Reproductive biology of the holothurian *Parastichopus regalis* in the Mediterranean Sea and its implications for fisheries management

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ABSTRACT

The sea cucumber *Parastichopus regalis* is a fished resource in the northwestern Mediterranean Sea but there are no studies about its reproductive biology. The aim of the present study is to characterize the reproductive ecology of *P. regalis* including its sex ratio, a description of the gonad and the reproductive cycle to help the implementation of a sustainable exploitation plan. A total of 1728 specimens were dissected over two years (April 2012–March 2014). The sex ratio of *P. regalis* was 1♀:1.2♂. The occurrence of gonads showed seasonal patterns, progressively decreasing from April to August/September and increasing from November, with a peak in March. *P. regalis* had an annual reproductive cycle in the northwestern Mediterranean Sea, with gonad maturation during spring and spawning in summer. No gonads were found in autumn, thus the gonadal cycle started in winter. Gonadal tubules had a larger diameter in spring in females, but seasonal differences were not found in the tubule diameter for males. The maximum percentage of *P. regalis* specimens with gonad per 1 cm size class did not exceed 24%. These results characterize the reproduction of *P. regalis* filling a key information gap for future management of the commercial fishery, which is currently not regulated. As a fishery management strategy, we propose one closed season from April to June to protect the reproductive period. The influence of evisceration in the calculations of basic fishery parameters based on the presence or weight of the gonad (size at first maturity, gonadal index) further emphasizes the importance of this study.

1. Introduction

Holothuroids are echinoderms found throughout the marine environment, from intertidal areas and shallow seas to abyssal depths. They are a conspicuous and diverse group in the world's oceans, and provide important ecosystem services as their bioturbation and deposit feeding activities enhance nutrient cycling and local productivity in sediments (Purcell et al., 2016). At least 60 species of sea cucumbers are harvested worldwide, including in the Pacific, Atlantic, and Indian Oceans, and the Mediterranean, Caribbean, Arabian, and North Seas (Purcell et al., 2014, 2016). Holothurians are mainly consumed in Asia, where they are regarded as traditional medicine and a delicacy. Sea cucumbers may be sold live, fresh, frozen, and as "*trepang*" (gutted, boiled, and dried). Their edible part is usually the thick body tegument although other tissues may be consumed (Purcell et al., 2012). The rising demand in these markets has resulted in declines of many sea cucumber populations worldwide (Purcell et al., 2012; Toral-Granda et al., 2008). Growing interest in stock restoration has led to significant advances in culture

techniques for the more valuable sea cucumber species (Lovatelli, 2004).

The sea cucumber *Parastichopus regalis* (Cuvier, 1817) belongs to the family Stichopodidae and its distribution area includes the Mediterranean, the eastern Atlantic from the south of the Canary Islands to the north of Ireland, and the Antilles and Gulf of Mexico in the western Atlantic (Míguez-Rodríguez, 2009; Tortonese, 1965). It is a benthic species found at a wide range of depths (5–800 m according to Tortonese, 1965), being more abundant between 50 and 300 m in the Mediterranean (Ramón et al., 2010). *P. regalis* is commercialized for human consumption in Catalonia, Valencia and the Balearic Islands (Western Mediterranean), where it is captured by trawling and consumed locally (Ramón et al., 2010). It is the most expensive sea product in the Catalan market, where prices in the auction ranged between 64 and 129€/kg in 2019. The first study to evaluate the potential of *P. regalis* as a fishery resource in Galicia (northwest Spain) was carried out by Míguez-Rodríguez (2009). In the Sole Bank (northeast Atlantic), the exploitation of *P. regalis* by the Spanish fishing fleet began in 2016

(González-Wangüemert et al., 2018). The fishery of holothurians has been extended to other Mediterranean countries such as Turkey, where sea cucumbers are not consumed domestically, but instead are exported and consumed in Asia (Aydin, 2008). The edible part of *P. regalis* is not the body tegument but the five longitudinal muscle bands, which are sold fresh. To our knowledge, there is only one other species harvested for its muscle strips, the sea cucumber *Apostichopus californicus* in Alaska (Bechtel et al., 2013).

