

**Sea cucumbers, *Holothuria arguinensis* and *H. mammata*, from the southern Iberian Peninsula: variation in reproductive activity between populations from different habitats.**

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1 **Abstract**

2 New fisheries in the western Mediterranean and north eastern Atlantic target the sea cucumbers  
3 *Holothuria arguinensis* and *H. mammata*; however, lack of biological information hinders  
4 management decisions. Here, the reproductive biology of populations the two species was  
5 investigated in the southern Iberian Peninsula. Different populations located along a narrow  
6 latitudinal range displayed the same general reproductive pattern of summer-autumn spawning.  
7 However, significant differences in size, gonadal production and maturity profile between  
8 locations suggests the influence of site-specific factors. In Sagres and Ria Formosa *H.*  
9 *arguinensis* individuals were larger and had larger gonads than in Olhos de Água, which had  
10 relatively more immature animals. The spawning and active gametogenesis periods were also  
11 longer in Sagres, possibly linked to specificity of food availability and tidal conditions. Ria  
12 Formosa also had larger *H. mammata* individuals with larger gonads than in Murcia and Olhos  
13 de Água, possibly reflecting differences in feeding activity in different substrates (muddy/sandy  
14 vs rocky). Gametogenesis in *H. arguinensis* may be triggered by decreasing photoperiod and  
15 temperature, and spawning by increasing temperature. Altogether, these results, which include  
16 fecundity and size at first maturity, provide an important basis for the scientific management of  
17 sea cucumber fisheries in the region.

18

19 **Keywords:** Holothurian, reproduction, first maturity, fecundity

## 20        **1. Introduction**

21    Sea cucumbers are bottom-dwelling echinoderms found in all regions of the ocean, from  
22    intertidal to deep-sea and from polar to tropical regions (Conand, 1989, 2004). As sea  
23    cucumbers are mostly deposit feeders and bioturbators, they have a key role in maintaining  
24    healthy marine ecosystems by mixing of sediments, recycling of nutrients, stimulating algal  
25    growth, and regulating both carbonate content and water pH (Massin, 1982; Purcell, 2004;  
26    Purcell et al., 2016; Schneider et al., 2011; Uthicke, 2001a, 2001b; Wolkenhauer et al., 2010).  
27    Besides their ecological importance, sea cucumbers represent an important fishery resource  
28    mainly exported to Asian countries (Bordbar et al., 2011; Chen, 2003; Chen, 2004; Conand,  
29    1989). Currently, at least 66 sea cucumber species are fished worldwide in more than 70  
30    countries (Purcell, 2010; Purcell et al., 2013).

31    Sea cucumber populations have been subjected to increased exploitation which, in combination  
32    with ineffective fisheries management, has led to severe overfishing throughout the world,  
33    particularly in the Indo-Pacific region (Anderson et al., 2011; Kinch et al., 2008; Purcell, 2010;  
34    Purcell et al., 2013). This has been compounded by the low recruitment, slow growth rate, late  
35    maturity and density-dependent reproductive success of sea cucumbers that make them  
36    especially vulnerable to overexploitation (Bruckner et al., 2003; Conand, 2006a, 2006b;  
37    Uthicke et al., 2004).

38    New sea cucumber fisheries are also being developed rapidly in the Northeastern Atlantic  
39    Ocean and Mediterranean Sea, in Turkey, Italy, Spain, Greece and Portugal, in response to the  
40    strong Chinese market demand. *Holothuria arguinensis* (Koelher and Vaney, 1906) and  
41    *Holothuria mammata* (Grube, 1840) are two of the target species for these fisheries with prices  
42    ranging from 70 to 350 euros per kilo and could reach 1-2 million \$US of total annual revenue  
43    (González-Wangüemert and Borrero-Pérez, 2012; González-Wangüemert et al., 2013b;  
44    González-Wangüemert et al., 2016). *H. arguinensis* is present from the Berlengas Islands

45 (Portugal) to Morocco and Mauritania, including the Canary Islands (Costello, 2001;  
46 Rodrigues, 2012). In the Mediterranean, it has recently been registered on the eastern coast of  
47 Spain and on Algerian coast (González-Wangüemert and Borrero-Pérez, 2012; Mezali and  
48 Thandar, 2014). This species is found from the intertidal zone to 52 m depth, and is frequently  
49 observed on macroalgal-dominated beds and sea grass meadows of *Cymodocea nodosa* and  
50 *Zostera noltii* (González-Wangüemert and Borrero-Pérez, 2012; Navarro et al., 2012; Navarro  
51 et al., 2014; Siegenthaler et al., 2015). *H. mammata* is distributed widely throughout the  
52 Mediterranean Sea and northeast Atlantic Ocean, including the coast of Portugal and the  
53 Macaronesian Islands of the Azores, Madeira and the Canary Islands (Borrero-Perez et al.,  
54 2011). It is found from the intertidal zone to 25 m depths, and is mainly associated with rocky  
55 shores though it can also be found on muddy/sandy substrate dominated by sea grass meadows  
56 (Borrero-Perez et al., 2011; Borrero-Pérez et al., 2009; González-Wangüemert et al., 2013a;  
57 González-Wangüemert et al., 2016; Siegenthaler et al., 2015).

58 Understanding the reproductive biology of a species is central to sound fisheries management,  
59 such as the establishment of a closure season during spawning and a minimum capture size.  
60 Potential productivity of a fisheries, an essential parameter to define the resilience of a  
61 population under exploitation or in the face of human activity disturbances, can also be  
62 determined by studying reproductive processes (Morgan, 2008). Finally, knowledge about  
63 reproductive characteristics can help to restore and enhance wild stocks and are essential for  
64 breeding and aquaculture programs (Mercier and Hamel, 2009; Wang et al., 2015).

65 Sea cucumbers display reproductive cycles typically associated to predictable fluctuations in  
66 environmental factors maximizing the fertilization success through synchronization between  
67 individuals (Levitan, 1995; Mercier and Hamel, 2009). Reproductive activity is thought to be  
68 regulated by endogenous and exogenous cues such as photoperiod, water temperature, salinity,  
69 food availability, tidal flow, light intensity and phytoplankton blooms (Conand, 1981; Drumm

70 and Loneragan, 2005; Hamel et al., 1993; Hamel and Mercier, 1996b; Ramofafia et al., 2000;  
71 Ramofafia et al., 2003). However, the specific influence of each of these factors has rarely been  
72 explicitly tested (Mercier and Hamel, 2009).

73 In order to promote the scientific management of *H. arguinensis* and *H. mammata* it is essential  
74 to acquire baseline knowledge about their ecology, population dynamics, and reproduction.  
75 Therefore, the objectives of the present study are: (1) to provide a detailed description of the  
76 morphology of reproductive structures in relation to the reproductive cycle of the two species;  
77 (2) to obtain an insight of the seasonal and inter-population variability of the reproductive cycle;  
78 (3) to determine the size at first sexual maturity; and (4) to examine possible relationships  
79 between the reproductive cycle and environmental parameters.

80

## 81 **2. Materials and Methods**

### 82 *2.1. Sampling locations and collection of specimens*

83 Individuals of *H. arguinensis* were collected at three locations along the Algarve coast (southern  
84 Portugal) of similar latitude: Ria Formosa (RF; 37°00'35.02''N; 7°59'46.10''O), Sagres (SA;  
85 37°00'44.78''N; 8°55'49.51''O), and Olhos de Água (OD; 37°05'18.76''N; 8°11'34.86''O;  
86 Fig. 1). At each location, between 10 and 15 individuals were collected monthly from May  
87 2013 to April 2014. The collection program was extended until December 2014 in Sagres for  
88 inter-annual variability analysis, and this location was chosen because it had a denser population  
89 of sea cucumbers in this area. Individuals of *H. mammata* were collected in Ria Formosa and  
90 in Olhos de Água in the Atlantic Ocean, and in Murcia at Los Cocedores (MU; 37°24'20.39''N;  
91 1°37'02.27''O; Fig. 1) in the Mediterranean Sea. Collections of *H. mammata* were more spaced  
92 in time than that of *H. arguinensis*, and around 20 individuals were collected each season,  
93 because of logistical factors and their low density in the Ria Formosa (González-Wangüemert  
94 et al., 2013a; Siegenthaler, 2013).

95 Ria Formosa is a sheltered mesotidal coastal lagoon extending for about 55 km along the south  
96 coast of Portugal, with a mean depth of 1.5 m. Sea cucumbers were collected in the intertidal  
97 zone composed of mud and muddy-sand flats where perennial seagrasses such as *Cymodocea*  
98 *nodosa*, *Zoltera noltii* and *Z. marina* and green mat-forming macroalgae (Ulvaes) dominate  
99 (Asmus et al., 2000). Olhos de Água is 20 km to the west of the Ria Formosa, a sheltered  
100 mesotidal coast moderately exposed to the WSW prevailing waves. The intertidal zone where  
101 the sea cucumbers were collected had rock pools and platforms alternating with sandy sediment  
102 areas (Moura et al., 2006; Rosa et al., 2013). Sagres, 70 km to the west of Olhos de Água, is a  
103 mesotidal moderately exposed coastal area (Bettencourt et al., 2004). Sea cucumbers were  
104 collected in the subtidal zone (about 2 m depth) off Praia da Baleeira, next to Sagres harbor,  
105 that is characterized by sandy and rocky areas. This area is affected by summer upwelling, due  
106 to a dominant coastal northerly wind, that supplies nutrients to the euphotic zone (Relvas and  
107 Barton, 2002; Sousa and Bricaud, 1992; Wooster et al., 1976). Los Cocedores is located in a  
108 sheltered bay where sea cucumbers were collected subtidally (1.5 – 2m depth) from rocky  
109 substrate close to sandy patches covered by *C. nodosa* and *Posidonia oceanica* meadows.

## 110 2.2. Biometric measurements

111 Immediately upon arrival at the laboratory, total length (TL), total weight (TW), body wet  
112 weight after dissection and removal of internal organs and coelomic fluid (guttated body weight,  
113 GBW) and gonad weight were measured for each individual. For histological analysis, a small  
114 piece (around 1cm) in the tubule's mid region was removed and fixed in Bouin's solution for  
115 24h and was then stored in 70% ethanol. The remaining gonad was fixed in 10% buffered  
116 formaldehyde for measurement of the gonadal tubules and to estimate fecundity (see below).  
117 The gonad index (GI) was calculated for each individual as  $GI / GBW * 100$  (Conand, 1981).  
118 Length-weight relationships (LWR) were inferred for each population according to  $GBW = a$

119  $TL^b$  (Keys 1928), where GBW is the gutted weight in g, TL is the total length in mm, a is the  
120 regression intercept on the Y-axis and b is the regression slope.

### 121 2.3. *Maturity stages of gonads*

122 A scale of gonadal maturity was established based on the morphology and histological analysis  
123 following the criteria of Conand (1981) modified by Ramofafia et al. (2000): I. Immature, II.  
124 Recovery, III. Growing, IV. Mature, V. Partly-spawned, VI. Spent. The length and diameter of  
125 10 to 15 tubules, taken randomly or from the most representative cohorts, were measured under  
126 the microscope using a ruler and a microscope eyepiece graticule. For histology, paraffin  
127 sections of the testes were stained with Masson's trichrome (Humason, 1972) and the ovaries  
128 with V.O.F. (brilliant yellow-green, Orange G and acid fuchsin) (Gutiérrez, 1967).

### 129 2.4. *Fecundity and sexual maturity*

130 Absolute fecundity was determined for 63 *H. arguinensis* and 27 *H. mammata* individuals from  
131 a small piece of gonad fixed in 10% formalin as described by Muthiga et al. (2009). The  
132 size/weight at first sexual maturity, defined as the size/weight when gonads of 50% of  
133 individuals were undergoing gametogenesis (stage 2, 3, 4, 5 and 6) (Conand, 1981), was  
134 determined from 92 *H. arguinensis* (49 from 2013 and 43 from 2014) collected in SA between  
135 May and June (see Supplementary materials). This was not estimated for *H. mammata* due to  
136 lack of small sized individuals.

### 137 2.5. *Environmental factors*

138 Monthly averaged sea surface temperature (SST) and chlorophyll a concentration with a 4 km  
139 resolution were retrieved from the Moderate Imaging Spectroradiometer-Aqua (MODIS-Aqua)  
140 dataset available from the National Aeronautics and Space Administration (NASA) Goddard  
141 Earth Sciences Data and Information Services Center (GESDISC) between May 2013 and April  
142 2014 for all studied locations, and until December 2014 for SA. Visualization was performed  
143 using Giovanni, a web-based application developed by the GESDISC (Acker and Leptoukh,

144 2007). As complementary information, the upper layer of the surface sediment where sea  
145 cucumbers feed was sampled in March 2014 at each location to determine (i) the organic matter  
146 content and (ii) carbonate content (see Supplementary material 1).

