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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ABSTRACT

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

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1. Introduction

Sea cucumbers are bottom-dwelling echinoderms found in all regions of the ocean, from intertidal to deep-sea and from polar to tropical regions (Conand, 1989, 2004). As sea cucumbers are mostly deposit feeders and bioturbators, they have a key role in maintaining healthy marine ecosystems by mixing of sediments, recycling of nutrients, stimulating algal growth, and regulating both carbonate content and water pH (Massin, 1982; Purcell, 2004; Purcell et al., 2016; Schneider et al., 2011; Uthicke, 2001a,b; Wolkenhauer et al., 2010). Besides their ecological importance, sea cucumbers represent an important fishery resource mainly exported to Asian countries (Bordbar et al., 2011; Chen, 2003; Chen, 2004; Conand,

1989). Currently, at least 66 sea cucumber species are fished worldwide in more than 70 countries (Purcell, 2010; Purcell et al., 2013).

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

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

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

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

Sea cucumbers display reproductive cycles typically associated to predictable fluctuations in environmental factors maximizing the fertilization success through synchronization between individuals (Levitan, 1995; Mercier and Hamel, 2009). Reproductive activity is thought to be regulated by endogenous and exogenous cues such as photoperiod, water temperature, salinity, food availability, tidal flow, light intensity and phytoplankton blooms (Conand, 1981; Drumm and Loneragan, 2005; Hamel et al., 1993; Hamel and Mercier, 1996b; Ramofafia et al., 2000, 2003). However, the specific influence of each of these factors has rarely been explicitly tested (Mercier and Hamel, 2009).

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

2. Materials and methods

2.1. Sampling locations and collection of specimens

Individuals of *H. arguinensis* were collected at three locations along the Algarve coast (southern Portugal) of similar latitude: Ria Formosa (RF; 37°00′35.02″N; 7°59′46.10″O), Sagres (SA; 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; Fig. 1). At each location, between 10 and 15 individuals were collected monthly from May 2013 to April 2014. The collection program was extended until December 2014 in Sagres for inter-annual variability analysis, and this location was chosen because it had a denser population of sea cucumbers in this area. Individuals of *H. mammata* were collected in Ria Formosa and in Olhos de Água in the Atlantic Ocean, and in Murcia

at Los Cocedores (MU; 37°24′20.39″N; 1°37′02.27″O; Fig. 1) in the Mediterranean Sea. Collections of *H. mammata* were more spaced in time than that of *H. arguinensis*, and around 20 individuals were collected each season, because of logistical factors and their low density in the Ria Formosa (González-Wangüemert et al., 2013a; Siegenthaler, 2013).

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

2.2. Biometric measurements

Immediately upon arrival at the laboratory, total length (TL), total weight (TW), body wet weight after dissection and removal of internal organs and coelomic fluid (gutted body weight, GBW) and gonad weight were measured for each individual. For histological analysis, a small piece (around 1 cm) in the tubule's mid region was removed and fixed in Bouin's solution for 24 h and was then stored in 70% ethanol. The remaining gonad was fixed in 10% buffered formaldehyde for measurement of the gonadal tubules and to estimate fecundity (see below). The gonad index (GI) was calculated for each individual as GI/GBW * 100 (Conand, 1981). Length-weight relationships (LWR) were inferred for each population according to GBW = a TL^b (Keys 1928), where GBW is the gutted weight in g, TL is the total length in mm, a is the regression intercept on the Y-axis and b is the regression slope.

2.3. Maturity stages of gonads

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

2.4. Fecundity and sexual maturity

Absolute fecundity was determined for 63 *H. arguinensis* and 27 *H. mammata* individuals from a small piece of gonad fixed in 10% formalin as described by Muthiga et al. (2009). The size/weight at first sexual maturity, defined as the size/weight when gonads of 50% of individuals were undergoing gametogenesis (stage 2, 3, 4,
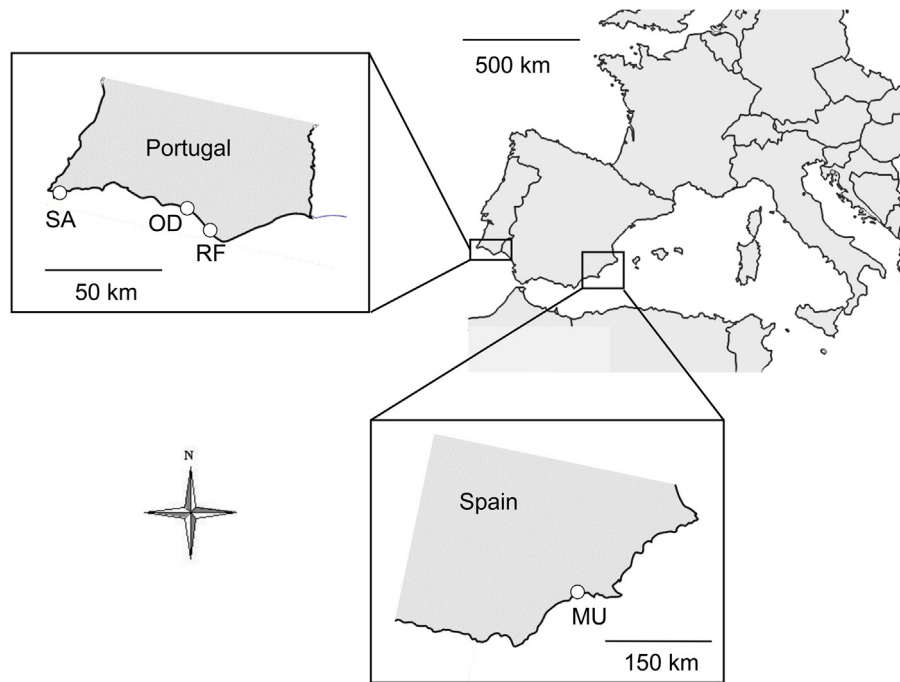
Fig. 1. Sampling locations of *H. arguinensis* (SA, OD and RF) and *H. mammata* (OD, RF and MU).

