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HISTOMORPHOLOGICAL STUDY OF OVARIAN ATRESIA OVER THE
REPRODUCTIVE CYCLE OF *OCTOPUS VULGARIS* FROM GALICIAN
WATERS (NW SPAIN)

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Abstract

Atresia has been poorly examined in cephalopods. We here provide a histological description of this process along the whole ovary development for *Octopus vulgaris*. Additionally, we related its occurrence to morphometric parameters, and its seasonal cycle was further analysed. Atresia occurred all year round in immature and mature females and in previtellogenic and vitellogenic oocytes. However, more mature females were more prone of being atretic. This occurred mainly in spring when females had atretic previtellogenic oocytes in mature macrostages. By contrast, vitellogenic atresia occurred mainly from spawning to post-spawning females. Furthermore, two types of phagocytic cells were identified as responsible for the reabsorption during atresia. The phagocytic follicle cells only occurred in yolk-bearing oocytes; and within the two haemocyte populations only the smaller ones seemed to be involved in engulfing atretic oocytes. Additionally, advanced atresia in post-spawning females showed yellow-brown bodies as a possible result of follicle cell apoptosis and highlighting the end of the reproductive cycle. Given the pattern of atresia, the reproductive strategy of this species

26 is based on an asynchronic ovary development and a synchronous ovulation during
27 spawning. We further suggest that potential fecundity for this species should be
28 measured on late vitellogenic oocytes in pre-spawning females.

29 INTRODUCTION

30 Ovarian atresia is a degenerative and resorptive process whereby oocytes and
31 post-ovulatory follicles are reabsorbed from the ovary. It regulates egg production
32 reducing potential fecundity and allowing females to recover part of the energy invested
33 in the formation of oocytes (Guraya, 1986). The study of this phenomenon allows (i) the
34 estimation of crucial reproductive traits such as fecundity and spawning biomass
35 (Ganias et al., 2003); and (ii) the assessment of the physiological condition due to
36 external factors. Thus, since ovarian atresia affects fertility rates, its determination
37 allows to differentiate between potential and total fecundity and to identify at what stage
38 of sexual maturity fecundity is reduced (Boyle & Chevis, 1992). In the case of
39 iteroparous species such as fishes, defining atretic stages and the subsequent assignment
40 of females to different spawning status (e.g. active, inactive/immature) are of great
41 importance for later estimation of spawning biomass (Hunter & Macewicz, 1985;
42 Hunter & Lo, 1997). Moreover, the study of prevalence and intensity of histological
43 stages of the atretic oocytes allows predicting the cessation of spawning for a given
44 population (Kurita et al., 2003; Ganias et al., 2003). Furthermore, atresia is essential for
45 the maintenance of ovarian homeostasis; however, a number of factors have been
46 described as potential causes of increased ovarian atresia such as marine pollution or
47 reduced food supply (Cabrera-Páez et al., 2009; Ortiz-Zarragoitia et al., 2011;
48 Yamamoto et al., 2011). Thus, besides indicating a poorer physiological condition for
49 reproduction, elevated atretic indices could also reflect an environmental impact.

50 Gonadal atresia can appear at any stage of oocyte development; however, it is
51 mainly described in vitellogenic oocytes in both marine vertebrates and invertebrates.
52 By contrast, atresia in previtellogenic oocytes is less evident because oocytes at this
53 stage are smaller and unyolked. In general, atresia has been widely studied in marine

fishes (Valdebenito et al., 2011), however, little is known for other marine organisms. The study of this process is particularly poor in marine invertebrates such as cephalopod species, and only in a few cases detailed histological analyses have been used in order to identify and/or describe the atretic oocytes. Examples include studies for octopods such as *Octopus rubescens*, *Octopus hubbsorum* and *Octopus ocellatus* (López-Peraza et al., 2013; Alejo-Plata & Gómez-Plata, 2015; Wang et al., 2015); loliginids such as *Loligo gahi* and *Lolliguncula panamensis* (Laptikhovsky & Arkhipkin, 2001; Arizmendi-Rodríguez et al., 2012); chranchiids such as *Galiteuthis glacialis* (Nesis et al., 1998), lycoteuthids such as *Lycoteuthis lorigera* (Hoving et al., 2014), and ommastrephids such as *Dosidicus gigas* (Hernández-Muñoz et al., 2016).