#### 147 2.6. Statistical analyses

148 Data are presented as mean  $\pm$  standard error of the mean (SEM), unless otherwise stated (see  
149 Supplementary material 2 for more detailed information about the statistical analyses).  
150 PERMANOVA (Anderson, 2001) was used to test differences in: 1) TL, GBW, length and  
151 diameter of gonadal tubules between location with as fixed factors location (3 levels) and sex  
152 (2 levels); 2) GI between location with as fixed factors location (3 levels) and month (12 levels  
153 for *H. arguinensis* and 4 levels for *H. mammata*); 3) GI between years in SA for *H. arguinensis*  
154 with as fixed factors year (2 levels) and month (8 levels). A theoretical 1:1 sex ratio of each  
155 population was tested using a chi-square test ( $\chi^2$ ). Sexual dimorphism in each location for each  
156 species was tested with the Mann-Whitney test.

157 Length-weight relationships were used to determine whether the growth of each sea cucumber  
158 population was isometric or allometric (Ricker, 1973). The significance of the regression was  
159 assessed with the F-statistic, and the slope b for each population was tested with a Students t-  
160 test for deviations from the isometric coefficient  $b=3$  (Sokal and Rohlf, 1995). Pearson's  
161 correlation was used to determine relationships between GW and GBW before spawning in all  
162 locations.

163 Absolute fecundity was compared between locations for each species using analysis of  
164 covariance (ANCOVA) with absolute fecundity as a dependent variable and gutted body weight  
165 as a covariate followed by the Tukey HSD post-hoc test for pairwise comparisons and unequal  
166 sample size. Spearman's correlation was used to estimate the relationship between fecundity  
167 and GI in each species. To estimate the size and weight at first sexual maturity, data were fitted  
168 to a logistic curve, using the Levenberg-Marquardt algorithm (Marquardt, 1963).



169 *H. arguinensis* monthly mean GI was cross-correlated (1 month lag interval) with monthly  
170 mean sea surface temperature, chlorophyll *a* concentration and length of photophase over 12  
171 months in 2013 at the three locations and during the 8 months in common between 2013 and  
172 2014 (from May to December) in SA, using GI as the lagged variable. Organic and carbonate  
173 content were analyzed using one-way ANOVA followed by Tamahane and SNK post-hoc tests  
174 for pairwise comparisons.

### 175 **3. Results**

#### 176 *3.1. Population characteristics*

177 There were significant differences in TL among locations (PERMANOVA,  $df = 2$ ,  $n = 552$  for  
178 *H. arguinensis*,  $n = 165$  for *H. mammata*,  $p(\text{perm}) < 0.001$  for each species) and in GBW  
179 (PERMANOVA,  $df = 2$ ,  $p(\text{perm}) < 0.001$  for each species) (Fig. 2). For *H. arguinensis*, TL and  
180 the GBW varied significantly and had the following order: SA > RF > OD (Post-hoc pair-wise  
181 tests,  $p(\text{perm}) < 0.001$  for all combinations; Fig. 2a, b). *H. mammata* had similar TL (Post-hoc  
182 pair-wise tests,  $p(\text{perm}) = 0.97$ ) and GBW ( $p(\text{perm}) = 0.84$ ) at MU and OD, but at RF they were  
183 longer and heavier ( $p(\text{perm}) < 0.001$  for all combinations; Fig. 2c, d). There was no sexual  
184 dimorphism in TL (*H. arguinensis* PERMANOVA,  $df = 2$ , interaction location x sex,  $p(\text{perm})$   
185 = 0.37 and *H. mammata*  $p(\text{perm}) = 0.08$ ) or GBW (*H. arguinensis* PERMANOVA,  $df = 2$ ,  
186 interaction location x sex,  $p(\text{perm}) = 0.25$  and *H. mammata*  $p(\text{perm}) = 0.08$ ). The sex ratio in  
187 each population did not differ significantly from 1:1 (Chi-squared tests, *H. arguinensis*,  $df = 1$ ,  
188 SA:  $\chi^2 = 0.002$ ,  $p = 0.97$ ; OD:  $\chi^2 = 0.136$ ,  $p = 0.71$ ; RF:  $\chi^2 = 0.501$ ,  $p = 0.48$ ; *H. mammata*, RF:  
189  $\chi^2 = 1.976$ ,  $p = 0.16$ ; OD:  $\chi^2 = 0.134$ ,  $p = 0.71$ ; MU:  $\chi^2 = 0.439$ ,  $p = 0.51$ ).

190 Log(TL) was positively correlated to Log(GBW) with correlation coefficients of 0.529, 0.753,  
191 0.483 at SA, OD and RF, respectively, for *H. arguinensis* (ANOVA,  $p < 0.001$  for each  
192 population; Tab. 1) and 0.717, 0.679 and 0.623 at MU, OD and RF, respectively, for *H.*  
193 *mammata* ( $p < 0.001$ ; Tab. 1). The slope *b* varied from 0.980 at RF to 1.437 at OD for *H.*

194 *arguinensis* and from 0.924 at MU to 1.175 at OD for *H. mammata*. The slope  $b$  was  
195 significantly lower than 3 (Students t-test,  $p < 0.001$  in each case), indicating negative  
196 allometry.

### 197 3.2. Gonad morphology

198 The gonad in both *H. arguinensis* and *H. mammata* contains a multitude of tubules joined at  
199 their base and attached to a dorsal mesentery leading to a gonopore from which the gametes are  
200 released. The development of the tubules during gametogenesis follows the same pattern in the  
201 different locations. The length and the diameter of tubules increased until reaching a maximum  
202 when individuals were fully mature, and became shorter and thinner after the spawning period.  
203 The six maturity stages based on the morphology and the histological characteristics of the  
204 gonadal tubules are detailed in Supplementary material 3, 4 and 5.

205 In *H. arguinensis*, cohorts of tubules at different stages were observed in a single gonad, varying  
206 in relative abundance and type between individuals and, in some cases, all tubules were at the  
207 same stage. Pre-vitellogenic oocytes lining the germinal epithelium were also observed side by  
208 side with mature oocytes (Supplementary material 4). After spawning, the spent tubules were  
209 completely resorbed while other categories of tubules persisted in the gonad. The length and  
210 the diameter of the most represented category of tubules varied significantly between  
211 geographical locations according to the following order: SA > RF > OD (PERMANOVA,  $df =$   
212 2,  $p(\text{perm}) < 0.05$  for each case; Post-hoc pair-wise tests,  $p(\text{perm}) < 0.001$  for each combination;  
213 Supplementary material 6). No sexual dimorphism was observed in tubule length  
214 (PERMANOVA,  $df = 2$ , location and sex interaction,  $p(\text{perm}) = 0.65$ ) or diameter ( $p(\text{perm}) =$   
215 0.15).

216 In *H. mammata*, in each individual all tubules were at the same stage in all gonads analyzed.  
217 However, as in *H. arguinensis*, smaller oocytes were observed lining the tubular epithelium  
218 side by side with mature oocytes (Supplementary material 4). All tubules regressed after

219 spawning. Tubule length and diameter of individuals from RF was significantly larger than  
220 those from MU and OD (PERMANOVA,  $df = 2$ ,  $p(\text{perm}) < 0.001$  in each case; Post-hoc pair-  
221 wise tests,  $p(\text{perm}) < 0.01$  in each case; Supplementary material 7) but there were no significant  
222 differences in tubule length ( $p(\text{perm}) = 0.12$ ) and diameter ( $p(\text{perm}) = 0.96$ ) between individuals  
223 from the two latter locations. No sexual dimorphism was observed in tubule diameter  
224 (PERMANOVA,  $df = 2$ , location and sex interaction,  $p(\text{perm}) = 0.92$ ). However, males had  
225 significantly longer tubules than females in RF and MU (PERMANOVA,  $df = 2$ , location and  
226 sex interaction,  $p(\text{perm}) < 0.05$ ; Post-hoc pair-wise tests,  $p(\text{perm}) < 0.05$  in both cases).

### 227 3.3. GI and gametogenesis

228 *H. arguinensis* - The reproductive pattern of *H. arguinensis* was similar in the three sampled  
229 locations. Four main phases were distinguished: (1) a growth phase characterized by a gradual  
230 increase of GI (2) a final maturation phase ending by the peak of GI, (3) a spawning phase  
231 characterized by a drastic decrease of the GI, and (4) a post-spawning resting phase  
232 characterized by a low and stable GI. Within these broad phases, differences in GI and  
233 gametogenic state were observed between SA, OD and RF (Fig. 3a-c). While the GI was not  
234 statistically different between SA and RF (PERMANOVA,  $df = 2$ ,  $n = 506$ , location,  $p(\text{perm}) <$   
235  $0.001$ ; Post-hoc pair-wise tests,  $p(\text{perm}) = 0.14$ ), both differed from OD ( $p(\text{perm}) < 0.001$  for  
236 RF and SA). No significant differences in GI was found between males and females at the three  
237 locations (Mann-Whitney U-tests, SA:  $U = 8588$ ,  $p = 0.13$ ; OD:  $U = 2028$ ,  $p = 0.51$ ; RF:  $U =$   
238  $2025$ ,  $p = 0.06$ ).

239 At SA, mature individuals were already present in January 2014, but in OD and RF were only  
240 observed in March and April, respectively. The GI peak at SA ( $24.53 \pm 3.88\%$ ) and RF ( $32.16$   
241  $\pm 5.53\%$ ) was reached in June 2013 at similar levels (PERMANOVA,  $df = 22$ , interaction  
242 location x month,  $p(\text{perm}) < 0.01$ ; Post-hoc pair-wise tests,  $p(\text{perm}) = 0.26$ ), but it was a month

243 earlier and significantly smaller at OD ( $8.63 \pm 2.00\%$ :  $p(\text{perm}) < 0.001$  for all combinations).  
244 However, almost 30% of OD individuals were immature in June 2013.  
245 During the spawning phase there was a decrease of more than 85% in mean GI at the three  
246 locations. This phase lasted over 6 months at SA, although it was particularly intense from July  
247 to October 2013 with a peak in August 2013 (68.75% of spent individuals). At OD and RF, the  
248 spawning phase extended over 4 months from July to October 2013, and was less intense than  
249 at SA and the highest percentage of spent individuals (46.67%) occurred in September 2013.  
250 After release of the gametes, individuals entered a resting phase, the duration of which was  
251 considerably different between SA and RF. At SA, the individuals were predominantly in  
252 resting phase (60%) only in October 2013, and from then on most individuals (> 60%) were  
253 already at the growing stage (GI ca. 4%). In contrast, at RF most individuals (> 50%) were in  
254 the resting phase for four months, with a mean GI around 1%, from October 2013 to January  
255 2014. At OD, the GI stayed low, at around 1%, from October to December 2013 with around  
256 20% of individuals at the resting stage and more than 60% immature.  
257 Gametogenesis started earlier at SA (October 2013) than at OD (December 2013) and RF  
258 (November 2013). Individuals at the gonad growth stage were predominant (> 50%) for longer  
259 at SA (5 months) compared to OD (3 months) and RF (2 months). During gonad growth the GI  
260 increased by 342%, 388% and 227% at SA, OD and RF, respectively.  
261 Comparison of the breeding season at SA between 2013 and 2014 over the 8-month overlap  
262 between May and December (Fig. 3a) revealed the monthly GI was significantly different  
263 between the two years (PERMANOVA,  $df = 1$ ,  $n = 228$ , interaction year  $\times$  month  $p(\text{perm}) <$   
264  $0.001$ ). In particular, post-hoc PERMANOVA tests revealed that the GI in May, July, August  
265 and September was significantly different between the two years ( $p < 0.05$  for all combinations).  
266 The GI peak in 2013 was reached a month earlier (June) than in 2014 (July). However, the  
267 spawning phase started two months later in 2014 and a similar decrease of GI occurred in the

268 two years (88.38% in 2013 and 86.27% in 2014). The spawning period extended from August  
269 to December in 2014, with 45.33% of spent individuals detected during that period compared  
270 to 41.38% in 2013.