5 and 6) (Conand, 1981), was determined from 92 *H. arguinensis* (49 from 2013 and 43 from 2014) collected in SA between May and June (see Supplementary materials). This was not estimated for *H. mammata* due to lack of small sized individuals.

2.5. Environmental factors

Monthly averaged sea surface temperature (SST) and chlorophyll a concentration with a 4 km resolution were retrieved from the Moderate Imaging Spectroradiometer-Aqua (MODIS-Aqua) dataset available from the National Aeronautics and Space Administration (NASA) Goddard Earth Sciences Data and Information Services Center (GESDISC) between May 2013 and April 2014 for all studied locations, and until December 2014 for SA. Visualization was performed using Giovanni, a web-based application developed by the GESDISC (Acker and Leptoukh, 2007). As complementary information, the upper layer of the surface sediment where sea cucumbers feed was sampled in March 2014 at each location to determine (i) the organic matter content and (ii) carbonate content (see Supplementary material 1).

2.6. Statistical analyses

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

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

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

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

3. Results

3.1. Population characteristics

There were significant differences in TL among locations (PERMANOVA, df = 2, n = 552 for *H. arguinensis*, n = 165 for *H. mammata*, p(perm) < 0.001 for each species) and in GBW (PERMANOVA, df = 2, p(perm)<0.001 for each species) (Fig. 2). For *H. arguinensis*, TL and the GBW varied significantly and had the following order: SA > RF > OD (Post-hoc pair-wise tests, p(perm) < 0.001 for all combinations; Fig. 2a, b). *H. mammata* had similar TL (Post-hoc pair-wise tests, p(perm) = 0.97) and GBW (p(perm) = 0.84) at MU and OD, but at RF they were longer and heavier (p(perm) < 0.001 for all combinations; Fig. 2c, d). There was no sexual dimorphism in TL (*H. arguinensis* PERMANOVA, df = 2, interaction location
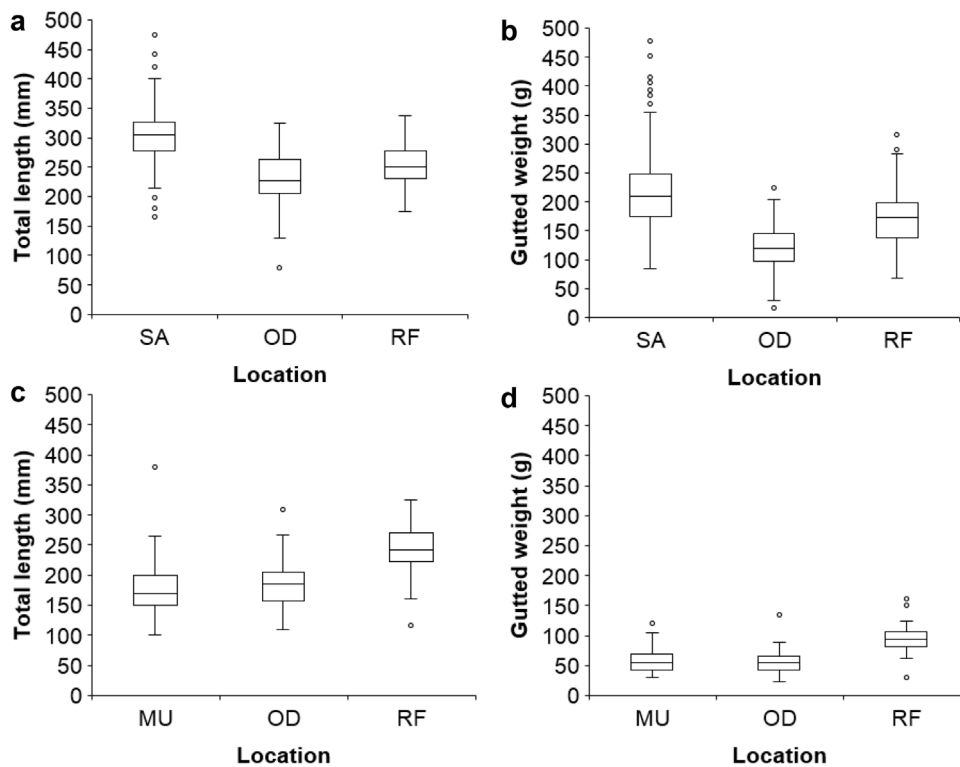
Fig. 2. Box-whisker plots of morphometric characteristics of the studied populations: (a, c) mean total length and (b, d) gutted weight of (a, b) *H. arguinensis* (SA: n = 288; OD: n = 165; RF: n = 178) and (c, d) *H. mammata* (MU: n = 80; OD: n = 100; RF: n = 45).