Two structures, post-ovulatory follicles (POFs) and haemocytes, are associated with the ovarian atresia. POFs are generated as a consequence of ovulation when oocytes are released to the ovarian cavity with their innermost layer, the chorion. Meanwhile, the follicular envelopes of those oocytes, now called POFs, continue attached to the connective tissue strand. They start to degenerate in a similar way as atretic oocytes do, although more rapidly, being easily confounded with atretic oocytes in advanced resorptive stages. The classification of POFs in deteriorated stages over time has allowed to estimate spawning frequency in fishes (e.g. Ganas, 2012), and, to a lesser extent, in cephalopods (e.g. Melo & Sauer, 2007). For any considered species, the degree of POFs deterioration decreases with decreasing temperatures increasing the time that POFs can be detected within the ovary (Ganas et al., 2007; Laptikhovsky, 2013). POFs have been histologically identified in several cephalopod species, however, POF staging have only been determined in the loliginid species *Loligo reynaudii* (Melo & Sauer, 2007) and *Doryteuthis opalescens* (Macewicz et al., 2004).

On other hand, haemocytes are haemolymph circulating cells that are involved in several functions such as wound repair, nutrient digestion, transport and excretion. Moreover, in some molluscs, they have an important role as a defence cells against pathogens (Cheng, 1975). Furthermore, haemocytes are essential for the resorption of the atretic oocytes through phagocytosis. This process has been identified in several marine organisms such as bivalve molluscs (Le Pennec et al., 1991; Suárez-Alonso et al., 2007; Camacho-Mondragón et al., 2012), crustaceans (Zara et al., 2013), and fishes, where these immune cells are called granulocytes (Bruslé-Sicard et al., 1992; Besseau & Faliex, 1994; Miranda et al., 1999). Regarding cephalopods, haemocytes, as well as their phagocytic activity, have been detected in the hemolymph of *Eledone cirrhosa* (Malham et al., 1997), *Sepia officinalis* (Le Pabic et al., 2014), *Euprymna scolopes* (Nyholm et al., 2009), and *Octopus vulgaris* (Rodríguez-Domínguez et al., 2006). However, the identified cell types vary among these species, and in some cephalopods' ovary development studies other terms such as amoebocytes, granulocytes or lymphoid cells have been previously used instead of haemocyte. Buckley (1977) described the presence of occasional amoebocytes in the blood vessels towards the end of vitellogenesis, and massive amoebocytes immediately prior to and afterwards egg laying in common octopus. Melo & Sauer (1998) suggested the presence of lymphoid cells, and observed occasional granulocytes in the thecal stroma, both occurring in atretic previtellogenic oocytes of *Loligo reynaudii*. However, these authors did not attribute any phagocytic role to those cells during ovarian atresia.

The common octopus (*Octopus vulgaris* Cuvier, 1797) is one of the most commercially important cephalopods worldwide and especially in European waters (Pierce et al., 2010). As for most cephalopods, it has a short life cycle of less than two years; it grows rapidly to maturity, spawns once, often seasonally, at the end of its life,

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103 and is an ecological opportunist with labile populations (Guerra, 2006). Earlier
104 described as a simultaneous terminal spawner with a synchronous ovulation (Rocha et
105 al., 2001), its reproductive strategy has been recently reconsidered through detailed
106 histological analyses of ovary development (Gonçalves et al., 2002; Cuccu et al., 2013;
107 Sieiro et al., 2014). These works suggest that oogenesis is an asynchronic process.
108 However, ovulation and spawning patterns, though presumably also asynchronic,
109 remain unresolved. The study of gonadal atresia as a normal process in the ovary
110 development would help to determine which maturity microstages are affected, and
111 therefore, would allow further understanding of its reproductive strategy. Though
112 previous studies have already identified the presence of atretic oocytes in the species
113 (Di Cosmo et al., 2001; Cuccu et al., 2013), there is no other detailed information such
114 as the morphological changes that occur in the atretic oocytes, their classification, the
115 occurrence of other cell structures such as (atretic) POFs and haemocytes, or the
116 relationship between atresia and morphometric variability.

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117 Therefore, the objectives of this work were (i) to describe gonadal atresia in female
118 common octopus according to different degenerative oocytes (i.e. different atretic
119 microstages) found throughout the whole reproductive cycle and its categorization
120 based on previtellogenesis and vitellogenesis phases; (ii) to identify haemocytes as
121 phagocytic cells, together with follicle cells, executing the atretic process; (iii) to relate
122 the presence of atresia with morphometric parameters; and (iv) to analyse its seasonal
123 cycle.

