abstract** Early life stages of cephalopods are somewhat complex due to the life  
16 history strategy or species specificity of generalized ontogenetic patterns and  
17 processes. This work aimed to determine the time length of embryonic development  
18 at different temperatures, and if the egg size is a determinant of hatchling size in  
19 *Sepiola atlantica* d'Orbigny, 1839-1842. Successful hatching occurred in 98.5 to 100 %  
20 of the eggs for each female. As seen in other coleoid cephalopods, temperature  
21 determines the amount of time for embryonic development in *S. atlantica*, and the

22 obtained data was very similar to other coleoid cephalopods. Developmental times  
23 for temperatures at  $13 \pm 0.4^\circ\text{C}$ ,  $18 \pm 0.3^\circ\text{C}$  and  $16.4 \pm 1.1^\circ\text{C}$  were  $61.8 \pm 3.8$ ,  $22.6 \pm 1.7$   
24 and  $40.1 \pm 4.8$  days. The duration of embryonic development and hatchling mantle  
25 length was not strictly related. The egg volume was positively related to hatchling  
26 mantle length. Our results provide new records on the duration of embryogenesis  
27 and other information on reproductive patterns in this species. Some hatching and  
28 post-hatching behaviour are shown and discussed.

29

30 **Key words** Cephalopoda, *Sepiolo atlantica*, reproduction, development, hatchling

31

## 32 **Introduction**

33

34 **Development and reproductive** trait analysis provide examples for most adaptive  
35 relationships and responses to intrinsic and extrinsic forces of selection acting  
36 throughout the life cycle (Boletzky 1993; Boletzky 2003a). In natural populations,  
37 offspring size and egg size are classical life history traits which identify effects on  
38 hatchling size and competitive performance (Boletzky 1997; Boletzky 2003b; Marshall  
39 and Keough 2007). **However, most** data is from empirical studies provided from  
40 laboratory observations, since field studies are lacking due to the logistical  
41 difficulties of experimentally manipulating population densities in natural settings  
42 (Svensson and Sinervo 2000).

43           In marine invertebrates, a change in hatchling size can be obtained by the  
44 adoption of intracapsular feeding on stored nutritive material or by the production of  
45 larger eggs (Boletzky 1997). Larger hatchlings are supposed to reduce susceptibility  
46 to predation (Steer et al. 2003) and starvation due to enhanced swimming ability and  
47 predatory competence in comparison to smaller hatchlings. This trade-off has been  
48 identified in different animal taxa, such as lizards (Svensson and Sinervo 2000), many  
49 marine invertebrates (Marshall and Keough 2007 a review) and cephalopods  
50 (Laptikhovsky and Nigmatullin 1993; Rodrigues et al. in press; Steer et al. 2003; 2004;  
51 see also Boletzky 2003b and Villanueva and Norman 2008 for reviews). Moreover,  
52 differences in egg size and hatchling size seem to reflect ecosystem fluctuations,  
53 mainly temperature and food availability, such as those found during different  
54 spawning groups in the squid *Illex argentine*s (Laptikhovsky and Nigmatullin 1993).

55           In cephalopods, the duration of embryonic development is dependent not  
56 only on species but on temperature, as documented for several species (Boletzky  
57 2003a; Villanueva and Norman 2008). In contrast to many other marine invertebrates  
58 (references in Marshall and Keough 2007), there is very little information on how  
59 maternal conditions affect the hatchling condition in cephalopods (Boletzky 2003b;  
60 Rodrigues et al. in press; Steer et al. 2004). Therefore, it is beneficial to investigate  
61 whether egg size is a determinant of hatchling size in *S. atlantica* and if temperature  
62 has an effect on this trait.

63           During embryonic development, prevailing environmental conditions may  
64 affect the physiological state of the developing larva (Giménez and Torres 2002),  
65 particularly in cephalopod paralarvae where the inner yolk sac provides energy for

66 **metabolism in learning activities such as predation** (Boletzky 1974; 1993). Several  
67 authors have suggested that the transition from yolk utilization to active predation  
68 represents a critical period in the early life history of cephalopods (Boletzky 2003a;  
69 Vidal et al. 2002; Villanueva and Norman 2008). In paralarvae of chokka squid, *Loligo*  
70 *reynaudii*, rates of inner yolk utilization **may** be influenced by environmental  
71 temperature (Martins et al. 2010).