x sex, p(perm) = 0.37 and *H. mammata* p(perm) = 0.08) or GBW (*H. arguinensis* PERMANOVA, df = 2, interaction location x sex, p(perm) = 0.25 and *H. mammata* p(perm) = 0.08). The sex ratio in each population did not differ significantly from 1:1 (Chi-squared tests, *H. arguinensis,* df = 1, 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: $\chi^2 = 1.976$, p = 0.16; OD: $\chi^2 = 0.134$, p = 0.71; MU: $\chi^2 = 0.439$, p = 0.51).

Log(TL) was positively correlated to Log(GBW) with correlation coefficients of 0.529, 0.753, 0.483 at SA, OD and RF, respectively, for *H. arguinensis* (ANOVA, p < 0.001 for each population; Table 1) and 0.717, 0.679 and 0.623 at MU, OD and RF, respectively, for *H. mammata* (p < 0.001; Table 1). The slope *b* varied from 0.980 at RF to 1.437 at OD for *H. arguinensis* and from 0.924 at MU to 1.175 at OD for *H. mammata*. The slope *b* was significantly lower than 3 (Students *t*-test, p < 0.001 in each case), indicating negative allometry.

3.2. Gonad morphology

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

In *H. arguinensis*, cohorts of tubules at different stages were observed in a single gonad, varying in relative abundance and type between individuals and, in some cases, all tubules were at the same stage. Pre-vitellogenic oocytes lining the germinal epithelium were also observed side by side with mature oocytes (Supplementary material 4). After spawning, the spent tubules

were completely resorbed while other categories of tubules persisted in the gonad. The length and the diameter of the most represented category of tubules varied significantly between geographical locations according to the following order: SA > RF > OD (PERMANOVA, df = 2, p(perm) < 0.05 for each case; Post-hoc pairwise tests, p(perm) < 0.001 for each combination; Supplementary material 6). No sexual dimorphism was observed in tubule length (PERMANOVA, df = 2, location and sex interaction, p(perm) = 0.65) or diameter (p(perm) = 0.15).

In *H. mammata*, in each individual all tubules were at the same stage in all gonads analyzed. However, as in *H. arguinensis,* smaller oocytes were observed lining the tubular epithelium side by side with mature oocytes (Supplementary material 4). All tubules regressed after spawning. Tubule length and diameter of individuals from RF was significantly larger than those from MU and OD (PERMANOVA, df = 2, p(perm) < 0.001 in each case; Post-hoc pair-wise tests, p(perm) < 0.01 in each case; Supplementary material 7) but there were no significant differences in tubule length (p(perm) = 0.12) and diameter (p(perm) = 0.96) between individuals from the two latter locations. No sexual dimorphism was observed in tubule diameter (PERMANOVA, df = 2, location and sex interaction, p(perm) = 0.92). However, males had significantly longer tubules than females in RF and MU (PERMANOVA, df = 2, location and sex interaction, p(perm) < 0.05; Post-hoc pair-wise tests, p(perm) < 0.05 in both cases).

3.3. GI and gametogenesis

H. arguinensis – The reproductive pattern of *H. arguinensis* was similar in the three sampled locations. Four main phases were distinguished: (1) a growth phase characterized by a gradual increase of GI (2) a final maturation phase ending by the peak of GI, (3) a spawning phase characterized by a drastic decrease of the GI, and (4) a post-spawning resting phase characterized by a low and stable GI. Within these broad phases, differences in GI and gametogenic

Table 1
Characteristics and parameters of the length – gutted body weight relationships in each population for *H. arguinensis* (1) and *H. mammata* (2).

Species	Location	N	Length range (mm)		Gutted weight range (g)		a	b	S.E. b	r	t-value	p-value
			Min	Max	Min	Max						
1	SA	288	165	474	84.21	478.44	0.565	1.038	0.098	0.529	10.565	p < 0.0001
1	OD	178	80	325	17.47	223.68	0.047	1.437	0.094	0.753	15.177	p < 0.0001
1	RF	165	175	337	68.77	323.61	0.737	0.980	0.139	0.483	7.034	p < 0.0001
2	MU	80	100	380	33.00	104.00	0.474	0.924	0.102	0.717	9.098	p < 0.0001
2	OD	100	109	310	38.11	134.58	0.119	1.175	0.128	0.679	9.162	p < 0.0001
2	RF	45	117	325	29.57	162.07	0.373	1.002	0.192	0.623	5.227	p < 0.0001