271 *H. mammata* - The same four main reproductive phases were found for *H. mammata* as for *H.*  
272 *arguinensis* (Fig. 4a-c): (1) an increase of GI from spring to summer, (2) a drastic decrease of  
273 GI from summer to autumn, (3) a stabilization of GI from autumn to winter, and (4) a gradual  
274 increase of GI from winter to spring. The GI was significantly different between locations  
275 (PERMANOVA,  $df = 2$ ,  $n = 225$ ,  $p(\text{perm}) < 0.001$ ). Individuals from OD and MU had the same  
276 GI (Post-hoc pair-wise tests,  $p(\text{perm}) = 0.77$ ) which was significantly smaller than at RF  
277 ( $p(\text{perm}) < 0.001$  for all combination). The GI peak in summer was significantly higher at RF  
278 ( $18.93 \pm 4.15$  %) than at OD ( $6.23 \pm 1.71$  %) and MU ( $3.54 \pm 0.72$  %) (PERMANOVA,  $df =$   
279  $6$ , interaction location  $\times$  season,  $p(\text{perm}) < 0.01$ ; Post-hoc pair-wise tests,  $p < 0.01$  for all  
280 combination) with no differences between the latter two locations (Post-hoc pair-wise tests,  $p$   
281  $= 0.65$ ). No differences in GI between males and females was detected at any of the three  
282 locations (Mann-Whitney U-tests, MU:  $U = 395.50$ ,  $p = 0.90$ ; OD:  $U = 414$ ,  $p = 0.07$ ; RF:  $U =$   
283  $182$ ,  $p = 0.63$ ).

284 From spring to summer, the mean GI increased by 30%, 78% and 51% in RF, OD and MU,  
285 respectively, (Fig. 4a-c) and most individuals ( $> 60\%$ ) became mature. From summer to  
286 autumn, the GI decreased 74%, 75%, and 88% at RF, OD and MU, respectively. At RF and  
287 OD, more than 15% released their gametes in summer while at MU spawning occurred in the  
288 autumn. At RF spawning individuals (including partly-spawned and spent) were found in  
289 summer and winter, at OD in summer and autumn and at MU in autumn and winter. The highest  
290 percentage of spent individuals was found in autumn at all locations: 50%, 26% and 60% at RF,  
291 OD and MU, respectively. From autumn to winter, the mean GI stabilized at around 1% at OD  
292 and MU, while it was still decreasing from 5% to 2% at RF. Most individuals were at the

293 recovery stage (> 55%) in winter with some at the growing stage at OD (5%) and MU (20%).  
294 Interestingly, at MU only 5 individuals out of 20 had visible gonads in winter. Also at MU, the  
295 gonad growth stage was predominant in spring (65%) while at OD and RF during the same  
296 period was around 37% and mature were 45%.

#### 297 3.4. Soma-gonad relationships

298 For both species, there was a positive correlation between log(GW) and log(GBW) before  
299 spawning ( $p < 0.05$  in each case, Pearson's correlation; Fig. 5a-b). In *H. arguinensis*, the  
300 correlation coefficients were 0.50, 0.67 and 0.52 for SA (n = 77), OD (n = 37) and RF (n = 27)  
301 respectively. In *H. mammata*, the correlation coefficients were 0.55 for MU (n = 37), 0.59 for  
302 OD (n = 31) and 0.52 for RF (n = 23).

#### 303 3.5. Fecundity and sexual maturity

304 The absolute fecundity in *H. arguinensis* varied from 0.27 to  $12.77 \times 10^6$  oocytes/female with  
305 a mean of  $5.09 \pm 0.42 \times 10^6$  oocytes/female (Fig. 6a) while in *H. mammata* fecundity ranged  
306 from 0.10 –  $6.21 \times 10^6$  oocytes/female with a mean of  $1.32 \pm 0.30 \times 10^6$  oocytes/female (Fig.  
307 6b). For *H. arguinensis*, individuals from RF and SA has similar fecundities (ANCOVA, square  
308 root transformation,  $df = 2$ ,  $n = 63$ ,  $p < 0.01$ ; Unequal Tukey HSD,  $p = 0.99$ ), but both had a  
309 higher absolute fecundity than those from OD (ANCOVA, square root transformation,  $df = 2$ ,  
310  $n = 63$ ,  $p < 0.01$ ; Unequal Tukey HSD,  $p < 0.01$ ). In *H. mammata*, individuals from RF had a  
311 higher absolute fecundity than those from OD and MU (ANCOVA, log transformation,  $df = 2$ ,  
312  $n = 27$ ,  $p < 0.05$ ; Unequal Tukey HSD,  $p < 0.01$ ) with no differences between the latter two ( $p$   
313  $= 0.94$ ). Absolute fecundity was positively correlated (Spearman correlation;  $p < 0.05$ ) to GI  
314 both in *H. arguinensis* (SA:  $r = 0.81$ , RF:  $r = 0.88$ , OD:  $r = 0.80$ ) and *H. mammata* (RF:  $r =$   
315  $0.84$ , OD:  $r = 0.87$ ).

316 The length at first sexual maturity ( $TL_{50}$ ) for *H. arguinensis* was estimated to be between 210  
317 and 230 mm, while the GBW at first maturity ( $GW_{50}$ ) was between 110 and 130 g. The TW at  
318 first sexual maturity ( $TW_{50}$ ) was between 220 and 260 g (Supplementary material 8).

### 319 3.6. Reproductive activity and environmental parameters

320 The mean monthly sea surface temperatures showed a clear seasonal cycle at the three locations  
321 with a maximum in September and a minimum in February (Fig. 7a). The mean sea surface  
322 temperature was slightly higher at RF ( $17.8 \pm 0.95$  °C) than at OD ( $17.6 \pm 0.97$  °C) or SA ( $16.7$   
323  $\pm 0.66$  °C). Significant cross-correlations were found for *H. arguinensis* in 2013 between the  
324 mean GI and mean sea surface temperature in the three sites (Fig. 8a). The largest positive  
325 correlations were detected between lags +1 and +4, meaning that maximal GI values were  
326 reached from 1 to 4 months before the temperature peaked, while minimal GI values were  
327 reached from 1 to 4 months before the temperature minima. For the common 8-month sampling  
328 period, from May to December, in 2013 and 2014, at SA, significant cross-correlation between  
329 these two parameters was observed in 2013 at lag -1 while no correlation was found in 2014.  
330 The latter may reflect the difference in temperature between years with a decrease of  
331 temperature from June to July in 2014 when it increased gradually in 2013. In all locations  
332 gametogenesis started when temperature was decreasing (October-November), with the SA  
333 individuals started earlier. In contrast, gonad maturation was observed when temperature was  
334 increasing at OD and RF (March-April) whereas it started earlier at SA (January) when the  
335 temperature was still decreasing. Spawning started when temperature increased rapidly (June-  
336 July), with the highest percentage of spent individuals identified when the temperature was  
337 maximal at RF and OD (September). At SA, the highest percentage of spent individuals was  
338 found a month before the peak temperature (August). The difference in water temperature a  
339 month before and a month after the GI peak was 4.67 °C, 5.94 °C and 6.05 °C at SA, OD and  
340 RF, respectively. Although the peak of water temperature at SA was in September for both

341 years, the GI peak and the onset of spawning differed between years, possibly related to  
342 differences in summer water temperatures (Fig. 7a).

343 The longest photophase in June was  $14.66 \pm 0.03$  h and the lowest in December was  $9.66 \pm 0.01$   
344 h (Fig. 7b). In 2013, significant positive correlations were observed between the mean GI and  
345 mean photophase at 0 and +2 months lag including all locations, meaning that GI correlated  
346 with the photophase or preceded the photophase by 1 to 2 months (Fig. 8b). The peak GI  
347 coincided with the summer solstice. At the three locations, gametogenesis initiated after the  
348 autumnal equinox, during short days (October-December) whereas spawning occurred after the  
349 summer solstice (June-July). At SA, from May to December, significant positive correlations  
350 were found between the mean GI and the mean monthly photophase 0 and +1 month later in  
351 2013 and +1 month later in 2014.

352 The pattern of chlorophyll *a* did not show clear seasonal variation at the three locations (Fig.  
353 7c). The mean concentration of chlorophyll *a* was similar at RF ( $1.78 \pm 0.31$  mg/m<sup>3</sup>) and OD  
354 ( $1.70 \pm 0.35$  mg/m<sup>3</sup>), but both had a higher mean value than SA ( $0.95 \pm 0.13$  mg/m<sup>3</sup>). All three  
355 locations had a peak of chlorophyll *a* in November 2013, corresponding to four to five months  
356 after the beginning of spawning in all locations. No significant cross-correlations were found  
357 between mean monthly GI and chlorophyll *a* concentration at any of the locations analyzed  
358 (Fig. 8c) or during the 8 months in common between both sampling years (2013 and 2014) at  
359 SA. However, the largest mean chlorophyll peak was in August 2014 ( $4.76 \pm 0.01$  mg/m<sup>3</sup>)  
360 coinciding with the beginning of the spawning (Fig. 7c).

361 The complementary information on sediment analysis at each location showed that the organic  
362 matter was significantly lower at OD ( $0.63 \pm 0.03$  %) relative to RF ( $1.35 \pm 0.18$  %), SA ( $1.07$   
363  $\pm 0.04$  %) and MU ( $1.73 \pm 0.03$  %) (ANOVA, *df* = 3, *n* = 48; Tamhane test: 35.72, *p* < 0.001  
364 for all combinations), with no differences among the latter. Mean values in carbonate content  
365 varied significantly between all four locations according to the following order: SA ( $52.46 \pm$



366 1.98 %) > OD ( $27.46 \pm 1.77$  %) > MU ( $17.95 \pm 0.47$  %) > RF ( $8.90 \pm 0.98$  %; ANOVA, arcsin  
367 transformation,  $df = 3$ ,  $n = 48$ ; SNK test,  $p < 0.01$  in each case, Supplementary material 9).

368 **4. Discussion**

369 Separate sea cucumbers populations of *H. arguinensis* and *H. mammata* have similar seasonal  
370 reproductive patterns largely correlated to temperature and photoperiod. However, significant  
371 differences in average size/weight, fecundity and timing of reproductive activity for the sea  
372 cucumber populations studied was reflected in the size and maturity of the gonad, and this was  
373 most likely linked to local conditions.

374 The two species of sea cucumber studied are gonochoric, without sexual dimorphism,  
375 exhibiting a balanced sex ratio as commonly observed in many Aspidochirote holothurians (e.g.  
376 Asha and Muthiah, 2007; Conand, 1981; Despalatovic et al., 2004; Kazanidis et al., 2014;  
377 Mezali et al., 2014; Navarro et al., 2012). The studied populations had negative allometric  
378 growth, with the strongest positive correlations between TL and GBW generally observed in  
379 populations of *H. mammata*, reflecting a difference in body shape between the two species  
380 (Cone, 1989; Herrero-Pérezrul and Reyes-Bonilla, 2008). Such a pattern has been already  
381 described for most of holothurians (e.g. Bulteel et al., 1992; Conand, 1993; Herrero-Pérezrul et  
382 al., 1999; Kazanidis et al., 2010; Poot-Salazar et al., 2014) where negative allometry was  
383 explained by the cylindrical shape of the body (Conand, 1989) and the fact that the thickness  
384 of some part of the body wall was independent the size of the individuals (Ramón et al., 2010).

385 The size distribution of individuals varied between locations suggesting the importance of local  
386 environmental conditions. However, population structure may also reflect differing recruitment  
387 dynamics between locations, particularly at OD where the proportion of immature individuals  
388 was highest for both species. The individuals of both species at this location, where the organic  
389 matter was the lowest, were also smaller. Intertidal conditions at RF may also be potentially  
390 more stressful (air exposure, variable temperature) compared to the more stable conditions in  
391 the subtidal SA where the individuals can spend more energy on growth rather than on  
392 physiological changes linked to survival during air exposure. For *H. mammata*, however, tidal

393 constraints and food availability may not be the main factors affecting the inter-population size  
394 differences, since small individuals could be found in the subtidal zone at MU where the organic  
395 matter is high. Conversely, the smaller sizes at MU and OD could be a consequence of  
396 preference of this species for rough substrates where individuals adopt nocturnal feeding  
397 behavior (Aydin and Erkan, 2015; Navarro, 2012; Navarro et al., 2013a; Navarro et al., 2013b)  
398 in contrast to the soft substrate at RF where feeding activity may occur day and night. Although  
399 carbonate content has in some cases been associated with organic carbon productivity, the  
400 amount of carbonate in the sediments at the experimental locations does not seem to influence  
401 the size/weight of the sea cucumbers since it was the lowest at RF where larger individuals for  
402 both species were found.