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125 MATERIALS AND METHODS

126 Morphometrical and histological analyses

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127 A total of 359 females of *Octopus vulgaris* from the creel fishery were sampled at three
128 ports in Galicia (NW Spain) from 2004 to 2007. In order to sample all maturity stages, 5
129 wild individuals were below the minimum legal catch size (1 kg), and 26 specimens
130 were spawning females obtained from cage ongrowing (see the cages display in Chapela
131 et al., 2006). All females were used for subsequent morphometric and histological
132 analyses. Maturation and reproduction were assessed using a macroscopic maturity
133 scale proposed by Inejih (2000) and six reproductive measurements. These included:
134 body weight (BW), digestive gland weight (DGW), ovary weight (OW), oviducal
135 complex weight (OCW), and longitudinal (LD_{OG}) and transversal (TD_{OG}) diameters of
136 the oviducal gland. A suite of three morphological indices were also used: the
137 gonadosomatic (GSI), Hayashi (HI), and digestive gland (DGI) index (see Otero et al.,
138 2007 for calculations). Based on histological analyses, maturation was further assessed
139 using a microscopic scale and a histological maturity index (HMI) (see further details in
140 Sieiro et al., 2014).

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141 Ovarian preparations were evaluated using a light microscope (Leica DM5500
142 B; 12.5–1000× magnification) coupled with a Leica DFC 310 FX digital video camera.
143 The software used was Leica Microsystems CMS GmbH, LAS v. 4.1 (Build 1264) (©
144 2003–2012). The histological analyses consisted of, first, a microscopic staging of
145 ovarian maturity based on Sieiro et al. (2014). Second, ovaries were further classified
146 according to the presence or absence of atresia based on the following criteria: (1) the
147 arrangement and hypertrophy of follicular envelope, mainly follicle cells; (2) the
148 presence of chromatin condensation; (3) the identification of haemocytes; (4) the
149 phagocytosis of yolk; and, (5) the degree of vascularisation and presence of yellow–
150 brown bodies. If present, atresia was further subcategorised in previtellogenic,
151 vitellogenic, or the presence of both types. In some cases, fluorescence reaction was

used to differ between previtellogenic and vitellogenic atretic oocytes, as well as between POFs and atretic oocytes, using a fluorescence narrow bandwidth-filter set (©Leica GFP-Plant; 470/40 nm excitation filter; 495 nm dichromatic beam splitter; and 525/50 nm barrier filter). Third, in atretic ovaries, haemocytes were morphologically identified following Castellanos-Martínez et al. (2014). Measures of total diameter, nucleus diameter and the ratio nucleus/cytoplasm (N/C) were taken from a sample of 303 haemocytes found in blood vessels of the ovarian stroma, and from 95 haemocytes found in initial atretic oocytes.

Statistical analyses

Generalized Linear Models (GLMs) were used to relate the presence of atresia to the set of morphometric variables described above. A binomial distribution with a logit link was used. To study the seasonal cycle of atresia a Generalized Additive Model was fit to the data. A penalized cyclic cubic regression spline was used. Differences in average values taken from within the population of haemocytes were evaluated using an analysis of variance (ANOVA).

RESULTS

Morphological characterization of ovarian atresia

Based on observed relevant morphological changes in the oocytes we have identified three atretic histological stages in both previtellogenic and vitellogenic oocytes. These stages are named and described as follows:

1. Initial stage (Fig. 1). We have observed two substages:

176 1.1. There is an apparent increase of the area between the cell layers of the
177 follicular envelope, that is, the outer flattened and elongated cells (outer cells) and inner
178 cuboidal cells (follicle cells). There is a generalized disorganization of the inner cell
179 layer losing its peculiar linearity. This is more obvious from the folding oocytes (FO)
180 microstage onwards (see Sieiro et al., 2014), in the proximity of foldings where large
181 blood vessels are formed between both cell layers.

182 1.2. Through these blood vessels, haemocytes appear between cells of the
183 follicular envelope mainly near the foldings. Haemocytes push the follicle cells to the
184 ooplasm and invade it. If the chorion was previously formed (Late Vitellogenic
185 microstage, LV, Sieiro et al., 2014) it is disintegrated into fragments that are also
186 displaced inwards.

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188 2. Medium stage (Fig. 2). We have observed two substages:

189 2.1. The ooplasm appears to be invaded by phagocytic cells, that is, haemocytes
190 and follicle cells, whereas the outer cells remain as an organized layer. Phagocytosis
191 occurs in the same way as haemocytes invade the ooplasm, from the outer to the inner
192 side of it. As a consequence, many of these cells are larger than in the previous phase
193 with pyknotic and basophilic nuclei mainly regarding to previtellogenic atretic oocytes
194 (Fig. 2A–D). The arrangement of both types of cells is different in vitellogenic atretic
195 oocytes, where patches of haemocytes and follicle cells are found over the yolk, and no
196 hypertrophied haemocytes occur (Fig. 2E–G). The oocyte nucleus remains intact.