Note: a and b are the parameters from equation $GBW = a\,TL^b$ in which GBW is gutted body weight and TL is total length. S.E. is the standard error.
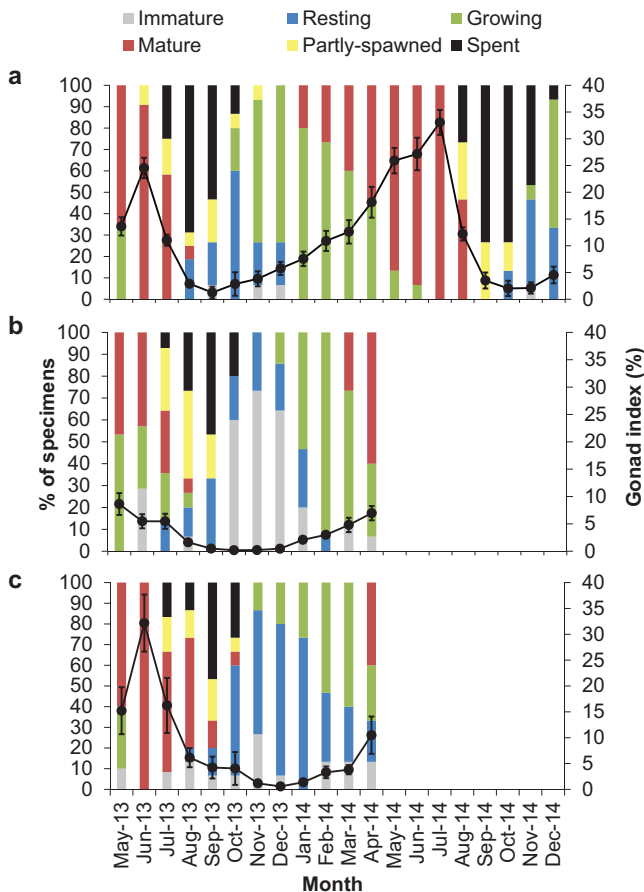

Fig. 3. Gametogenic cycle and mean gonad index ± SEM (line, right Y-axis) of *H. arguinensis* at (a) SA, (b) OD and (c) RF. Histograms show relative frequencies of gonad stages per month from May 2013 to April 2014 for OD and RF, and to December 2014 for SA (n = 10–15 per month). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

state were observed between SA, OD and RF (Fig. 3a–c). While the GI was not statistically different between SA and RF (PERMANOVA, df = 2, n = 506, location, p(perm) < 0.001; Post-hoc pair-wise tests, p(perm) = 0.14), both differed from OD (p(perm) < 0.001 for RF and SA). No significant differences in GI was found between males and females at the three locations (Mann-Whitney *U* tests, SA: U = 8588, p = 0.13; OD: U = 2028, p = 0.51; RF: U = 2025, p = 0.06).

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

at OD (8.63 ± 2.00%: p(perm) < 0.001 for all combinations). However, almost 30% of OD individuals were immature in June 2013.

During the spawning phase there was a decrease of more than 85% in mean GI at the three locations. This phase lasted over 6 months at SA, although it was particularly intense from July to October 2013 with a peak in August 2013 (68.75% of spent individuals). At OD and RF, the spawning phase extended over 4 months from July to October 2013, and was less intense than at SA and the highest percentage of spent individuals (46.67%) occurred in September 2013.

After release of the gametes, individuals entered a resting phase, the duration of which was considerably different between SA and RF. At SA, the individuals were predominantly in resting phase (60%) only in October 2013, and from then on most individuals (>60%) were already at the growing stage (GI ca. 4%). In contrast, at RF most individuals (>50%) were in the resting phase for four months, with a mean GI around 1%, from October 2013 to January 2014. At OD, the GI stayed low, at around 1%, from October to December 2013 with around 20% of individuals at the resting stage and more than 60% immature.

Gametogenesis started earlier at SA (October 2013) than at OD (December 2013) and RF (November 2013). Individuals at the gonad growth stage were predominant (>50%) for longer at SA (5 months) compared to OD (3 months) and RF (2 months). During gonad growth the GI increased by 342%, 388% and 227% at SA, OD and RF, respectively.

Comparison of the breeding season at SA between 2013 and 2014 over the 8-month overlap between May and December (Fig. 3a) revealed the monthly GI was significantly different between the two years (PERMANOVA, df = 1, n = 228, interaction year × month p(perm) < 0.001). In particular, post-hoc PERMANOVA tests revealed that the GI in May, July, August and September was significantly different between the two years (p < 0.05 for all combinations). The GI peak in 2013 was reached a month earlier (June) than in 2014 (July). However, the spawning phase started two months later in 2014 and a similar decrease of GI occurred in the two years (88.38% in 2013 and 86.27% in 2014). The spawning period extended from August to December in 2014, with 45.33% of spent individuals detected during that period compared to 41.38% in 2013.