P. regalis is a large, common sea cucumber of ecological and economic importance. However, information on its biology and ecology is scarce, with some exceptions on basic biology, food selection, and genetics. A first study reported a multimodal length-frequency and an aggregated spatial distribution in Balearic Sea populations, providing information on abundance by depth, length-weight relationship, market value, and catch per unit effort (Ramón et al., 2010). Later studies have addressed additional topics. For example, Maggi and González-Wangüemert (2015) analyzed the genetic differentiation among populations along the Western Mediterranean Sea and found lower genetic diversity and few recent and exclusive haplotypes in *P. regalis* specimens collected in Catalonia compared with locations that lack fishery pressure. Those authors suggested that the loss of haplotypes may be a consequence of fishing pressure and proposed, among other measures, studying the reproductive cycle for appropriate management of the resource. Galimany et al. (2018) analyzed the immune response of *P. regalis* when exposed to different temperatures and demonstrated that the species has a maximum thermal tolerance below 23 °C. Recently, an analysis of feeding behavior by Ramón et al. (2019) showed that *P. regalis* is able to select the sediment ingested by choosing the smaller particles available. Considering the increasing interest in the *P. regalis* fishery and that a proper assessment of any fishery requires a complete understanding of the biology, including reproduction, life cycle, and distribution, efforts must be made to expand the limited existing knowledge on its life history traits.

Holothurians reproduce sexually even though some species are also capable of asexual reproduction (Conand et al., 2002). They are mainly gonochoric and have a single gonad comprising one or two tufts of elongate tubules attached to the dorsal mesentery (Conand, 1989). When spawning, gametes are freely released to the sea water. In some species, when gonads are mature, the tubules of males and females can be distinguished by their different color (Hamel et al., 1993; Navarro et al., 2012). It has been described that the thickness of the gonadal tubules may be an indicator of sexual maturation (thicker tubules have higher numbers of oocytes), and can show a seasonal pattern, such as in *Holothuria whitmaei* (Shiell and Uthicke, 2006). However, there is no information on the reproductive biology of *P. regalis*. Therefore, this study analyzes important aspects of the species related to reproduction, such as gonad characteristics, reproductive cycle, population sex ratio, and size at first maturity. This knowledge will assist in the development of sustainable management actions for this target species, such as a closed fishery season along with a minimum landing size.

2. Material and methods

2.1. Collection of samples

Parastichopus regalis were collected monthly on board a commercial otter trawler, between April 2012 and March 2014, in the fishing grounds of Arenys de Mar (41.5768°N, 2.5593°E, NW Mediterranean), at 50–150 m depth. On seven occasions (May and October 2012, and January, April, May, August and October 2013), all the specimens caught at each of the 3–4 hauls performed during the day were dissected on board (Table 1). Length (L) was measured dorsally from the mouth to the anal orifice. A longitudinal incision was made along the lateral surface to record the presence of gonadal tubules. In addition, throughout the two years of study, about thirty holothurians were kept monthly in coolers with seawater and brought to the Aquaria and

Table 1

Details of the studied samples: date of the collection, total number of holothurians analyzed, average length (cm), average body weight (g), occurrence of gonads, and special observations. Av. L: average length; Av. G. W: average gutted weight; G. n: number of gonads; G. O: gonad occurrence.

Date	Ind	Av. L.	Av. G. W.	G. n.	G. O. (%)	Observations
12/04/2012	31	17.2	142.14	13	41.94	
15/05/2012	30	19.1	142	17	56.67	
19/06/2012	23	19.98	162.13	5	21.74	
28/06/2012	127	15.29	–	1	0.79	Dissection on board
30/07/2012	32	17.8	100.72	4	12.50	
28/08/2012	25	19.1	116.78	5	20.00	
25/09/2012	36	15.56	97.59	0	0	
04/10/2012	198	14.26	–	0	0	Dissection on board
24/10/2012	30	17.83	116	0	0	
28/11/2012	32	16.39	121.68	0	0	
19/12/2012	30	16.5	136.3	3	10.00	
24/01/2013	30	14.97	128.28	4	13.33	
31/01/2013	243	13.11	–	0	0	Dissection on board
13/02/2013	30	15.83	212.48	10	33.33	
23/03/2013	30	16.42	299.66	19	63.33	
15/04/2013	228	14.6	–	51	22.37	Dissection on board
02/05/2013	25	14.42	167.83	3	12.00	
23/05/2013	78	17.05	–	19	24.36	Dissection on board
13/06/2013	30	17.2	284.93	6	20.00	
25/07/2013	31	18.11	268.9	4	12.90	
07/08/2013	154	17.48	–	1	0.65	Dissection on board
09/2013						No sampling
04/10/2013	109	14.02	–	0	0	Dissection on board
24/10/2013	26	12.54	108.27	0	0	
11/2013						No sampling
11/12/2013	30	13.8	171	0	0	
16/01/2014	30	16.03	238.37	5	16.67	
13/02/2014	30	16.35	235.13	16	53.33	
26/03/2014	30	16.12	235.93	22	73.33	