72 As observed in other sepiolids, *Sepiola atlantica* d'Orbigny, 1839-1842, transfers  
73 spermatophores to the female, who stores them in the bursa copulatrix, a large pouch  
74 lying on the visceral mass that allows the spermatangia to become attached  
75 (Rodrigues et al. 2009). This species is an intermittent terminal spawner (see Rocha et  
76 al. 2001) and **this** spawning strategy comprises group-synchronous ovary  
77 maturation, multiple **egg laying** and deposition of egg clutches in different locations.  
78 Furthermore, **the egg size** shows a positive correlation with maternal size, and **the**  
79 **female** does not take parental care of its progeny (Rodrigues et al. in press).

80 The **goals of this study were to** evaluate the influence of temperature on  
81 development in *S. atlantica* and to determine if hatchling size is dependent on the  
82 duration of development, **along with** providing new information on post-hatching  
83 behaviour and condition.

84

## 85 **Material and Methods**

86

87 A total of twelve mated adult females were obtained during dedicated dives in the  
88 Ría of Vigo (NW Iberian Peninsula) (42° 14' N; 8° 47' W). The field collection and

89 laboratory study were carried out between January and September 2009. Live  
90 specimens were immediately with care to the Marine Station of the University of  
91 Vigo in Toralla Island (ECIMAT-UVIGO). Collection depth was between 3 to 6 m in  
92 sandy substrate with no algae present.

93 Each individual was placed in a 20.2 l (30 cm long x 27 cm wide x 25 cm deep)  
94 glass tank with an open system of running seawater ( $16.4 \pm 1.1^\circ\text{C}$ ). The bottom of the  
95 tanks was covered with 0.5-1 cm of fine sand from the type locality. The system  
96 received a natural photoperiod according to the season, roughly: spring 12:12 LD,  
97 summer 15:9 LD, autumn 12:12 LD, winter 9:15 LD.

98 Sepiolid squids were fed daily *ad libitum* with freshly collected adult mysid  
99 shrimps, *Siriella armata* and *Leptomysis mediterranea*. Females were always kept in  
100 isolation in the tanks and were never exposed to males in captivity, but all of them  
101 produced eggs that initiated development, thus indicating that spermatangia were  
102 present in the females.

103 Twelve spawns in aquaria were obtained (753 eggs) from the captive females.  
104 Eggs were laid during the night or early morning, and were removed upon  
105 observation. Each clutch was divided into three groups exposed to different  
106 temperatures:  $13 \pm 0.4^\circ\text{C}$  (N= 247),  $18 \pm 0.3^\circ\text{C}$  (N= 251) and natural variation (NV=  
107 255)  $16.4 \pm 1.1^\circ\text{C}$ . Eggs from each female were maintained in small nylex mesh bags  
108 (0.5 mm mesh) where the spawning date, egg volume and “mother” were recorded.  
109 Bagged eggs were checked daily in routine controls at 8:00 - 9:00 a.m. and p.m.,  
110 allowing us to record roughly from each clutch the emergence of the first hatchling,

111 the diel hatching time and **rate** of successful hatchings for each female at the different  
112 temperatures.

113 After hatching, following Messenger (1985), a total of 721 specimens were  
114 anesthetized using a 1/1 ASW-MgCl<sub>2</sub> (7.14 %) solution for one minute in order to  
115 **reduce** their mobility. Dorsal mantle length (ML) and total length (TL= from  
116 posterior tip of the mantle to tip of the **longest** arm) were measured to 0.01 mm  
117 accuracy and weighed (BW) to 0.001 g immediately after being anesthetized. The  
118 same measurements were made **on** each female immediately after death. Once  
119 measured, hatchlings were maintained in the same conditions as the adults and  
120 juveniles of the mysid shrimps **mentioned above** were offered.

121 All measurements were made using a stereomicroscope (Nikon SMZ-1500).  
122 The image was calibrated on a video monitor and measurements were taken directly  
123 from the screen utilizing an image analyzer (Nikon, NIS-Elements).

124 Egg volume (EV) was approximated using the formula for the volume of a  
125 prolate ellipsoid,  $(4/3) \pi LW^2$  (within 0.01 mm<sup>3</sup>) (see Rodrigues et al. in press), where  
126  $L$ = egg length (mm) and  $W$ = egg width (mm). All statistical analyses were performed  
127 using SPSS software (SPSS statistics 17.0).

128 **In addition, a number of hatchlings (Fig. 1) were fixed in Bouin's fixative and**  
129 **preserved in 70 % EtOH (25 replicas in total).** These specimens were dehydrated in a  
130 graded series of ethanol, cleared with toluene and embedded in paraffin. Cross **and**  
131 **frontal** sections of 7 μm were mounted on slides and stained with haematoxylin and  
132 eosin.