H. mammata – The same four main reproductive phases were found for *H. mammata* as for *H. arguinensis* (Fig. 4a–c): (1) an increase of GI from spring to summer, (2) a drastic decrease of GI from summer to autumn, (3) a stabilization of GI from autumn to winter, and (4) a gradual increase of GI from winter to spring. The GI was significantly different between locations (PERMANOVA, df = 2, n = 225, p(perm) < 0.001). Individuals from OD and MU had the same GI (Post-hoc pair-wise tests, p(perm) = 0.77) which was significantly smaller than at RF (p(perm) < 0.001 for all combination). The GI peak in summer was significantly higher at RF (18.93 ± 4.15%) than at OD (6.23 ± 1.71%) and MU (3.54 ± 0.72%) (PERMANOVA, df = 6, interaction location x season, p(perm) < 0.01;
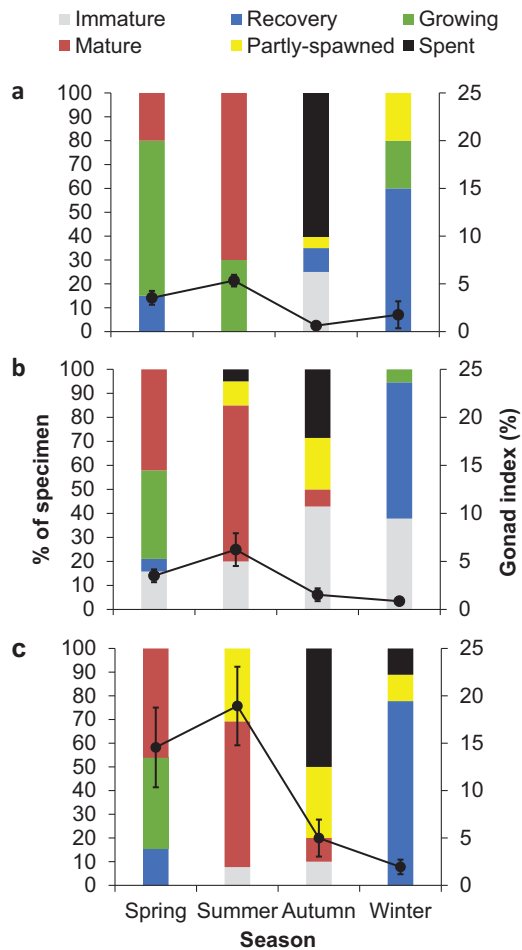
Fig. 4. Gametogenic cycle and mean gonad index ± SEM (line, right Y-axis) of *H. mammata* at (a) MU (n = 65), (b) OD (n = 90), RF (c, n = 45). Histograms show relative frequencies of gonad stages per season from Spring 2013 to Summer 2014. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Post-hoc pair-wise tests, p < 0.01 for all combination) with no differences between the latter two locations (Post-hoc pair-wise tests, p = 0.65). No differences in GI between males and females was detected at any of the three locations (Mann-Whitney *U* tests, MU: U = 395.50, p = 0.90; OD: U = 414, p = 0.07; RF: U = 182, p = 0.63).

From spring to summer, the mean GI increased by 30%, 78% and 51% in RF, OD and MU, respectively, (Fig. 4a–c) and most individuals (>60%) became mature. From summer to autumn, the GI decreased 74%, 75%, and 88% at RF, OD and MU, respectively. At RF and OD, more than 15% released their gametes in summer while at MU spawning occurred in the autumn. At RF spawning individuals (including partly-spawned and spent) were found in summer and winter, at OD in summer and autumn and at MU in autumn and winter. The highest percentage of spent individuals was found in autumn at all locations: 50%, 26% and 60% at RF, OD and MU, respectively. From autumn to winter, the mean GI stabilized at around 1% at OD and MU, while it was still decreasing from 5% to 2% at RF. Most individuals were at the recovery stage (>55%) in winter with some at the growing stage at OD (5%) and MU (20%). Interestingly, at MU only 5 individuals out of 20 had visible gonads in winter. Also at MU, the gonad growth stage was predominant in spring (65%) while at OD and RF during the same period was around 37% and mature were 45%.