403 Not only were there significant differences in size distribution between sea cucumber  
404 populations but their condition and fecundity varied. GW was positively correlated to GBW  
405 before spawning (when GIs were larger) which indicates that larger individuals have  
406 proportionally larger gonads and are potentially more fecund. *H. arguinensis* individuals at SA  
407 and RF not only were larger but also had larger absolute fecundity and GI than those at OD.  
408 Similarly, for *H. mammata*, individuals were larger and had larger absolute fecundity and GI at  
409 RF compared to OD or MU. This might be indicative of more favorable feeding and  
410 environmental conditions for both species at SA and RF allowing diversion of a higher  
411 proportion of energy to reproduction thus explaining their higher reproductive output (larger  
412 fecundity and GI) (Thompson, 1983).

413 First sexual maturity occurs later in *H. arguinensis* (for *H. mamata* it was not estimated) from  
414 SA (TL: 210-230mm; EW:110-130g; TW:220-260g) than in *H. sanctori* from Gran Canaria  
415 (TL:201-210mm; EW:101-110g; TW:176-200g (Navarro et al., 2012). *H. arguinensis* had on  
416 average 5-fold higher absolute fecundity than *H. mammata*, but both had lower fecundity  
417 compared to larger tropical species at about 9 to  $17 \cdot 10^6$  oocytes/female according to Conand

418 (1993). At equivalent sizes, our results obtained for *H. arguinensis* are similar to those  
419 estimated by Domínguez-Godino et al. (2015) for the same species, ranging from 1.5 to 9.6 x  
420 10<sup>6</sup> released eggs per female, and also those of the temperate *H. forskali* that varied between 2  
421 and 7 x 10<sup>6</sup> oocytes per female (Tuwo and Conand, 1994).

422 The difference in gonad morphology between *H. arguinensis* and *H. mammata* highlights the  
423 diversity of gametogenic processes that exists in the Holothuroidea. The gonads of *H. mammata*  
424 followed a uniform development, similar to what has been described in species such as *H.*  
425 *scabra* (Demeuldre and Eeckhaut, 2012), *H. fuscogilva* (Ramofafia and Byrne, 2001) and *H.*  
426 *spinifera* (Asha and Muthiah, 2007). The gonad development pattern in *H. arguinensis*  
427 generally followed the tubule recruitment model (TRM), with tubules organized in distinct  
428 cohorts, representing different maturity stages with a single generation of gametes within each  
429 tubule, and incomplete resorption of the gonad after spawning (Smiley, 1988). However, some  
430 specificities were observed: 1) some individuals had only tubules at one stage, 2) some tubules  
431 appeared to have more than one generation of oocytes. Exceptions to the TRM have been also  
432 described for other species (Foglietta et al., 2004; Gómez, 2011) and included variations in  
433 gonad structure and development which were not only found between species but also between  
434 locations and seasons for the same species (Sewell et al., 1997). Possible effects of local  
435 environmental conditions on tubular development have been suggested (Hamel and Mercier,  
436 1996b) but require systematic investigation.

437 The general pattern of reproduction was similar between the two species and populations in line  
438 with previous studies of sea cucumbers at narrow latitudinal ranges (Brewin et al., 2000; Byrne,  
439 1990; Byrne et al., 1998; Kazanidis et al., 2014). The reproductive cycle was seasonal with  
440 spawning during the warmer period (summer-autumn) as typically described for temperate sea  
441 cucumber species (Costelloe, 1988; Despalatovic et al., 2004; Kazanidis et al., 2014; Mezali et  
442 al., 2014; Navarro et al., 2012; Sewell, 1992; Sewell and Bergquist, 1990; Tuwo and Conand,

443 1992). Temperature and photoperiod correlated positively to GI, with some lag in *H.*  
444 *arguinensis*, indicating their potential role in the regulation of the reproductive cycle, as  
445 previously found for other sea cucumbers (e.g. Conand, 1993; Hamel and Mercier, 1996b;  
446 Muthiga, 2006; Navarro et al., 2012; Ramofafia et al., 2000; Santos et al., 2015; Shiell and  
447 Uthicke, 2006; Tuwo and Conand, 1992). In the locations studied, gametogenesis initiated after  
448 the autumnal equinox, under short days (< 12h) and decreasing temperature (below 20°C)  
449 whereas spawning started after the summer solstice when the photophase started to decrease  
450 and under increasing temperatures (above 20°C). The importance of temperature is highlighted  
451 by the variability in the timing of gametogenesis and spawning between 2013 and 2014 and  
452 between locations. Furthermore, temperature shocks have been used by the aquaculture industry  
453 and in the laboratory to induce spawning in sea cucumbers (e.g. Domínguez-Godino et al.,  
454 2015; Mercier and Hamel, 2009; Smiley et al., 1991). In addition, factors such as the lunar  
455 cycle, phytoplankton blooms, tidal flux, light intensity, and social cues (e.g. aggregative  
456 behavior, diffusible chemical signals) have also been suggested to trigger spawning (e.g. Giese  
457 and Kanatani, 1987; Hamel and Mercier, 1996a, 1999; Leite-Castro et al., 2016; Mercier and  
458 Hamel, 2009).

459 It is tempting to suggest that the photoperiod is a permissive factor for both gametogenesis  
460 initiation and spawning and that temperatures below 20°C are required for gametogenesis to  
461 develop and above 20°C for spawning in the species studied. Furthermore, environmental  
462 factors such as food abundance associated to the more stable conditions of the subtidal habitat  
463 may facilitate faster replenishment of gonads after the spawning and ensure an extended release  
464 of gametes owing to storage of more nutrients (Bourgoin and Guillou, 1990; Byrne, 1990;  
465 Byrne et al., 1998). The prolonged spawning at SA could also be size-related, as larger animals  
466 are more fecund and generally known to spawn earlier and over a longer period (Scott et al.,  
467 2006; Secor, 2000). In contrast, at RF and OD the sea cucumbers were intertidal and subjected

468 to repeated temperature and exposure to solar radiation that may have contributed to reduce the  
469 reproductive period and extended the recovery phase. Clearly, this is an area that requires  
470 investigation to pinpoint the specific contribution of the aforementioned environmental factors  
471 in the reproductive process.

472 Breeding periods coincide usually with optimal environmental conditions to maximize  
473 fertilization success and ensure offspring survival (Mercier and Hamel, 2009). Our results did  
474 not detect a relationship between the maturity stages or GI and chlorophyll *a* concentration. A  
475 sea surface chlorophyll *a* peak was only detected in November, 5 to 6 months after the  
476 beginning of the spawning, and was unlikely to influence larval development which in *H.*  
477 *arguinensis* has been estimated to be 18 days (Domínguez-Godino et al., 2015). Navarro et al.  
478 (2012) obtained a similar result for *H. sanctori* in the Canary Islands and suggested the larvae  
479 were able to develop in low-food environment. However, the satellite measurements used in  
480 the present study may not reflect local conditions and therefore more focused studies on the  
481 early life stages would be required to determine the proximate factors critical for larval survival  
482 and growth.

## 483 **5. Conclusion**

484 Our study showed that populations in *H. arguinensis* and *H. mammata* living in a narrow  
485 latitudinal range have the same general reproductive pattern with spawning during summer-  
486 autumn and a recovery phase in winter. This pattern was correlated to temperature and  
487 photoperiod in *H. arguinensis* which with small deviations fluctuated similarly in the studied  
488 locations. The differences in size/weight, gonadal production and maturity stages between  
489 locations most likely were influenced by the particular features of each location such as the  
490 food availability and tidal stress. Populations of *H. arguinensis* and *H. mammata* are not at  
491 present under official exploitation in Portugal, although unregulated fishing may be putting  
492 pressure on stocks. The reproductive parameters obtained in the present work will provide an

493 important basis for establishing regulatory measures for the management of sea cucumbers and  
494 preserve biodiversity.

495 **Compliance with Ethical Standards**

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501 **Ethical approval:** All applicable international, national and institutional guidelines for the care  
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503 **Contributors**

504 NM carried out the work, analyzed the data and wrote the manuscript; CC contributed to data  
505 interpretation and wrote the manuscript; DMP supervised histology and wrote the manuscript;  
506 AVMC supervised data analysis and wrote the manuscript; MGW designed the study and  
507 wrote the manuscript.

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- 801

802 **Figure legends**

803 **Fig. 1.** Sampling locations of *H. arguinensis* (SA, OD and RF) and *H. mammata* (OD, RF and  
804 MU).

805 **Fig. 2.** Box-whisker plots of morphometric characteristics of the studied populations: (a, c)  
806 mean total length and (b, d) gutted weight of (a, b) *H. arguinensis* (SA: n = 288; OD: n = 165;  
807 RF: n = 178) and (c, d) *H. mammata* (MU: n = 80; OD: n = 100; RF: n = 45).

808 **Fig. 3.** Gametogenic cycle and mean gonad index  $\pm$  SEM (line, right Y-axis) of *H. arguinensis*  
809 at (a) SA, (b) OD and (c) RF. Histograms show relative frequencies of gonad stages per month  
810 from May 2013 to April 2014 for OD and RF, and to December 2014 for SA (n = 10 to 15 per  
811 month).

812 **Fig. 4.** Gametogenic cycle and mean gonad index  $\pm$  SEM (line, right Y-axis) of *H. mammata*  
813 at (a) MU (n = 65), (b) OD (n = 90), RF (c, n = 45). Histograms show relative frequencies of  
814 gonad stages per season from Spring 2013 to Summer 2014.

815 **Fig. 5.** Relationship between gonad weight and gutted body weight before spawning in (a) *H.*  
816 *arguinensis* in SA (n = 77, r = 0.50), OD (n = 37, r = 0.67), and RF (n = 27, r = 0.52), and (b)  
817 *H. mammata* in MU (n = 37, r = 0.56), OD (n = 31, r = 0.55) and RF (n = 23, r = 0.52). All  
818 Pearson's correlations were significant at the  $p < 0.05$  level.

819 **Fig. 6.** Box-whisker plots of the absolute fecundity of mature females from (a) *H. arguinensis*  
820 collected at SA (n = 23), RF (n = 20) and OD (n = 20) and from (b) *H. mammata* collected in  
821 MU (n = 8), OD (n = 10) and RF (n = 9).

822 **Fig. 7.** Seasonal variations of environmental parameters: (a) sea surface temperature ( $^{\circ}$ C), (b)  
823 photophase duration (h) and (c) chlorophyll *a* ( $\text{mg}/\text{m}^3$ ) at SA, RF and OD. See Materials and  
824 Methods for details.



825 **Fig. 8.** Cross-correlation function analysis (CCF) between gonad index and (a) temperature, (b)  
826 photophase duration, and (c) chlorophyll *a* for *H. arguinensis* at each studied site. Red symbols  
827 indicate significant correlations at  $p = 0.05$ .

828 **Supplementary materials**

829

830 **Supplementary material 1.** Methodology used to characterize the sediment at the studied  
831 locations.

832 **Supplementary material 2.** Statistical analyses.

833 **Supplementary material 3.** Macroscopic and microscopic description of the gonadal tubules  
834 in *H. arguinensis* and *H. mammata* at the different stages of the gametogenesis.

835 **Supplementary material 4.** Histological characterization of maturity stages in females *H.*  
836 *arguinensis* and *H. mammata* with VOF stain **a** Recovering ovary with pre-vitellogenic oocytes  
837 (po); **b** Growing ovary with active vitellogenesis with early (evo) and mid (mvo) vitellogenic  
838 oocytes; **c, d** Mature ovary with large mature vitellogenic oocytes (mo) within follicles (f) with  
839 a large germinal vesicle (gv); **e** Partly-spawned ovary with loosely packed of unspawned  
840 oocytes; **f** Spent ovary with relict oocytes (ro) and phagocytes (p) degrading oocytes. Scale bars  
841 represent 100  $\mu\text{m}$ .