Experimental Chambers (ZAE) of the Marine Sciences Institute (ICM-CSIC) to be dissected the day after. Length, total weight before dissection (TW) and eviscerated weight (EW) were quantified, and the presence of gonads was examined.

2.2. Gonadal characterization

Gonads were carefully towel-dried, weighed (GW) to the nearest 0.01 g, and their color was recorded. Gonadal index (GI) was calculated monthly from April 2012 to March 2014, using the equation (Ramofafia et al., 2000):

GI = (GW/EW) × 100.

The diameter of the gonadal tubules was studied to identify differences by sex and season. Monthly samples of gonads from 4 to 10 individuals (depending on their availability along the reproductive cycle) were analyzed over one year (April 2012–March 2013). Five photographs were taken of each gonad in different areas with a binocular microscope (Leica M205C) equipped with a digital camera (Leica DFC450). Five tubules were chosen from each photograph and the diameter in three haphazard spots for each tubule was measured with the image processing program ImageJ (Schneider et al., 2012). Thus, for each gonad, a total of 75 measures were taken.

2.3. Histology

Gonadal tubules were removed and fixed in 4% buffered formaldehyde solution for 24 h. The tissues were then rinsed in tap water and transferred to 70% alcohol where they were kept until processing. The samples were progressively dehydrated in an ethanol series and finally embedded in paraffin. After processing, serial sections of 5-μm thickness were cut using a manual rotation microtome, placed on glass slides and stained using a hematoxylin-eosin staining procedure. Slides were examined under a light microscope at 10 ×, 40 × and 100 × magnifications, to identify the sex and assign a gonadal developmental stage.

Gonad development was classified using five stages, which were described based on previous studies performed with holothurian species of the F. Stichopodidae (Conand, 1993; Fajardo León et al., 2008).

2.4. Statistical analysis

The relationship between the variables gonad weight (GW) and length (L) was determined using the allometric model $W = aL^b$, selecting only animals that were collected during the periods with the highest frequency of mature gonads. The coefficients of determination (R^2) were also calculated to describe the proportion of the variation accounted for the independent variable.

Mean male and female length and weight were compared with a *t*-test. Sex ratio was tested for a theoretical 1:1 relation using a Chi-square (χ^2) test.

Differences in the variable tubule diameter by factors sex and season were tested using a two-way ANOVA. Seasons were defined as follows: Winter: December, January, and February; Spring: March, April, and May; Summer: June, July, and August; and Autumn: September, October, and November. Diameter data were checked for normality using a Kolmogorov-Smirnov test and homogeneity of variances using a Levene's test. From these results, three variables were log-transformed to achieve normality, i.e. length (Shapiro Wilk test, W = 0.978, p < 0.05), gonad tubule diameter (Shapiro Wilk test, W = 0.958, p < 0.001), and gonad weight (Shapiro Wilk test, W = 0.829, p < 0.001). Statistical analysis was performed with IBM SPSS statistics software.

3. Results

A total of 1.728 holothurians were dissected during the two sampling years. Specifically, 1137 individuals were dissected on board the vessel and 591 in the laboratory (Table 1). Of the 1728 holothurians, only 202 of them (12.04%) had visible gonads. The occurrence of gonads in the holothurians was seasonal, with a decrease from May or March (depending on the year) to August/September, and an increase between November–December and March–May (Fig. 1). The months with the highest gonad occurrences (> 50%) were May 2012, March 2013, and February and March 2014. No specimens with gonads were found from September to November–December in either year. Occasionally, gonad evisceration of some specimens was observed onboard, as well as in the acclimation tanks in the laboratory or after handling the sea cucumbers (Fig. 2).