3.4. Soma-gonad relationships

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

3.5. Fecundity and sexual maturity

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

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

3.6. Reproductive activity and environmental parameters

The mean monthly sea surface temperatures showed a clear seasonal cycle at the three locations with a maximum in September and a minimum in February (Fig. 7a). The mean sea surface temperature was slightly higher at RF ($17.8 \pm 0.95\,°C$) than at OD ($17.6 \pm 0.97\,°C$) or SA ($16.7 \pm 0.66\,°C$). Significant cross-correlations were found for *H. arguinensis* in 2013 between the mean GI and mean sea surface temperature in the three sites (Fig. 8a). The largest positive correlations were detected between lags +1 and +4, meaning that maximal GI values were reached from 1 to 4 months before the temperature peaked, while minimal GI values were reached from 1 to 4 months before the temperature minima. For the common 8-month sampling period, from May to December, in 2013 and 2014, at SA, significant cross-correlation between these two parameters was observed in 2013 at lag −1 while no correlation was found in 2014. The latter may reflect the difference in temperature between years with a decrease of temperature from June to July in 2014 when it increased gradually in 2013. In all locations gametogenesis started when temperature was decreasing (October–November), with the SA individuals started earlier. In contrast, gonad maturation was observed when temperature was increasing at OD and RF (March–April) whereas it started earlier at SA (January) when the temperature was still decreasing. Spawning started when temperature increased rapidly (June–July), with the highest percentage of spent individuals identified when the temperature was maximal at RF and OD (September). At SA, the highest percentage of spent individuals was found a month before the peak temperature (August). The difference in water temperature a month before and a month after the GI peak was $4.67\,°C$, $5.94\,°C$ and $6.05\,°C$ at SA, OD and RF, respectively. Although the peak of water temperature at SA was in September for both years, the GI
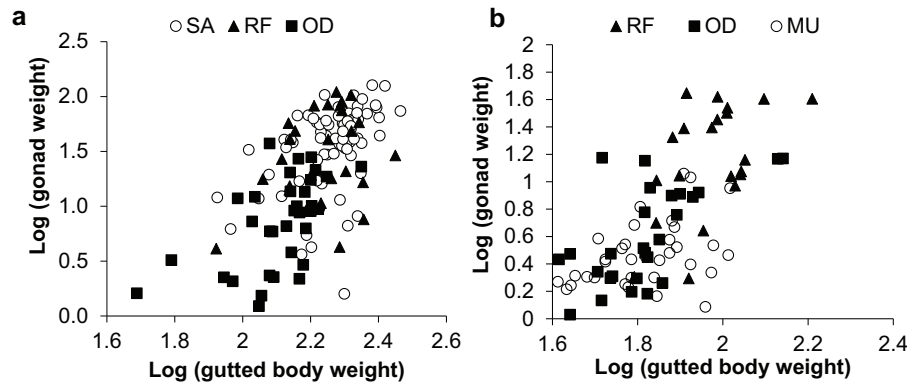
Fig. 5. Relationship between gonad weight and gutted body weight before spawning in (a) *H. arguinensis* in SA (n = 77, r = 0.50), OD (n = 37, r = 0.67), and RF (n = 27, r = 0.52), and (b) *H. mammata* in MU (n = 37, r = 0.56), OD (n = 31, r = 0.55) and RF (n = 23, r = 0.52). All Pearson's correlations were significant at the p < 0.05 level.
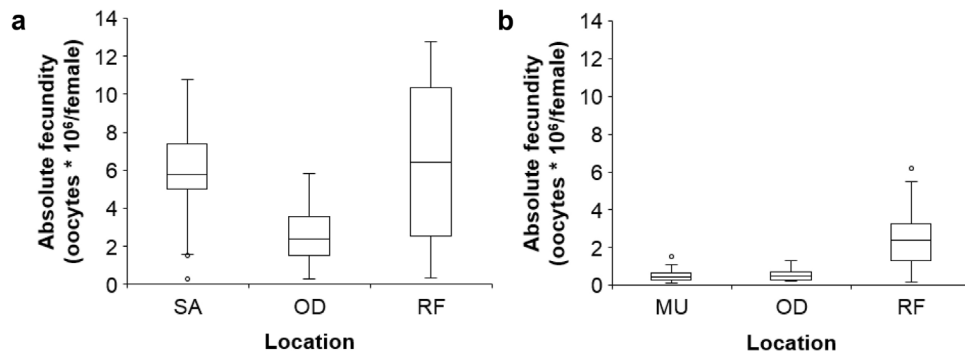

Fig. 6. Box-whisker plots of the absolute fecundity of mature females from (a) *H. arguinensis* collected at SA (n = 23), RF (n = 20) and OD (n = 20) and from (b) *H. mammata* collected in MU (n = 8), OD (n = 10) and RF (n = 9).
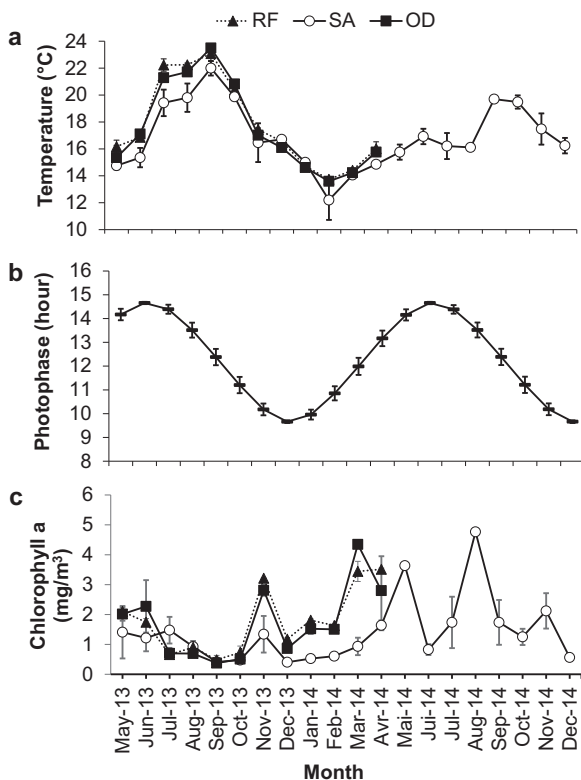

Fig. 7. Seasonal variations of environmental parameters: (a) sea surface temperature (°C), (b) photophase duration (h) and (c) chlorophyll *a* (mg/m³) at SA, RF and OD. See Materials and Methods for details.