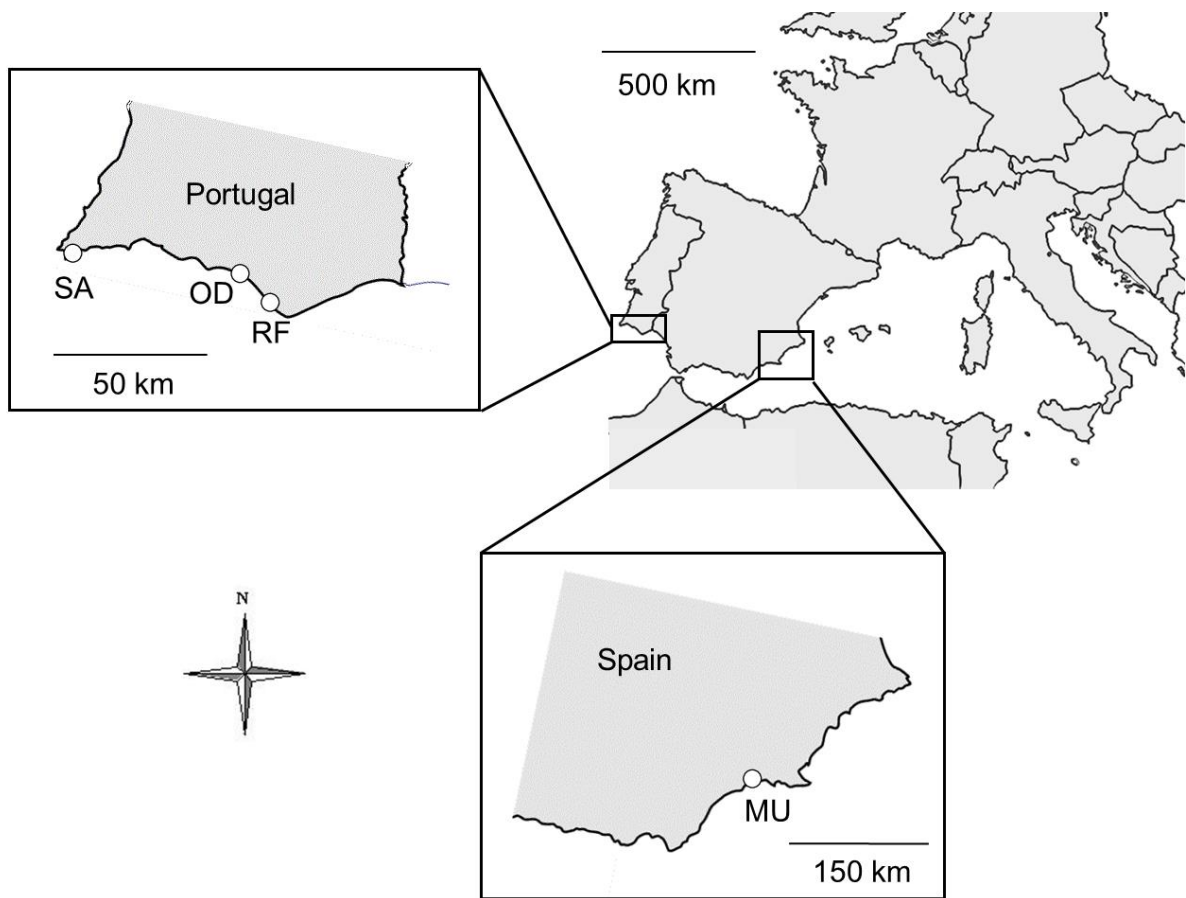
842 **Supplementary material 5.** Histological characterization using Masson's trichrome stain of  
843 the main maturity stages detected in males *H. arguinensis* and *H. mammata* gonads **a**  
844 Recovering testis with developing spermatocytes lining the germinal epithelium; **b** Growing  
845 testis with invaginations of the germinal epithelium and increasing abundance of spermatozoa  
846 in the lumen; **c** Mature testis with the lumen completely filled with spermatozoa (sz); **d** Partly-  
847 spawned testis with area less dense areas of spermatozoa (arrow); **e, f** Spent testis with  
848 unspawned spermatozoa (e) and an empty lumen (f). Scale bars represents 100  $\mu\text{m}$ .

849 **Supplementary material 6.** Morphometry of the gonadal tubules in the three populations of  
850 *H. arguinensis*.

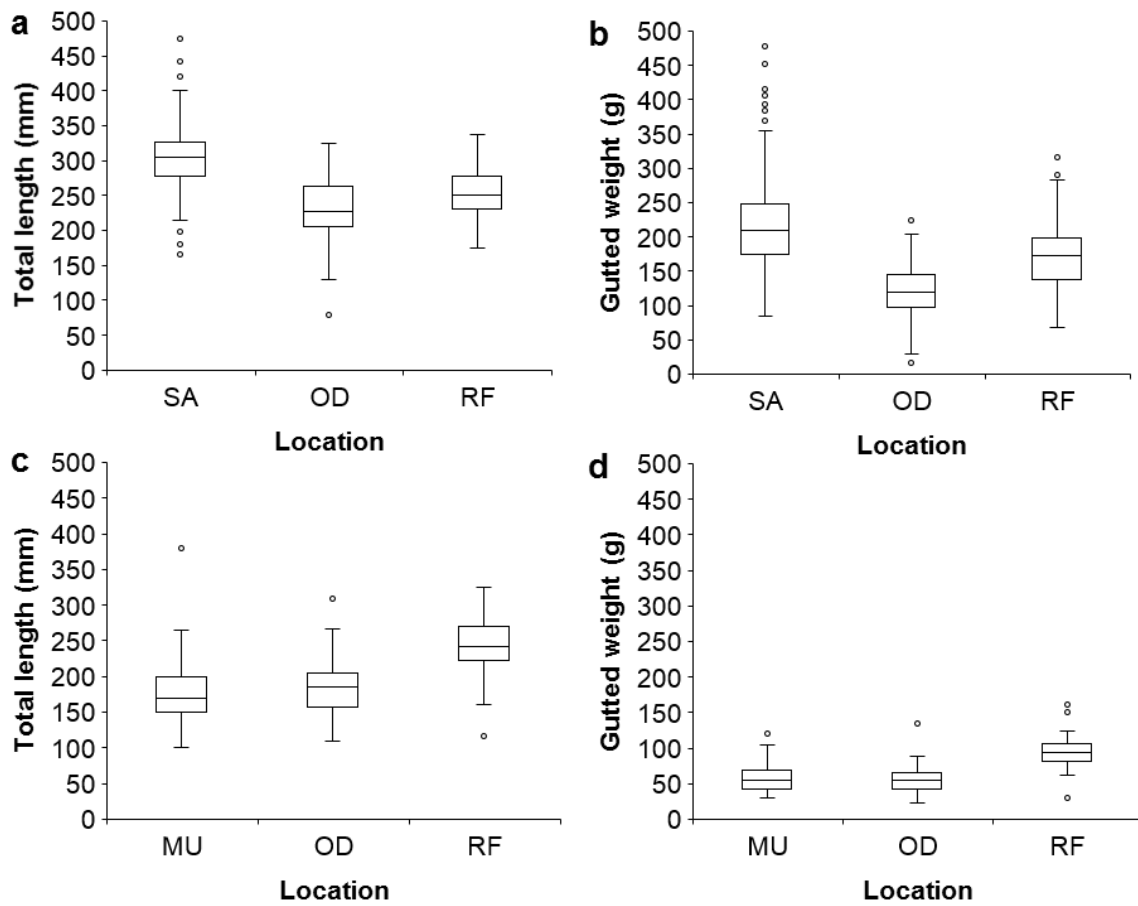
851 **Supplementary material 7.** Morphometry of the gonadal tubules in the three populations of  
852 *H. mammata*.

853 **Supplementary material 8.** Size (a), gutted weight (b) and total weight (c) at first sexual  
854 maturity in *H. arguinensis*.

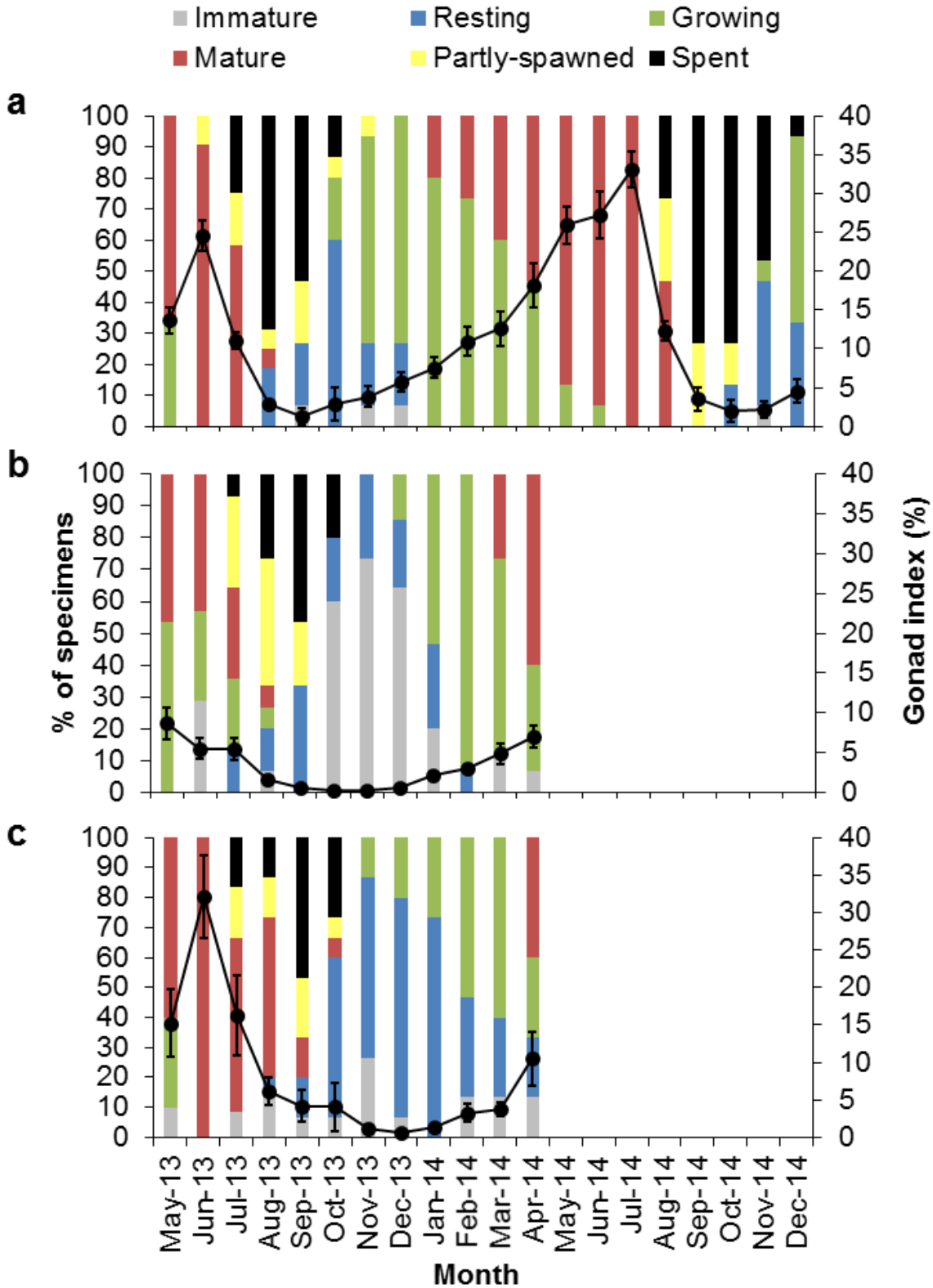
855 **Supplementary material 9.** Percentage of organic matter *versus* percentage of carbonates in  
856 each of the studied locations.