133

## 134 Results

135

136 Egg laying began between 67 and 104 days after females were collected. Of the  
137 twelve females, five deposited four egg clutches. The number of laid eggs per clutch  
138 was highly variable ranging between 2 and 86 eggs, with an apparent decrease in the  
139 number of eggs laid with time. The individual egg volume varied between 47.81 and  
140 182.45 mm<sup>3</sup> ( $53.29 \pm 27.12$  mm<sup>3</sup>; mean  $\pm$  standard deviation).

141 The embryonic development in terms of time duration was strictly dependent  
142 on temperature (Fig. 2). The slowest rate in developmental time occurred with  
143 samples incubated at 13 °C (N= 247),  $61.8 \pm 3.8$  days. Eggs at  $16.4 \pm 1.1$  °C (N= 251)  
144 hatched after  $40.1 \pm 4.8$  days. At 18 °C (N= 251) hatching occurred after  $22.6 \pm 1.7$   
145 days. Successful hatching was recorded in 98.5 to 100 % of all the eggs laid.

146 Hatchling's ML varied from 1.1 to 1.7 mm ( $1.5 \pm 0.3$  mm), and TL ranged  
147 between 2.6 and 3.6 mm ( $3.2 \pm 0.5$  mm). BW was 0.077 up to 0.098 g ( $0.081 \pm 0.02$  g).  
148 There was no relationship between hatchling length and weight ( $r^2= 0.36$ ,  $n= 721$ ,  
149  $p>0.05$ ).

150 Hatchling ML was positively related to EV ( $r^2= 0.69$ ,  $n= 721$ ,  $P<0.001$ ), which is  
151 represented by the following linear equation:  $ML=0.904+0.007EV$  (Fig. 3). However,  
152 hatchling ML was not affected by temperature, nor corresponding developmental  
153 time duration ( $F= 1.34$ ,  $n= 721$ ,  $p>0.05$ ).

154

155 Hatching and post-hatching behaviour



156

157 Of the 721 successful hatchings obtained, 96.3 % took place during the night or early  
158 morning, and the rest during daylight or early evening. Hatching was achieved with  
159 vigorous mantle contractions. To leave the egg case the hatchling possibly utilized  
160 the terminal spine as auxiliary equipment along with the hatching gland (see  
161 Boletzky 1991).

162 Newly hatched individuals showed expanded chromatophores of dark brown  
163 colour on a yellowish background along the whole body, and they often assumed a  
164 “flamboyant” arm display (as shown in Mauris 1989, figure 2- PB1). This posture  
165 consisted of the following arm patterns: i) the dorsal and laterodorsal arms were  
166 stretched upwards perpendicularly to the body axis; ii) the lateroventral and ventral  
167 arms together were stretched downward on either side; and iii) the tips of all the  
168 arms were rolled in.

169 Hatchlings were generally less active during daylight hours, and juveniles  
170 remained at the bottom of the tank attempting to bury themselves in the sand.  
171 However, after a few hours (4 - 6 hours) all the animals tried to bury in the substrate  
172 (sand grain size between 0.25 - 0.125 mm), but with varying levels of success. These  
173 attempts consisted in contractions and expansions of the mantle while the animal  
174 was sitting on the substrate (first phase, cf. Boletzky and Boletzky 1970). Subsequent  
175 attempts consisted of the juveniles gathering sand particles (second phase) with out-  
176 stretched laterodorsal arms, with varying success. A complete burying sequence (see  
177 Rodrigues et al. 2010) was observed after four days.

178           Although hatchlings possess yolk reserves (inner yolk sac) (Figure 4), they are  
179   poised to hunt mysid shrimp of the equal size one hour post-hatching. This initial  
180   innate predatory response was not always successful but gradually improved.  
181   Hatchlings showed expanded dark brown chromatophores when directing attention  
182   to the prey. Afterwards, they moved quickly toward the prey in order to attain  
183   appropriate attacking distance (Messenger 1968). Prior to attack, hatchlings  
184   contracted all chromatophores and consequently turned virtually transparent. Arms  
185   were spread to form a circular crown, to enable the tentacles to be rapidly shot out  
186   (generally to the dorsal side in the middle of the mysid shrimp). If the attack was  
187   successful, the tentacles were quickly retracted with the prey. When the tentacles  
188   were retracted (with or without prey), the animals resumed a dark brown colour.

189           Although most hatchlings had eaten mysid shrimp within the first few days  
190   post hatch, 97 % died between 3 to 6 days after hatching. One individual survived to  
191   24 days old.