There were significant differences in holothurian length by sex (t
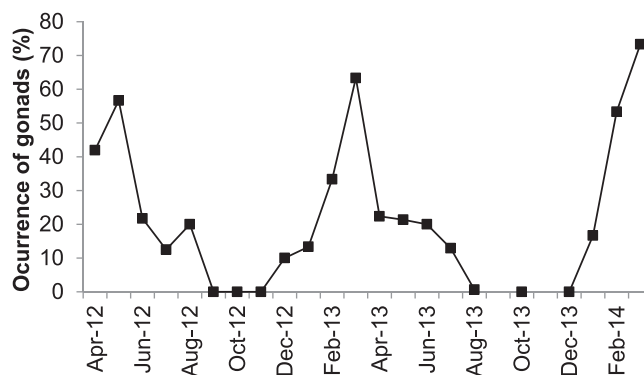

Fig. 1. Monthly evolution of the occurrence of gonads in the *P. regalis* individuals analyzed.

Fig. 2. Photograph of *P. regalis* specimens placed in acclimatization tanks after arriving at the laboratory. The evisceration of the gonads (arrow) can be seen in one of the individuals probably due to handling stress.

(142) = 2.145, p = 0.034), with males being smaller. Average length was 18.45 ± 3.16 cm for males and 19.67 ± 3.53 cm for females. Eviscerated weight also showed significant differences between sexes (t (112) = 2.694, p = 0.008), with males being lighter than females. Average gutted body weight was 178.03 ± 60.04 g for males and 209.21 ± 63.49 g for females.

There was no significant correlation between holothurian length and gonad weight for either of the sexes in individuals with the highest frequency of mature gonads (females: $Y = 0.302X^{0.257}$, $R^2 = 0.0009$, p = 0.887, n = 28; males: $Y = 0.573X^{0.069}$, $R^2 = 0.00008$, p = 0.957, n = 37) (Fig. 3A). The same was true for the gutted body weight and gonad weight (females: $Y = 0.0005X^{01.376}$, $R^2 = 0.047$, p = 0.268, n = 28; males: $Y = 0.000075X^{1.754}$, $R^2 = 0.163$, p = 0.014, n = 37) (Fig. 3B). Gonad weight had values between 0.09-4.18 g in males and 0.05–6.10 g in females.

Monthly values of the gonadal index showed high variability between individuals, in both males and females (Fig. 4A, B), especially in the months with high gonad occurrence (April and May 2012, March 2013 and February and March 2014).

3.1. Sex ratio

Of the 202 gonads, 183 were examined in the laboratory; 80 females, 98 males and 5 could not be sexed (indeterminate). The departure from a sex ratio of 1:1 was insignificant ($\chi^2 = 0.91245$, P = 0.339).

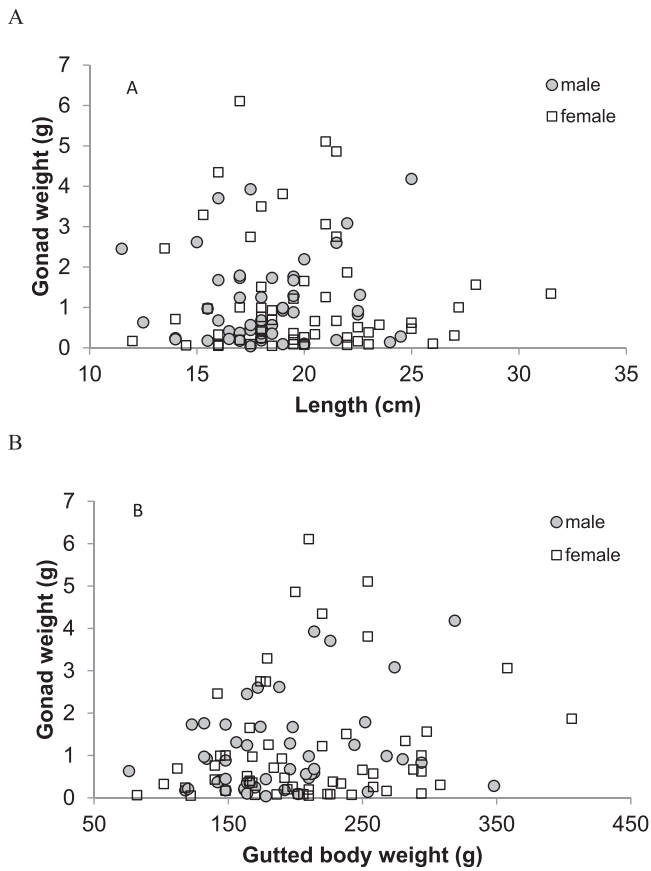Holothurian sizes examined ranged from 9 to 33 cm length. The size

Fig. 3. Relationship between the gonad weight of *P. regalis* to length (A) and dry body weight (B), by sex.