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

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

The pattern of chlorophyll *a* did not show clear seasonal variation at the three locations (Fig. 7c). The mean concentration of chlorophyll *a* was similar at RF (1.78 ± 0.31 mg/m³) and OD (1.70 ± 0.35 mg/m³), but both had a higher mean value than SA (0.95 ± 0.13 mg/m³). All three locations had a peak of chlorophyll *a* in November 2013, corresponding to four to five months after the beginning of spawning in all locations. No significant cross-correlations were found between mean monthly GI and chlorophyll *a* concentration at any of the locations analyzed (Fig. 8c) or during the 8 months in common between both sampling years (2013 and 2014) at SA. However, the largest mean chlorophyll peak was in August 2014 (4.76 ± 0.01 mg/m³) coinciding with the beginning of the spawning (Fig. 7c).

The complementary information on sediment analysis at each location showed that the organic matter was significantly lower at OD (0.63 ± 0.03%) relative to RF (1.35 ± 0.18%), SA (1.07 ± 0.04%) and MU (1.73 ± 0.03%) (ANOVA, df = 3, n = 48; Tamhane test: 35.72, p < 0.001 for all combinations), with no differences among the
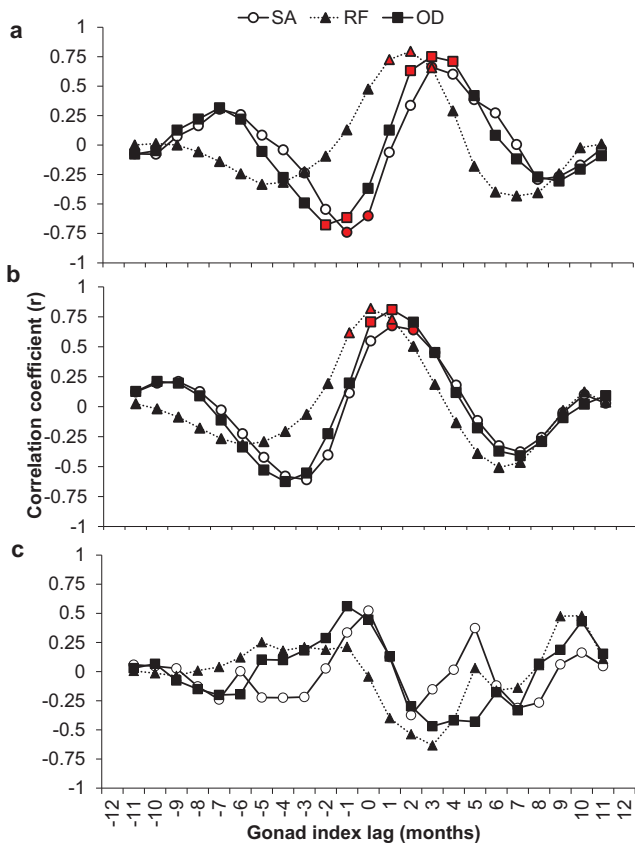
Fig. 8. Cross-correlation function analysis (CCF) between gonad index and (a) temperature, (b) photophase duration, and (c) chlorophyll *a* for *H. arguinensis* at each studied site. Red symbols indicate significant correlations at p = 0.05. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

latter. Mean values in carbonate content varied significantly between all four locations according to the following order: SA (52.46 ± 1.98%) > OD (27.46 ± 1.77%) > MU (17.95 ± 0.47%) > RF (8.90 ± 0.98%; ANOVA, arcsin transformation, df = 3, n = 48; SNK test, p < 0.01 in each case, Supplementary material 9).

4. Discussion

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

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

part of the body wall was independent the size of the individuals (Ramón et al., 2010).

The size distribution of individuals varied between locations suggesting the importance of local environmental conditions. However, population structure may also reflect differing recruitment dynamics between locations, particularly at OD where the proportion of immature individuals was highest for both species. The individuals of both species at this location, where the organic matter was the lowest, were also smaller. Intertidal conditions at RF may also be potentially more stressful (air exposure, variable temperature) compared to the more stable conditions in the subtidal SA where the individuals can spend more energy on growth rather than on physiological changes linked to survival during air exposure. For *H. mammata*, however, tidal constraints and food availability may not be the main factors affecting the inter-population size differences, since small individuals could be found in the subtidal zone at MU where the organic matter is high. Conversely, the smaller sizes at MU and OD could be a consequence of preference of this species for rough substrates where individuals adopt nocturnal feeding behavior (Aydin and Erkan, 2015; Navarro, 2012; Navarro et al., 2013a,b) in contrast to the soft substrate at RF where feeding activity may occur day and night. Although carbonate content has in some cases been associated with organic carbon productivity, the amount of carbonate in the sediments at the experimental locations does not seem to influence the size/weight of the sea cucumbers since it was the lowest at RF where larger individuals for both species were found.

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

First sexual maturity occurs later in *H. arguinensis* (for *H. mamata* it was not estimated) from SA (TL: 210–230 mm; EW:110–130 g; TW:220–260 g) than in *H. sanctori* from Gran Canaria (TL:201–210 mm; EW:101–110 g; TW:176–200 g) (Navarro et al., 2012). *H. arguinensis* had on average 5-fold higher absolute fecundity than *H. mammata*, but both had lower fecundity compared to larger tropical species at about $9–17.10^6$ oocytes/female according to Conand (1993). At equivalent sizes, our results obtained for *H. arguinensis* are similar to those estimated by Domínguez-Godino et al. (2015) for the same species, ranging from 1.5 to 9.6×10^6 released eggs per female, and also those of the temperate *H. forskali* that varied between 2 and 7×10^6 oocytes per female (Tuwo and Conand, 1994).