192           Histological analyses demonstrated that yolk was present in all hatchlings  
193   between 0-6 days old (N= 14). From 7 to 9 days old only 2 of 6 replicas (N=6) showed  
194   traces of yolk. The hatchlings 10 and 11 days old (N=4) had no traces of yolk.  
195   Nevertheless, the single juvenile that reached 24 days old showed traces of yolk (Fig.  
196   4).

197

198   **Discussion**

199

200 Spawning behaviour observed in this study was similar to those shown in previous  
201 studies (Rodrigues et al. in press). As in others coleoid cephalopods, *S. atlantica*  
202 showed an inverse relationship between temperature and developmental duration  
203 (Boletzky 2003a). The duration of development was similar to that observed by  
204 Boletzky (1983) in *Sepiolo robusta*. However, the mean time was 50 % lower than in  
205 the sepiolidae *Euprymna tasmanica* reared at the same temperature (18 °C, Steer et al.  
206 2004). Hatching lasted for 23 days in *S. atlantica* at a mean temperature of 14.4 °C in  
207 northern Welsh waters (Jones and Richardson, 2010), which is comparable to our  
208 results at 18 °C.

209 Our comparison between the three tested temperatures did not show apparent  
210 differences in hatching success (98.5 - 100 %). Since high hatching rates were  
211 obtained at all temperatures examined (with no malformations), our results suggest  
212 that *S. atlantica* is adapted to living in waters ranging between 13.0-18.0 °C. On the  
213 other hand, Steer et al. (2004) observed in *E. tasmanica* that hatching success is  
214 positively correlated with offspring size.

215 We do not have direct evidence relating to how long the female can store  
216 sperm in the *bursa copulatrix*. This is mainly because we do not know when the  
217 females in our experiments mated in the wild. However, we have evidence that  
218 female *S. atlantica* can store viable sperm for at least 104 days, which was the  
219 maximum time fertilized eggs were spawned in our experiment.

220 This study revealed a positive effect of egg size on hatchling size in captive *S.*  
221 *atlantica*. This result is supported by the positive trade-off between maternal fitness,  
222 which determines egg size and trade-offs previously identified by Rodrigues et al. (in

223 press) in the same laboratory conditions. Such **features are normally** found in direct  
224 marine developers which have no parental care (Marshall and Keough 2007).  
225 **However, as observed in the brighteye darters, *Etheostoma lynceum*, egg quality and**  
226 **size varies in successive spawning events (Heins et al. 2004).** Thus, the basis of the  
227 trade-off between female fitness and hatchling size could be influenced by other  
228 factors (such as temperature, food availability and salinity) in the field (Boletzky  
229 1997; **Heins et al. 2004**; Laptikhovsky and Nigmatullin 1993; Marshall and Keough  
230 2007; Steer et al. 2004; Svensson and Sinervo 2000).

231 *S. atlantica* hatchling ML was similar to that observed in other sepiolid  
232 **hatchling** species (Boletzky 1983; Jones and Richardson, **2010**; Nabhitabhata et al.  
233 2005), which ranged between 1.1 and 2.5 mm ML, with the exception of *Rossia*  
234 *macrossoma* where hatching ML was around 5 mm (Boletzky 1973).

235

236 Hatching and post-hatching behaviour

237

238 **Apparently in most cephalopod species, a decrease in natural light triggers hatching,**  
239 **but why this occurs in one particular is due at the physiological state that is attained**  
240 **by the animal** (Boletzky 1974). Summers (1985) observed laboratory hatching in  
241 *Rossia pacifica* that showed the **same diel hatching cycle as *S. atlantica*.** This type of  
242 **“hatching strategy”** is a form of minimizing vulnerability to predators. **We have now**  
243 **additional evidence which suggests that two new traits occur during the hatching**  
244 **strategy. This includes the presence of a dark brown pattern, and the flamboyant arm**  
245 **display.** The flamboyant posture especially allows the hatchling to convey a

246 confounding picture to a potential predator. This posture is perhaps not only related  
247 to prey capture (Mauris 1989). Both traits may improve camouflage, and may  
248 constitute part of the primary defence strategy of the juvenile sepiolid squid (Hanlon  
249 and Messenger 1996).

250 This study provides additional observations for the behavioural fitness of *S.*  
251 *atlantica* hatchlings without previous experience in burying and hunting. Juvenile  
252 cephalopods have a remarkable repertoire of actions that correspond to some “innate  
253 responses” similar to adult behaviour (Boletzky 1974; Villanueva and Norman, 2008).  
254 Although very vulnerable to predation, sepiolid hatchlings mainly behave in a  
255 defensive way by burying into the sand and by showing body patterns and displays  
256 adapted for concealment in the given environment (Boletzky and Boletzky 1973;  
257 Boletzky 1974). Although kept in a tank without sand for weeks, occasional  
258 observations demonstrated that sepiolid hatchlings of several *Sepiolo* and *Sepietta*  
259 species (Boletzky 1983; 2003a; Boletzky and Boletzky 1973) bury when sand is present  
260 using similar techniques observed in our study.