197 2.2. As cytoplasmic material is being reabsorbed, the nuclear chromatin
198 condenses and the nucleus disintegration begins. Some yolk granules and chorion
199 fragments remain in the ooplasm in full vitellogenic (FV) and LV atretic oocytes (see

200 Sieiro et al., 2014), respectively. Some size reduction in outer cells and in the whole
201 follicle can be appreciated.

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203 3. Advanced stage (Fig. 3). We have observed three substages:

204 3.1. The follicle shrinks, mainly across its transversal section, acquiring an
205 increasingly amorphous appearance. The nucleus has been completely disintegrated,
206 and the cytoplasmic material is scarcely observed being replaced by numerous blood
207 vessels. This blood network reduces the oocyte lumen and is arranged surrounding the
208 haemocytes (Fig. 3A-E).

209 3.2. A progressive decrease in the number and size of phagocytic cells occurs;
210 and the outer cells are hardly distinguishable. An increasing number of yellow-brown
211 bodies appear in the ooplasm of vitellogenic oocytes (Fig. 3F, G).

212 3.3. At this stage, the atretic oocytes begin to be easily confounded with ovarian
213 connective tissue, remaining like a scarce in the ovary. In the case of atretic vitellogenic
214 oocytes, they appear with a great lumen and small haemocytes, all surrounded by an
215 indistinguishable outer layer. Neither yellow-brown bodies nor follicle cells can be seen
216 (Fig. 3H, I).

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218 *Haemocyte analyses*

219 Different morphological characteristics were observed between haemocytes in
220 blood vessels and in initial atretic oocytes prior to the onset of phagocytosis.
221 Haemocytes in initial atretic oocytes were round, had a basophilic and centric nuclei,
222 and granules in their cytoplasm were not apparent (Fig. 4A-C). Whereas haemocytes in
223 blood vessels were U-shaped, had a basophilic and eccentric nuclei, and basophilic
224 granules occurred in the cytoplasm (Fig. 4D-F). Population sizes for haemocytes

225 sampled in both initial atretic oocytes and in blood vessels were unimodal (Fig. 5A).
226 Haemocytes in initial atretic oocytes had an average diameter of $5.12 \pm 0.93 \mu\text{m}$ (range
227 $3.40\text{--}7.40$), while haemocytes in blood vessels averaged $7.26 \pm 1.03 \mu\text{m}$ (range
228 $4.18\text{--}10.51$). This difference was statistically significant ($p < 0.0001$) (Fig. 5B). In
229 addition, the ratio N/C was also unimodal in both locations (Fig. 5C) with an average
230 value of $0.68 \pm 0.1 \mu\text{m}$ (range $0.45\text{--}0.91$) and $0.64 \pm 0.09 \mu\text{m}$ (range $0.41\text{--}0.95$) in
231 initial atretic oocytes and blood vessels, respectively. The slightly larger ratio in
232 haemocytes sampled within initial atretic oocytes was statistically significant ($p <$
233 0.0001) (Fig. 5D).

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235 *Occurrence of females with atretic oocytes and its seasonal cycle*

236 The maturity stage of the ovaries ranged from macrostages I to V, and from
237 microstages SO (Secondary Oocytes) to OV (Ovulated Oocytes). There were no ovaries
238 classified as either OO (Oogonia) or PO (Primary Oocytes). Almost 52% of the sampled
239 ovaries showed atresia, and this process occurred in previtellogenic (74.31%),
240 vitellogenic (13.19%) or both types of oocytes (12.50%) within the same ovary. The
241 percentage of atresia, understood as the percentage of females with ovarian atresia,
242 increased progressively from immature (macrostage I) to spawning females (macrostage
243 V), reaching a 100% in macrostage V (not shown). Similarly, regarding maturity
244 microstages, atresia increased from SO to OV ovaries reaching virtually a 100% in LV
245 ones (Fig. 6A). All microstages were affected by atresia, from PO to LV, with the
246 exception of OO and OV. When considering the type of atresia, there was a
247 predominance of previtellogenic atresia throughout the ovarian development with the
248 exception of OV microstage (Fig. 6B). However, atretic vitellogenic oocytes firstly
249 occurred in microstage FV reaching a maximum at the spawning stage, that is,

250 microstage OV (Fig. 6B). Finally, both types of atresia only occurred at microstages FV
251 and LV (Fig. 6B).