The difference in gonad morphology between *H. arguinensis* and *H. mammata* highlights the diversity of gametogenic processes that exists in the Holothuroidea. The gonads of *H. mammata* followed a uniform development, similar to what has been described in species such as *H. scabra* (Demeuldre and Eeckhaut, 2012), *H. fuscogilva* (Ramofafia and Byrne, 2001) and *H. spinifera* (Asha and Muthiah, 2007). The gonad development pattern in *H. arguinensis* generally followed the tubule recruitment model (TRM), with tubules organized in distinct cohorts, representing different maturity stages with a single generation of gametes within each tubule, and incomplete resorption of the gonad after spawning (Smiley, 1988). However, some specificities were observed: 1) some individuals had only tubules at one stage, 2) some tubules appeared

to have more than one generation of oocytes. Exceptions to the TRM have been also described for other species (Foglietta et al., 2004; Gómez, 2011) and included variations in gonad structure and development which were not only found between species but also between locations and seasons for the same species (Sewell et al., 1997). Possible effects of local environmental conditions on tubular development have been suggested (Hamel and Mercier, 1996b) but require systematic investigation.

The general pattern of reproduction was similar between the two species and populations in line with previous studies of sea cucumbers at narrow latitudinal ranges (Brewin et al., 2000; Byrne, 1990; Byrne et al., 1998; Kazanidis et al., 2014). The reproductive cycle was seasonal with spawning during the warmer period (summer-autumn) as typically described for temperate sea cucumber species (Costelloe, 1988; Despalatovic et al., 2004; Kazanidis et al., 2014; Mezali et al., 2014; Navarro et al., 2012; Sewell, 1992; Sewell and Bergquist, 1990; Tuwo and Conand, 1992). Temperature and photoperiod correlated positively to GI, with some lag in *H. arguinensis*, indicating their potential role in the regulation of the reproductive cycle, as previously found for other sea cucumbers (e.g. Conand, 1993; Hamel and Mercier, 1996b; Muthiga, 2006; Navarro et al., 2012; Ramofafia et al., 2000; Santos et al., 2015; Shiell and Uthicke, 2006; Tuwo and Conand, 1992). In the locations studied, gametogenesis initiated after the autumnal equinox, under short days (<12 h) and decreasing temperature (below 20 °C) whereas spawning started after the summer solstice when the photophase started to decrease and under increasing temperatures (above 20 °C). The importance of temperature is highlighted by the variability in the timing of gametogenesis and spawning between 2013 and 2014 and between locations. Furthermore, temperature shocks have been used by the aquaculture industry and in the laboratory to induce spawning in sea cucumbers (e.g. Domínguez-Godino et al., 2015; Mercier and Hamel, 2009; Smiley et al., 1991). In addition, factors such as the lunar cycle, phytoplankton blooms, tidal flux, light intensity, and social cues (e.g. aggregative behavior, diffusible chemical signals) have also been suggested to trigger spawning (e.g. Giese and Kanatani, 1987; Hamel and Mercier, 1996a, 1999; Leite-Castro et al., 2016; Mercier and Hamel, 2009).

It is tempting to suggest that the photoperiod is a permissive factor for both gametogenesis initiation and spawning and that temperatures below 20 °C are required for gametogenesis to develop and above 20 °C for spawning in the species studied. Furthermore, environmental factors such as food abundance associated to the more stable conditions of the subtidal habitat may facilitate faster replenishment of gonads after the spawning and ensure an extended release of gametes owing to storage of more nutrients (Bourgoin and Guillou, 1990; Byrne, 1990; Byrne et al., 1998). The prolonged spawning at SA could also be size-related, as larger animals are more fecund and generally known to spawn earlier and over a longer period (Scott et al., 2006; Secor, 2000). In contrast, at RF and OD the sea cucumbers were intertidal and subjected to repeated temperature and exposure to solar radiation that may have contributed to reduce the reproductive period and extended the recovery phase. Clearly, this is an area that requires investigation to pinpoint the specific contribution of the aforementioned environmental factors in the reproductive process.

Breeding periods coincide usually with optimal environmental conditions to maximize fertilization success and ensure offspring survival (Mercier and Hamel, 2009). Our results did not detect a relationship between the maturity stages or GI and chlorophyll a concentration. A sea surface chlorophyll *a* peak was only detected in November, 5–6 months after the beginning of the spawning, and was unlikely to influence larval development which in *H. arguinensis* has been estimated to be 18 days (Domínguez-Godino et al., 2015). Navarro et al. (2012) obtained a similar result for *H. sanctori* in the Canary Islands and suggested the larvae were able to

develop in low-food environment. However, the satellite measurements used in the present study may not reflect local conditions and therefore more focused studies on the early life stages would be required to determine the proximate factors critical for larval survival and growth.

5. Conclusion

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

Contributors

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

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national and institutional guidelines for the care and use of animals were followed.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fishres.2017.03.007.

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