261 This study demonstrates that *S. atlantica* possesses the innate predatory attack  
262 response one hour post-hatching while still possessing yolk reserves. This result is in  
263 disagreement with that observed in the same species by Jones and Richardson (2010),  
264 who found that individuals began hunting at six days old. In contrast, other studies  
265 have shown that newly hatched cuttlefish (*Sepia officinalis*) began their predatory  
266 attacks at 24-48 hours post hatching (Wells 1958), with the precise sequence of motor  
267 actions observed in mature adults (Messenger 1968).

268 High mortality was still observed, despite traces of yolk in juvenile *S. atlantica*  
269 (Fig. 4). This suggests the existence of a critical period (cf. Vidal et al. 2002) in the  
270 early life history in *S. atlantica*. Based on juvenile mortality, Jones and Richardson  
271 (2010) suggested that the first 40 days are critical in the life cycle of the species,  
272 although in their study no inner yolk was observed after 20 days old. The  
273 phenomenon of critical period was also observed in some squid species reared in  
274 laboratory up to date (eg. *Loligo opalescens*, Vidal et al. 2002). However some extrinsic  
275 factors can be affecting in this aspect, like food availability, substrate type and light  
276 cycle (Hanlon et al. 1997; Vidal et al. 2002).

277 Post-hatch conditions were similar for all hatchlings, despite being raised at  
278 different developmental temperatures. This may have changed the physiological  
279 consumption of the yolk (Giménez and Torres 2002). Unfortunately, we had no  
280 individual temperature information and, consequently, we cannot prove this. Yolk  
281 consumption was studied in crabs (*Chasmagnathus granulata*), with salinity as the  
282 variable factor and although salinity stress resulting in an increase in egg size  
283 (Giménez and Anger 2001) it was demonstrated that changes in salinity may have  
284 negative consequences in the growth rates at early juvenile stages (Giménez and  
285 Torres 2002). Future projects would need to address the variability in yolk  
286 consumption in *S. atlantica* hatchlings under various environmental conditions.

287 Although this is one of the first ecological trade-off investigations in the family  
288 Sepiolidae, our data and the previously reported correlation in maternal fitness and  
289 egg size (Rodrigues et al. in press), support the idea that maternal fitness is a  
290 determinant of hatchling size in *S. atlantica*. Moreover, the current results indicate

291 future areas of research in sepiolid squids, which may be fruitful towards  
292 understanding the innate behavioural response and the costs of metabolism during  
293 the transition from yolk utilization to active prey ingestion.

294

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391 **Figure Caption**

392 **Fig. 1** Scheme of the fixed post-hatching *Sepiolo atlantica* (N= 25) to make histological  
393 sections used in this study.

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410 **Figure Caption**

411 **Fig. 2** *S. atlantica*. Schematic representation of embryonic developmental duration at  
412 different temperatures. Line A represents the embryonic development at  $18 \pm 0.3$  °C;  
413 line B at  $16.4 \pm 1.1$  °C; line C at  $13 \pm 0.4$  °C.

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429 **Figure Caption**

430 **Fig. 3** *S. atlantica*. Relationship between egg volume (mm<sup>3</sup>) and hatchling mantle  
431 dorsal length (mm) fitted by a linearized calibration function, which explained 69 %  
432 (R<sup>2</sup>) of the associated variance.

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446 **Figure Caption**

447 **Fig. 4** Histological sections of *Sepiola atlantica*. **a** Cross section of a hatchling at 24  
448 days age on 10x magnification showing the rest of inner yolk sac; note the lateral lobe  
449 (ll) as two round structures embedded in the digestive gland (dg) and the transversal  
450 structure lying between them, on top of the ink sac (is), which is the middle part of  
451 the anterior lobe (al); **b** The same section at 60x magnification detailing the lateral  
452 lobe embedded admedially in the diverticula of the digestive gland, the middle part  
453 of anterior lobe is visible; **c** Frontal (horizontal) section of a hatchling 3 hours old at  
454 4x magnification. The dotted line indicates where the sections showed in figures **a**  
455 and **b** were made. *Scale bars:* 50  $\mu\text{m}$  (a), 100  $\mu\text{m}$  (b), 0.5 mm (c) sg = stellate ganglion;  
456 fp = fin pouch.

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