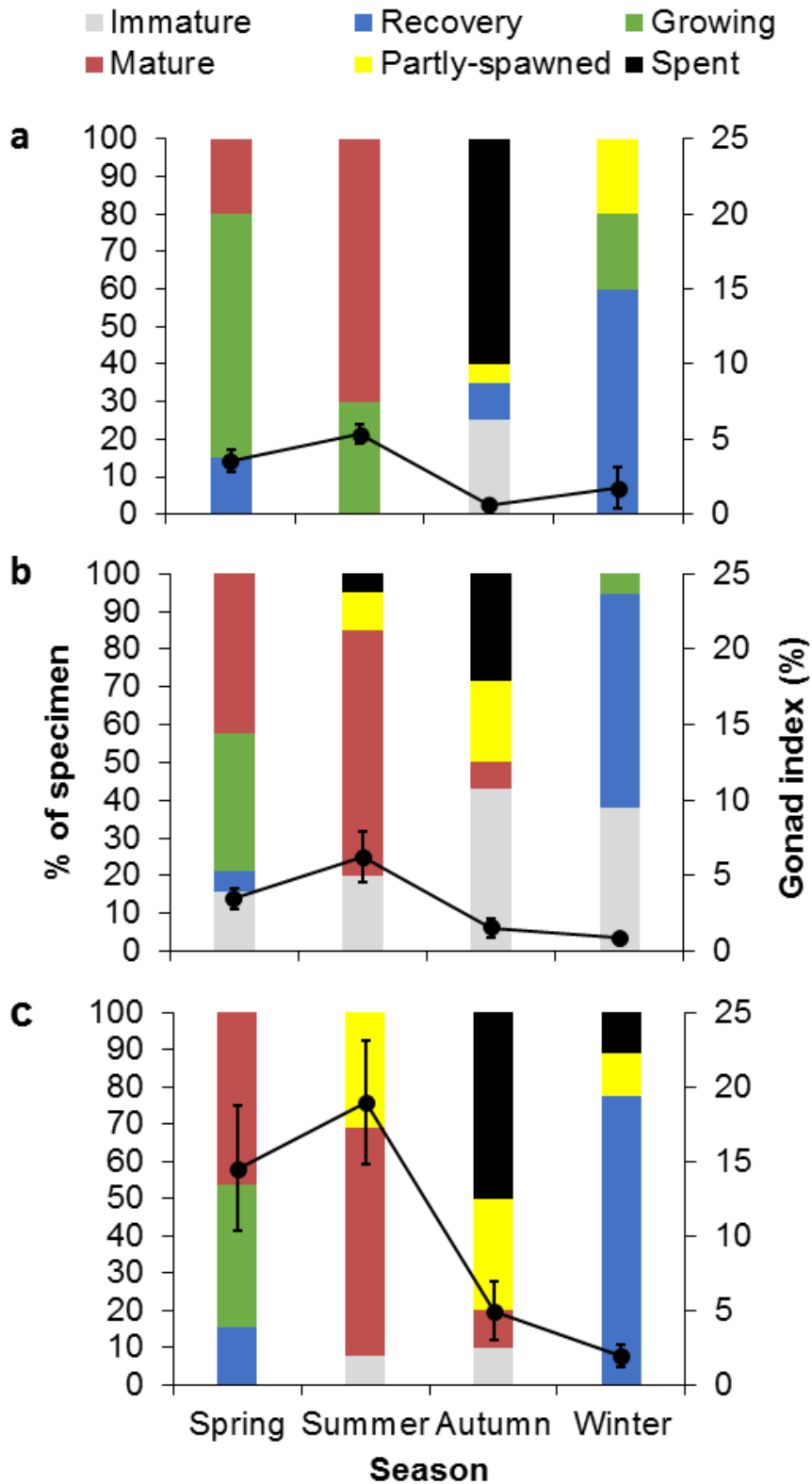
**Fig. 1.** Sampling locations of *H. arguinensis* (SA, OD and RF) and *H. mammata* (OD, RF and MU).



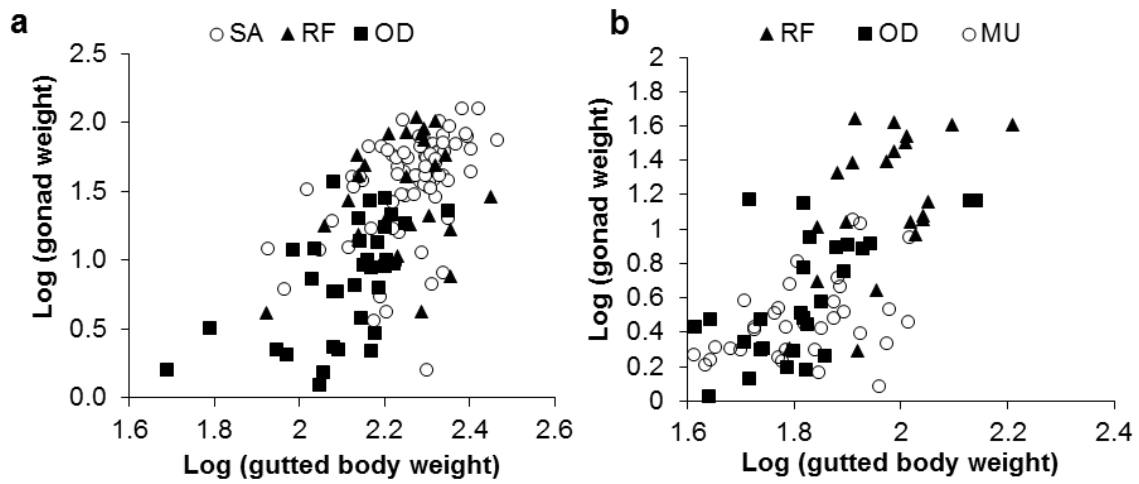
**Fig. 2.** Box-whisker plots of morphometric characteristics of the studied populations: (a, c) mean total length and (b, d) gutted weight of (a, b) *H. arguensis* (SA: n = 288; OD: n = 165; RF: n = 178) and (c, d) *H. mammata* (MU: n = 80; OD: n = 100; RF: n = 45).



**Fig. 3.** Gametogenic cycle and mean gonad index  $\pm$  SEM (line, right Y-axis) of *H. arguensis* at (a) SA, (b) OD and (c) RF. Histograms show relative frequencies of gonad stages per month from May 2013 to April 2014 for OD and RF, and to December 2014 for SA (n = 10 to 15 per month).

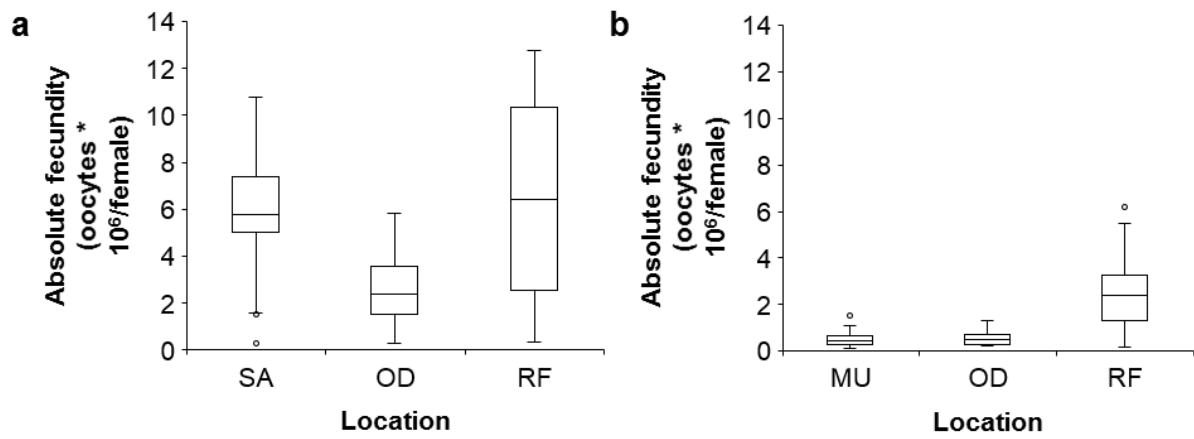


**Fig. 4.** Gametogenic cycle and mean gonad index  $\pm$  SEM (line, right Y-axis) of *H. mammata* at (a) MU (n = 65), (b) OD (n = 90), RF (c, n = 45). Histograms show relative frequencies of gonad stages per season from Spring 2013 to Summer 2014.

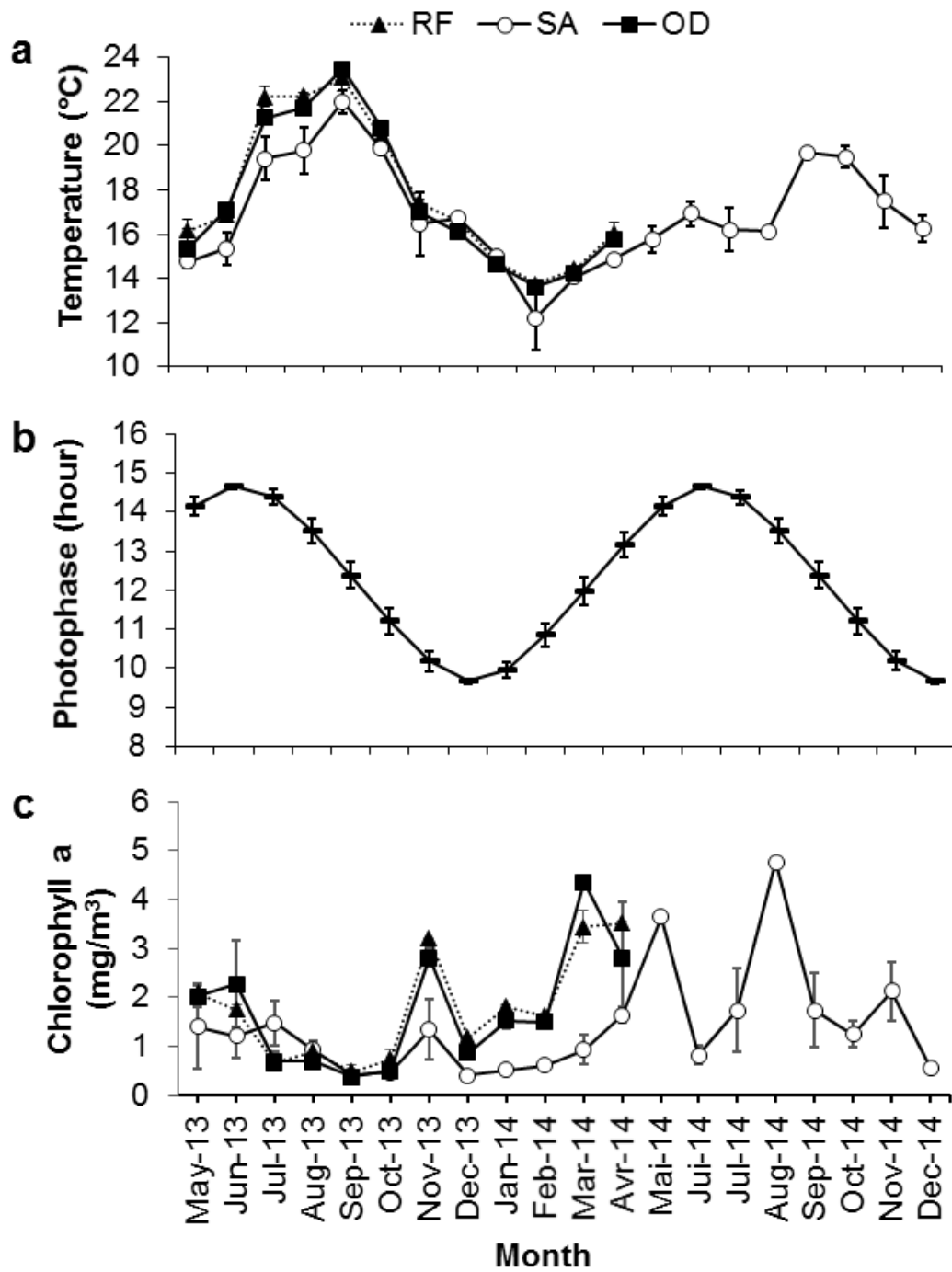


**Fig. 5.** Relationship between gonad weight and gutted body weight before spawning in (a) *H. arguinensis* in SA (n = 77, r = 0.50), OD (n = 37, r = 0.67), and RF (n = 27, r = 0.52), and (b) *H. mammata* in MU (n = 37, r = 0.56), OD (n = 31, r = 0.55) and RF (n = 23, r = 0.52). All Pearson's correlations were significant at the  $p < 0.05$  level.