252 The probability of occurrence of atresia, regardless of previtellogenic or
253 vitellogenic type, showed a significant seasonal cycle with a peak at the end of March
254 beginning of April and a trough in July (Fig. 7).

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256 *Relationship between atresia and morphometric parameters*

257 The probability of occurrence of atresia, regardless of the type, was related to
258 virtually all morphometric measurements and indices. With the exception of DGI, the
259 presence of atresia increased with octopus weight, reproductive organs' size and DGW
260 (Table 1). The probability of atresia occurrence further increased with morphological
261 indices. In particular, the presence of atresia strongly increased with GSI (Fig. 8A) and
262 HMI (Fig. 8B).

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264 DISCUSSION

265 *Morphological description of ovarian atresia in common octopus*

266 Atresia has been observed in several cephalopod species, though in most cases
267 neither using histological techniques nor throughout ovary development. Regarding
268 *Octopus vulgaris*, previous studies have suggested that the number of folds and the
269 presence of a scarce yolk in the oocytes could be a morphological indicators of atresia
270 (Di Cosmo et al., 2001). However, those facts might be confused given that the number
271 of folds (and their depth) depends on the oocyte orientation during the histological
272 sample preparation and cutting. In fact, in a previous study we only observed a
273 maximum of four foldings in fresh samples (Sieiro et al., 2014). Moreover, the scarce
274 yolk could be a symptom of an early vitellogenesis more than the yolk resorption if no

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275 other morphological change is observed such as a follicular envelope disorganization.
276 Furthermore, Cuccu et al. (2013) inferred the presence of atretic oocytes as structures
277 with disorganized follicular epithelium and chorion fragments. However, this work
278 neither provides a detailed description nor a classification of atresia for this species.
279 Therefore, given the lack of a baseline for common octopus atresia, our proposed
280 classification was based on histological characteristics as was similarly done in the
281 seminal work in the chokka squid *Loligo reynaudii* by Melo & Sauer (1998), and other
282 marine species such as fishes (e.g. Hunter & Macewicz, 1985; Guraya, 1986; Miranda
283 et al., 1999).

284 *Phagocytic cells: haemocytes and follicle cells*

285 Two morphological and functional populations were previously characterized in
286 *O. vulgaris* (Novoa et al., 2002; Castellanos-Martínez et al., 2014; Troncone et al.,
287 2015), though usually using differing names and cell diameters among those studies. In
288 our case, we also identified two haemocyte populations showing morphological
289 characteristics very similar to the above cited works. These would be, hyalinocytes and
290 granulocytes as called in Troncone et al. (2015), and large and small granulocytes as
291 named in Castellanos-Martínez et al. (2014). Moreover, our mean haemocyte diameters
292 and N/C ratios were in agreement with the ranges shown by Troncone et al. (2015) and
293 Castellanos-Martínez et al. (2014), respectively. The apparent differences might be due
294 to distinct sample processing techniques.

295 Castellanos-Martínez et al. (2014) and Troncone et al. (2015) found phagocytic
296 activity in both haemocyte types, being higher in the larger ones. Complementary to
297 this, we showed that haemocytes occurred in both atretic previtellogenic and
298 vitellogenic oocytes, and were massive in those ovaries and oviducal glands of
299 spawning and post-spawning females. Thus, we could conclude that these cells would

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300 digest the ooplasm content of atretic oocytes in *O. vulgaris*. This is one of the roles
301 proposed for these cells in other marine organisms such as molluscs, crustaceans and
302 fishes (see Introduction). Additionally, Troncone et al. (2015) postulated that the role of
303 granulocytes in phagocytosis would be linked to their capability for intracellular killing.
304 This could explain why we found small haemocytes within atretic oocytes and larger
305 haemocytes within blood vessels of the ovarian stroma. Both cells could then play
306 different roles, that is, an immune response to invading pathogens for the larger
307 haemocytes, and a phagocytic activity in gonadal atresia for the smaller ones.