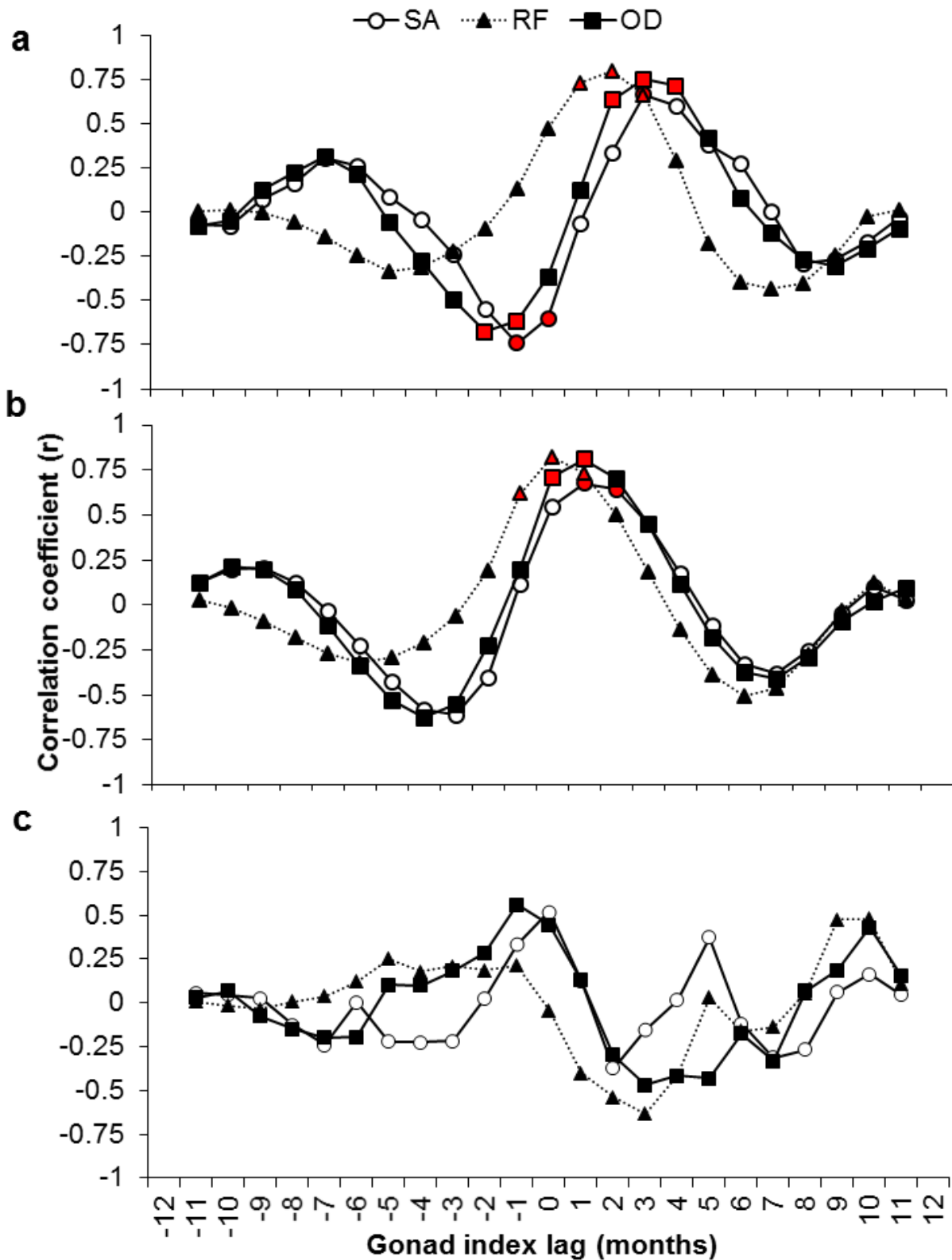




**Fig. 6.** Box-whisker plots of the absolute fecundity of mature females from (a) *H. arguinensis* collected at SA (n = 23), RF (n = 20) and OD (n = 20) and from (b) *H. mammata* collected in MU (n = 8), OD (n = 10) and RF (n = 9).



**Fig. 7.** Seasonal variations of environmental parameters: (a) sea surface temperature (°C), (b) photophase duration (h) and (c) chlorophyll *a* (mg/m<sup>3</sup>) at SA, RF and OD. See Materials and Methods for details.



**Fig. 8.** Cross-correlation function analysis (CCF) between gonad index and (a) temperature, (b) photophase duration, and (c) chlorophyll *a* for *H. arguensis* at each studied site. Red symbols indicate significant correlations at  $p = 0.05$ .

**Supplementary material 1.** Methodology used to characterize the sediment at the studied locations.

To characterize the sediment of each studied location, the upper layer of the surface sediment where sea cucumbers feed was sampled in March 2014. Twelve samples per location were dried in a drying oven at 60°C for 48 hours and processed to determine (i) organic matter content and (ii) carbonate content. The organic matter content was determined by carbonization at 450°C for 4 h. The organic matter content is the weight lost during carbonization (Gillan et al., 2005). Since the skeleton of sea cucumbers is composed of magnesium calcite, carbonates could be important for their development. To determine the carbonate content, dried sediments were decarbonated by dissolution with 37% HCl until effervescence disappeared. The decarbonated sediments were rinsed with distilled water, centrifuged and dried at 60°C during 48 hours. The carbonate content is the weight lost during the acid dissolution (Dean, 1974).

**References:**

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## **Supplementary material 2. Statistical analyses.**

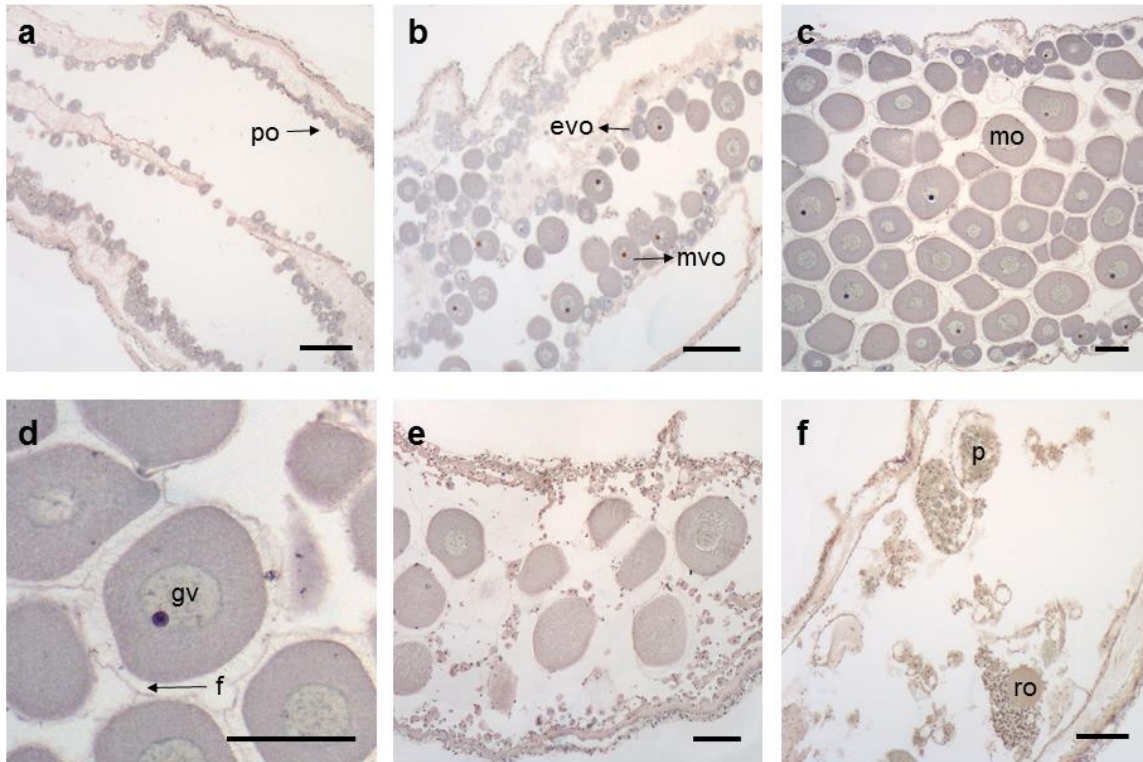
The protocol of Zuur et al. (2010) was followed to check for outliers, variance homogeneity, normality and relationships between variables before statistical analysis. Data were transformed when necessary to fulfill the prerequisites for parametric analysis (Shapiro-Wilk's and Cochran's tests) and were analyzed using SPSS version 17.0. If the prerequisites were not met even after data transformation, non-parametric tests were used. The significance level for all analyses was set at  $p < 0.05$ . When the design was unbalanced, non-parametric permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) with a type III (partial) sum of squares was applied as recommended by Anderson et al. (2008). Analyses were based on Euclidean distance and on 9999 permutations of residuals under a reduced model. When a factor was identified as statistically significant, post-hoc PERMANOVA pair-wise tests were conducted using 9999 permutations. Analyses were performed using Primer 6 with the add-on package PERMANOVA+ (Primer Ltd, Plymouth, UK).

## **References**

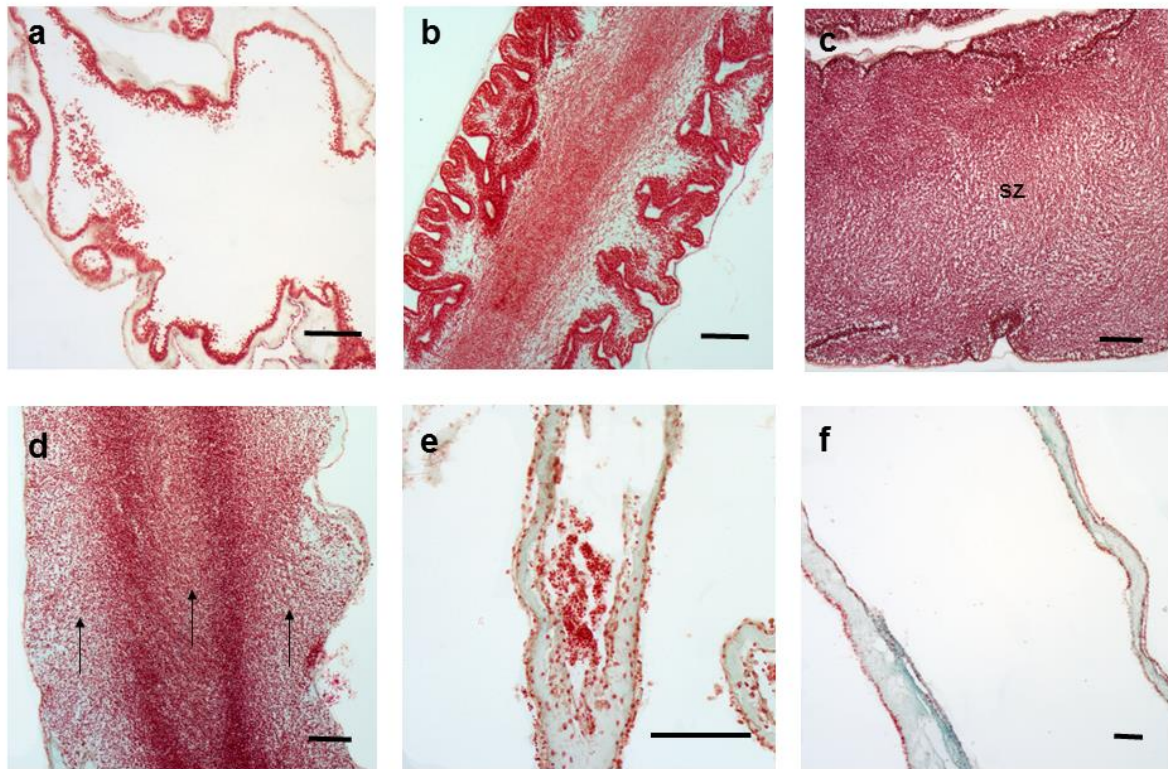
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**Supplementary material 3.** Macroscopic and microscopic description of the gonadal tubules in *H. arguinensis* and *H. mammata* at the different stages of the gametogenesis.

<b>Maturity stage</b>	<b>Sex</b>	<b>Description</b>
I. Immature	F/M	Short and thin tubules ending by a round shape. Distribution of the germinal cells along the tubule wall with a lumen completely empty.
II. Recovery	F	Extension and thickening of the tubules with previtellogenic oocytes (<20 µm) lining the germinal epithelium and an empty lumen. Degenerating oocytes and nutritive phagocytes can still be observed.
	M	Extension and thickening of the tubules with the beginning of invagination of germinal epithelium along which occurs layers of spermatogonia. Few spermatozoa can be observed in the lumen.
III. Growing	F	Thick and long tubules with still previtellogenic oocytes lining the germinal epithelium. Early (21-40 µm) and mid (41-80 µm) vitellogenic oocytes invade progressively the lumen of the tubule. No residual oocytes from the previous spawning.
	M	Thick and long tubules with abundant spermatogonia lining the numerous invaginations of the germinal epithelium and increasing of spermatozoa in the lumen.
IV. Mature	F	Maximal length and width of tubules with a thin wall and a lumen densely packed with mature oocytes (120-180 µm). Each oocyte is characterized by a well-defined germinal vesicle and germinal epithelium.
	M	Maximal length and width of tubules with a smooth and thin tubule wall and a lumen completely filled with mature spermatozoa. No early stages of spermatogenesis.
V. Partly-spawned	F	Shrinkage and wrinkling of tubules with loosely patches of mature oocytes and few disintegrated oocytes.
	M	Beaded appearance of shrunken tubules filled by patchy and low density of spermatozoa.
VI. Spent	F	Thin and wrinkled tubules with a lumen characterized by empty space, unspawned oocytes and some nutritive phagocytes.
	M	Thin and wrinkled tubules with empty area and relict spermatozoa in the lumen.