308 Follicle cells have been classically involved in atresia through morphological
309 changes such as hypertrophy, pyknosis, and arrangement around the yolk (e.g. Linares–
310 Casenave et al., 2002). In common octopus, only the inner cells (called here follicle
311 cells) within the follicular envelope seemed to have a phagocytic role during gonadal
312 atresia, whereas the outer cells experimented hypotrophy once atresia progresses in
313 vitellogenic oocytes. On the other hand, the different aspect observed for follicle cells
314 between reabsorbing previtellogenic and vitellogenic oocytes suggests a different
315 function. Thus, follicle cells hardly increased in size unlike pyknotic haemocytes
316 observed within atretic previtellogenic oocytes. In contrast, both enlarged follicle cells
317 and haemocytes showed pyknotic nuclei and were arranged over the yolk forming
318 basophilic patches in vitellogenic oocytes. This indicates that haemocytes have a clear
319 phagocytic role in all atretic oocytes, while it seems that follicle cells are only involved
320 in the phagocytic process if yolk is present. This could reinforce the hypothesis that
321 small haemocytes are implicated in the atretic process, and their phagocytic capability is
322 enhanced by follicle cells in vitellogenic atresia. Melo & Sauer (1998) assigned the
323 same phagocytic role to follicle cells in atretic previtellogenic and vitellogenic oocytes
324 for the chokka squid. However, they found thickened and hyperplasic outer cells

325 invading the follicle cells in degenerating previtellogenic and vitellogenic oocytes.
326 Regarding this topic, there is not a consensus in fishes (e.g. Saidapur, 1978; Guraya,
327 1998; McMillan, 2007; Morais et al., 2012; Sharma & Bhat, 2014).

328 *Yellow-brown bodies*

329 The occurrence of yellow-brown bodies in the ooplasm of atretic oocytes points
330 out the end of the atresia in various marine taxa such as fishes, echinoderms and
331 molluscs (e.g. Hunter & Macewicz, 1985; Blazer, 2002; Schäfer & Köhler, 2009;
332 Flores-Quintana et al., 2012; Cuevas et al., 2015). These pigmentary clusters are
333 characterized as chromolipoids, mainly lipofuscins/ceroids, as a result of the
334 degenerative process of proteins and lipids during atresia. However, their origin remains
335 unclear. In fish studies they seem to originate from oocyte, follicle envelope or
336 granulocyte degeneration (e.g. Besseau & Faliex, 1994; Miranda et al., 1999). In other
337 cases, they were particularly observed within the remaining granulosa cells at the end of
338 the atretic process (Santos et al., 2005). By contrast, these structures have not yet been
339 identified in cephalopod atretic ovaries. We have observed them within the high
340 vascularised ooplasm and near the haemocytes, coinciding with the disappearance of
341 follicle cells. Taking into account that these bodies are only observed in atretic ovaries
342 of spawning females, and that there is no ovary regeneration after breeding, we suggest
343 that follicle cell apoptosis results in the appearance of yellow-brown bodies that are
344 ultimately phagocytised by haemocytes at the end of atresia in post-spawning females.
345 In fact, in other marine organisms such as teleost fishes and other aquatic oviparous
346 vertebrates, follicular cells degenerate once yolk resorption is completed during atresia
347 (Wood & Van Der Kraak, 2001). The cell remnants after follicular cell apoptosis in fish
348 atretic oocytes and also POFs could be engulfed by normal follicle cells and/or
349 granulocytes (Drummond et al., 2000; Santos et al., 2008; Üçüncü & Çakici, 2009). In

any case, future works should be addressed to confirm the apoptosis occurrence in follicle cells once yolk resorption ends in spawning females of common octopus using for instance TUNEL assay as a molecular technique for DNA fragmentation in these cells.

Postovulatory follicles (POFs)

Cuccu et al (2013) ascribed the occurrence of POFs exclusively to post-spawning common octopus. However, POFs should occur as soon as mature females ovulate and spawn. In fact, in loliginids and other octopods, POFs were found from partially spent females (Melo & Sauer, 1999; Melo & Sauer, 2007; Macewicz et al., 2004; Olivares-Paz et al., 2001, Zamora & Olivares, 2004; Arizmendi-Rodríguez et al., 2012). We were, however, not able to identify POFs probably due to the low sample size of spawning females, and their advanced stage of spawning that hampered POF recognition due to their rapid resorption and probable misidentification with atretic oocytes. In fact, POFs were only reliably identifiable within 14h after spawning in *Loligo reynaudii*, being old postovulatory follicles difficult to distinguish from atretic oocytes afterwards (Melo & Sauer, 2007).

Atresia occurrence and reproductive strategy

Cuccu et al. (2013) found atretic oocytes restricted to the post-spawning stage in female common octopus. By contrast, we found ovarian atresia throughout the whole ovary development and affecting all common octopus females when spawning begins. In fact, the peak of atretic females was fairly coincident with the maximum of maturation in spring before spawning (Sieiro et al., 2014). Thus, the number of atretic females increased from immature to mature individuals. Moreover, atresia was predominately previtellogenic throughout sexual maturation with the exception of spawning when all females have atretic ovaries of vitellogenic type. Therefore, atresia

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375 in vitellogenic oocytes before spawning seems to be a rare event during the normal
376 ovary development. This is fairly similar to *Loligo reynaudii*, which has ovarian atresia
377 throughout the year with the vitellogenic type being particularly prevalent in fully spent
378 females (Melo & Sauer, 1998). In other octopods, apart from the post-spawning phase,
379 atretic oocytes were also found in earlier maturity stages such as the cases of *Eledone*
380 *cirrrosa* (Boyle & Chevis, 1992), *Octopus mimus* (Olivares-Paz et al., 2001), and
381 *Graneledone macrotyla* (Guerra et al., 2013).

382 Therefore the occurrence of ovarian atresia in *O. vulgaris* is a common and
383 regulative process inherent to the normal ovary development as observed in other
384 cephalopods such as *Dosidicus gigas* (Hernández-Muñoz et al., 2016), *Doroteuthis*
385 *opalescens* (Macewicz et al., 2004), and *Loligo reynaudii* (Melo & Sauer, 1998).
386 However, ovarian atresia in common octopus has not yet been considered as an
387 important physiological process for the species, which would have further implications
388 for the reproductive strategy and the potential fecundity. *O. vulgaris* has an asynchronic
389 ovary development since all microstages were identified over the whole ovary
390 maturation (Gonçalves et al., 2002; Cuccu et al., 2013; Sieiro et al., 2014). Additionally,
391 all previtellogenic oocytes end up being reabsorbed before spawning begins,
392 particularly in LV ovaries. Moreover, FV microstage starts to degenerate at the
393 spawning onset contrary to LV and OV oocytes. Once egg-laying ends, atresia
394 continues in those LV and OV oocytes that will remain within the spawned female. This
395 is in contrast to Mangold-Wirz (1963), who stated that a total egg laying occurs in this
396 species. Given these elements we can confirm histologically that ovulation follows a
397 synchronic pattern unlike ovary maturation. All these facts would explain why
398 spawning duration, that has been recently estimated at around 35 days into the wild
399 (Garci et al., 2016), is shorter than ovary maturation, and why common octopus would

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400 differ from other cephalopods such as the intermittent terminal spawner *Loligo*
401 *reynaudii* (Melo & Sauer, 1999), or the multiple spawner jumbo squid, *Dosidicus gigas*
402 (Hernández-Muñoz et al., 2016).

403 Our findings also have implications concerning potential fecundity, that is, only
404 LV oocytes in pre-spawning ovaries of common octopus should be considered in
405 fecundity estimations since this microstage does not experience atresia until spawning
406 ends. Another plausible method would be to apply a correction factor if percentages of
407 atretic oocytes could be calculated for each type of ovary. In this regard, we propose for
408 future works to calculate the intensity of atresia for merely the FV microstage during
409 ovary development of common octopus since atresia is predominately previtellogenic
410 during maturation in this species. This would allow shortening the time required when
411 an appropriate stereological method would be applied for this purpose.

412

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7 427 Compliance with ethical standards

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9 428 Conflict of interest: The authors declare that they have no conflict of interest.

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11 429 Ethical approval: We have worked with individuals obtained from the commercial

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13 430 fishery that were captured according to the legal standards established in our region.

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15 431 Informed consent: This article does not contain any studies with human participants

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644 TABLES

645 Table 1 Relationships between presence/absence of atresia and all morphometric
646 measurements and indices (see the main text for the abbreviations). In each case, data

647 were fit to a logistic model of the form: $\pi_i = \frac{e^{\alpha + \beta \times X_i}}{1 + e^{\alpha + \beta \times X_i}}$ were π_i is the probability of

648 atresia, X_i is a morphometric measurement or index, and α and β are parameters to be

649 estimated. SE = standard error, DE = deviance explained, N = number of data. Note that