**Supplementary material 4.** Histological characterization of maturity stages in females *H. arguinensis* and *H. mammata* with VOF stain **a** Recovering ovary with pre-vitellogenic oocytes (po); **b** Growing ovary with active vitellogenesis with early (evo) and mid (mvo) vitellogenic oocytes; **c, d** Mature ovary with large mature vitellogenic oocytes (mo) within follicles (f) with a large germinal vesicle (gv); **e** Partly-spawned ovary with loosely packed of unspawned oocytes; **f** Spent ovary with relict oocytes (ro) and phagocytes (p) degrading oocytes. Scale bars represent 100  $\mu\text{m}$ .



**Supplementary material 5.** Histological characterization using Masson's trichrome stain of the main maturity stages detected in males *H. arguinensis* and *H. mammata* gonads **a** Recovering testis with developing spermatocytes lining the germinal epithelium; **b** Growing testis with invaginations of the germinal epithelium and increasing abundance of spermatozoa in the lumen; **c** Mature testis with the lumen completely filled with spermatozoa (sz); **d** Partly-spawned testis with area less dense areas of spermatozoa (arrow); **e, f** Spent testis with unspawned spermatozoa (e) and an empty lumen (f). Scale bars represents 100  $\mu$ m.

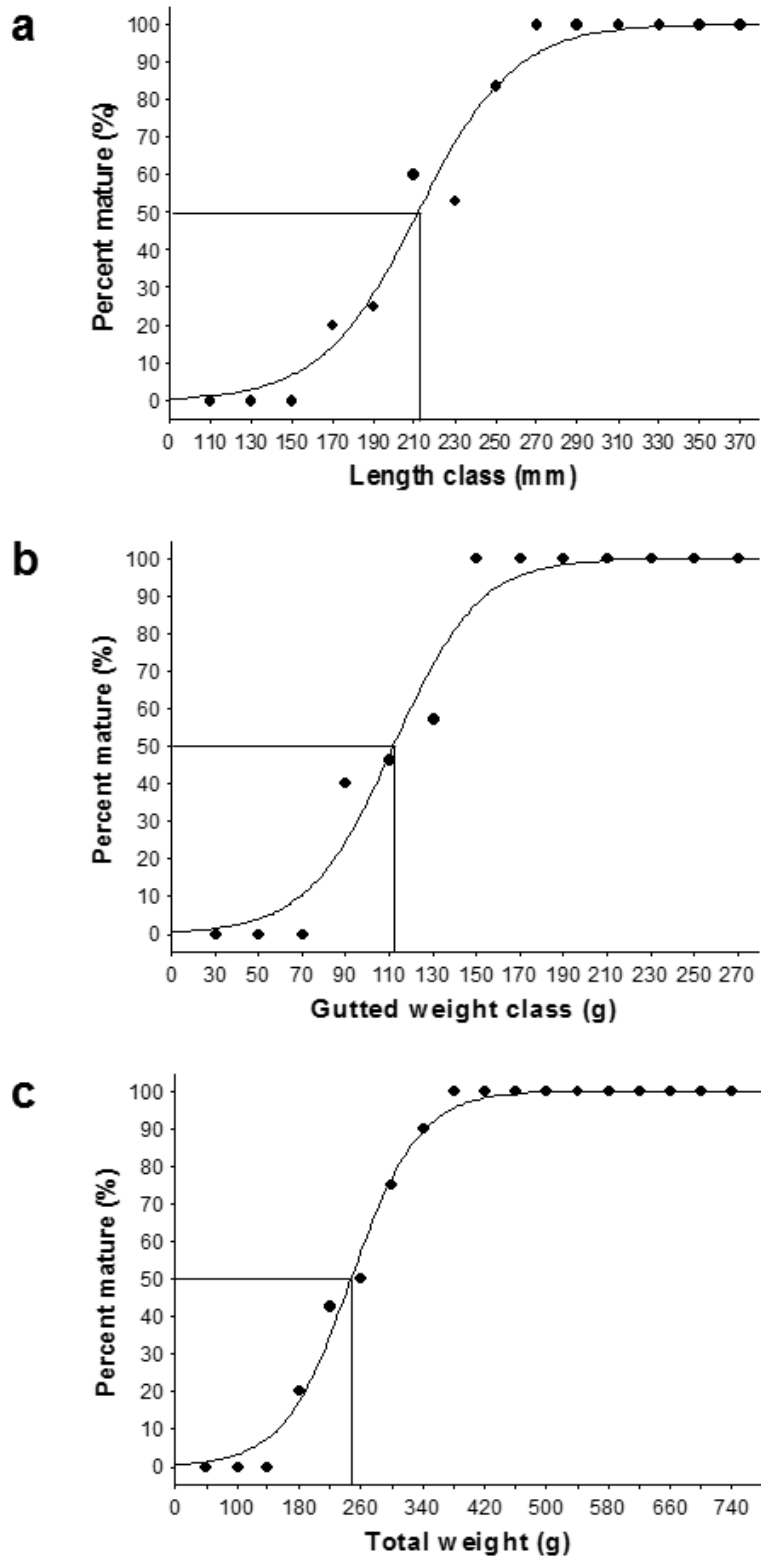


**Supplementary material 6.** Morphometry of the gonadal tubules in the three populations of *H. arguinensis*.

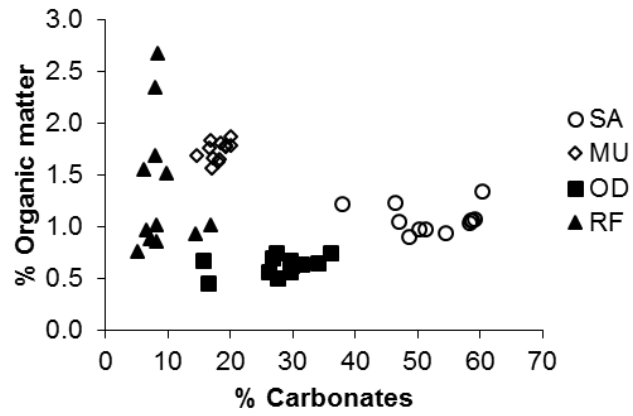
Maturity stage	Sex	Site	N	Tubules characteristics		
				Length (mm)	Diameter (mm)	Colour
I. Immature	F/M	SA	60	9.90 ± 5.73	0.24 ± 0.11	Translucent
		OD	615	10.41 ± 3.02	0.34 ± 0.09	
		RF	240	16.02 ± 78.10	0.31 ± 0.12	
II. Recovery	F	SA	285	53.00 ± 25.46	0.75 ± 0.23	Translucent to whitish
		OD	195	20.70 ± 4.98	0.55 ± 0.10	
		RF	450	28.21 ± 36.22	0.63 ± 0.36	
	M	SA	225	45.95 ± 25.28	0.60 ± 0.29	Translucent to whitish
		OD	165	21.53 ± 6.32	0.57 ± 0.13	
		RF	360	30.18 ± 46.31	0.59 ± 0.58	
III. Growing	F	SA	465	77.34 ± 41.32	1.15 ± 0.34	Light pink to to light orange
		OD	165	39.22 ± 6.35	1.06 ± 0.33	
		RF	300	54.84 ± 56.46	1.31 ± 1.24	
	M	SA	540	102.31 ± 51.67	0.89 ± 0.34	White to cream
		OD	210	47.87 ± 13.34	0.80 ± 0.18	
		RF	195	66.23 ± 91.58	0.74 ± 0.47	
IV. Mature	F	SA	750	111.27 ± 39.20	1.57 ± 0.51	Light orange to to reddish orange
		OD	165	67.78 ± 21.36	1.85 ± 0.36	
		RF	300	101.99 ± 38.05	1.87 ± 0.44	
	M	SA	660	124.24 ± 59.18	1.37 ± 0.58	Cream to beige
		OD	60	100.32 ± 8.97	1.43 ± 0.17	
		RF	285	119.56 ± 38.05	1.45 ± 0.42	
V. Partly-spawned	F	SA	75	47.00 ± 28.84	0.95 ± 0.11	Pale orange
		OD	92	29.55 ± 6.51	0.76 ± 0.08	
		RF	60	62.27 ± 22.93	1.09 ± 0.19	
	M	SA	210	67.08 ± 29.96	0.89 ± 0.54	Beige with brown patches
		OD	107	46.39 ± 5.60	0.67 ± 0.08	
		RF	60	71.28 ± 67.52	0.88 ± 0.23	
VI. Spent	F	SA	345	60.17 ± 34.25	0.54 ± 0.19	Brownish to translucent with rust blotches.
		OD	75	36.34 ± 4.45	0.44 ± 0.05	
		RF	45	20.71 ± 5.33	0.41 ± 0.07	
	M	SA	435	50.94 ± 20.89	0.41 ± 0.12	Brownish to translucent with rust blotches.
		OD	75	21.73 ± 6.35	0.47 ± 0.08	
		RF	105	17.36 ± 10.27	0.43 ± 0.17	

**Supplementary material 7.** Morphometry of the gonadal tubules in the three populations of *H. mammata*.

Maturity stage	Sex	Site	N	Tubules characteristics		
				Length (mm)	Diameter (mm)	Colour
I. Immature	F/M	MU	67	6.10 ± 2.80	0.38 ± 0.08	Translucent
		OD	136	6.48 ± 6.13	0.37 ± 0.08	
		RF	30	7.30 ± 1.39	0.40 ± 0.08	
II. Recovery	F	MU	44	6.63 ± 1.78	0.86 ± 0.96	Translucent to whitish
		OD	191	10.17 ± 9.91	0.83 ± 0.62	
		RF	75	27.34 ± 10.11	0.78 ± 0.11	
	M	MU	75	13.88 ± 5.30	0.78 ± 0.25	Translucent to whitish
		OD	135	12.57 ± 12.93	0.85 ± 0.29	
		RF	30	33.17 ± 7.05	0.89 ± 0.13	
III. Growing	F	MU	95	18.76 ± 6.46	1.14 ± 0.25	Light orange to to intense orange
		OD	89	16.74 ± 8.62	1.03 ± 0.36	
		RF	61	40.06 ± 16.16	1.18 ± 0.12	
	M	MU	167	21.59 ± 6.60	0.89 ± 0.23	White to cream
		OD	86	19.05 ± 4.92	0.88 ± 0.23	
		RF	15	36.13 ± 2.75	0.84 ± 0.05	
IV. Mature	F	MU	112	25.30 ± 5.96	1.42 ± 0.37	Intense red
		OD	97	25.29 ± 10.93	1.42 ± 0.35	
		RF	137	47.60 ± 22.67	2.39 ± 0.51	
	M	MU	126	30.80 ± 14.32	1.21 ± 0.43	Cream to beige
		OD	134	28.21 ± 7.17	1.22 ± 0.42	
		RF	90	61.54 ± 10.25	2.35 ± 0.31	
V. Partly-spawned	F	MU	15	17.73 ± 9.03	1.42 ± 0.19	Pale orange to yellow
		OD	11	16.91 ± 9.00	1.43 ± 0.20	
		RF	60	26.52 ± 4.98	1.95 ± 0.18	
	M	MU	15	12.93 ± 4.92	0.79 ± 0.06	Beige with brown patches
		OD	35	12.20 ± 6.36	0.89 ± 0.15	
		RF	60	49.03 ± 52.72	1.51 ± 0.30	
VI. Spent	F	MU	91	7.22 ± 1.92	0.45 ± 0.06	Brownish to translucent with rust blotches
		OD	10	5.20 ± 2.53	0.39 ± 0.14	
		RF	45	12.00 ± 1.11	0.34 ± 0.06	
	M	MU	30	12.33 ± 1.51	0.45 ± 0.06	Brownish to translucent with rust blotches
		OD	6	6.33 ± 1.21	0.40 ± 0.04	
		RF	15	23.73 ± 1.03	0.25 ± 0.06	



**Supplementary material 8.** Size (a), gutted weight (b) and total weight (c) at first sexual maturity in *H. arguensis*.



**Supplementary material 9.** Percentage of organic matter *versus* percentage of carbonates in each of the studied locations.