650 with the exception of DGI and HMI all variables were natural log-transformed

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	Variable	α (SE)	β (SE)	DE (%)	N
	BW	-8.90 (1.93)***	1.19 (0.27)***	6.3	278
	OW	-1.76 (0.27)***	0.81 (0.11)***	21.8	273
	TDog	-5.44 (0.72)***	2.73 (0.35)***	23.7	273
	LDog	-6.62 (0.92)***	3.57 (0.49)***	20.7	273
	DGW	-1.90 (0.81)*	0.45 (0.18)*	1.72	272
	GSI	0.74 (0.18)***	0.99 (0.13)***	22.2	273
	HI	-2.45 (0.39)***	-1.35 (0.20)***	16	273
	DGI	-0.04 (0.41) ^{ns}	0.03 (0.08) ^{ns}	0.1	272
	HMI	-6.52 (1.37)***	1.56 (0.34)***	22.8	91
652	*** p-value < 0.0001; ** p-value < 0.001; * p-value < 0.01; ^{ns} non-significant				

FIGURE CAPTIONS

Fig. 1 Initial atresia. Irruption of haemocytes in the follicular envelope as well as in the ooplasm causing a generalized disorganization of the inner cell layer that is more apparent near the folds in the FO microstage (A and B). Panel C shows the disorganized layer and the haemocytes in the SO microstage. Abbreviations: *cc*, cuboidal cells; *fc*, flat cells; *f*, fold; *h*, haemocytes; *N*, nucleus; *Nl*, nucleolus.

Fig. 2 Medium stage of atresia in previtellogenic and vitellogenic oocytes. Panels A-D present haemocytes with pyknotic and basophilic nuclei that appear hypertrophied compared to follicle cells (inner cuboidal cells) in previtellogenic oocytes. At this stage chromatin condensation and nucleus disintegration starts. Panels A and B show the medium-atretic SO microstage with visible chromatin condensation, whereas panels C and D illustrate the medium-atretic FO microstage. Panels E-G show haemocytes and follicular cells disposed over the yolk forming patches in medium-atretic vitellogenic oocytes. Panels F and G show the chorion and yolk under fluorescence reaction in a normal (LV microstage) and atretic (FV microstage) oocyte, respectively. Arrows in E indicate atretic oocytes. Abbreviations: *ch*, chorion; *Chr*, chromatin condensation; *Y*, yolk; the other abbreviations as in Fig. 1.

Fig. 3 Advanced atresia in previtellogenic (A-C) and vitellogenic oocytes (D-I). Panels A and B show superficial cuts of atretic oocytes arranged as a mixture of cells from the follicular envelope with haemocytes. Some haemocytes are still hypertrophied. Panel C presents an atretic oocyte showing a lumen with few cells and numerous blood vessels. Panels D-F show atretic oocytes in spawning females with chorion fragments (E) and yellow-brown bodies (F). Panel G shows yellow-brown bodies and many wide blood

678 vessels in a post-spawning female. Panels H and I show the final reproductive cycle of
679 a post-spawning female with many wide blood vessels in the connective tissue (arrows
680 in H) and the last stages of atresia (arrowheads in H). This stage displays a large lumen,
681 few cells inside (presumably haemocytes) and indistinguishable flat cells (I).
682 Abbreviations: *bv*, blood vessel; *ch*, chorion; *OV*, ovulated oocyte; *yb*, yellow-brown
683 bodies; the other abbreviations as in Fig. 1.

684

685 Fig. 4 Haemocytes within atretic oocytes (A-C) and blood vessels (D-F). Panel A
686 shows haemocytes within an atretic previtellogenic oocyte (arrows); panels B and C
687 show haemocytes in atretic vitellogenic oocytes forming patches over the yolk (arrows);
688 panels D and E present haemocytes within blood vessels of the ovarian connective
689 tissue showing the U-shaped nuclei (arrows). In panel F, haemocytes occur within a
690 blood vessel of the oviducal gland in a spawning female showing basophilic granules
691 inside (arrows). Abbreviations: *y*, yolk; the other abbreviations as in Fig. 1.

692

693 Fig. 5 Haemocyte data. Density plots (A, C) and average values ($\pm 95\%$ CI) (B, D) of
694 haemocyte diameter (A, B) and ratio nucleus/cytoplasm (C, D) measured in haemocytes
695 found in oocytes (dark grey) and blood vessels (light grey).

696

697 Fig. 6 Frequency distribution of presence of atresia among microscopic maturity stages
698 (A). Frequency distribution of the different types of atresia among microscopic maturity
699 stages (B).

700

Fig. 7 Seasonal cycle of the probability of presence of atresia resulted from fitting a binomial generalized additive model to the data (see text). The rugs indicate the sampling days.

704

Fig. 8 Probability of presence of atresia as a function of the gonadosomatic index (A) and the histological maturity index (B) as obtained from Sieiro et al. (2014). See Table 1 for the coefficients of each model.