Fig. 4. Mean monthly gonad index of *P. regalis*. A: Males. B: Females.

Fig. 5. Length frequency distribution of the *P. regalis* individuals analyzed throughout the study period, by sex or with no gonad.

of the smallest holothurian identified as a male was 11 cm, whereas females were all larger than 13 cm (Fig. 5), thus all sizes below these are unsexed individuals. The size of the smallest sea cucumber with well developed gonads (Stage 3) was 15 cm for females and 16 cm for males. No males larger than 27 cm were found but the largest individual was a female of 33 cm in length. The proportion of mature individuals by size class increased from 15 cm (2.2%) to 25 cm, when the maximum percentage found in the study (24%) was reached (Fig. 6).

3.2. Gonad description

Parastichopus regalis is a gonochoric species with no external evidence of sexual dimorphism. The gonad consisted of a pair of tubule tufts arising from the gonad base (Fig. 7A), that was contiguous with the dorsal wall of the esophagus. The tubules were elongated and branched. Branching of tubules was by bifurcation, which occurred on previously branched segments, with branches varying in length (Fig. 7B, C). As maturation progressed the tubule coloration changed from translucent to cream in males and females, and the female gametes could be seen inside the tubules under a stereo microscope (Fig. 7E).

Tubule diameter in *P. regalis* ranged between 365 and 783 μm in males and 238–1111 μm in females (Fig. 8), with average values of 571 ± 165 μm and 688 ± 303 μm, respectively. The log transformed tubule diameter measurements were tested for differences in diameter by sex and season. A two-way ANOVA revealed that there was a statistically significant interaction between season and sex (F = 3.398, p = 0.042) (Table 2). Due to the significant interaction, the effect of season on the tubule diameters was analyzed for males and females separately with a one-way ANOVA. Simple main effects analysis showed that season did not find a statistically significant effect on the tubule diameter of males (F: 1.795, p = 0.193, Table 3A) but it was significant
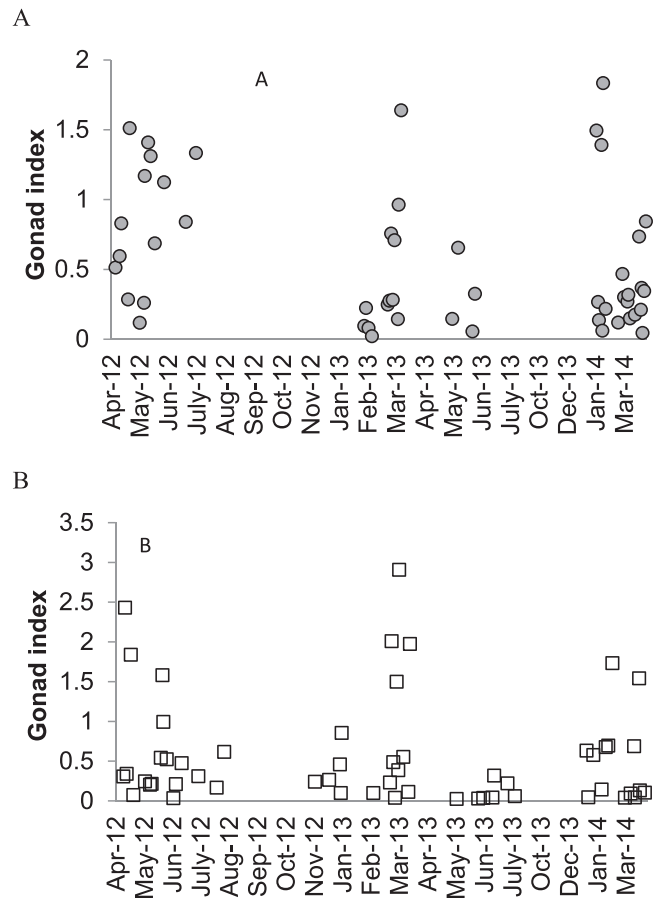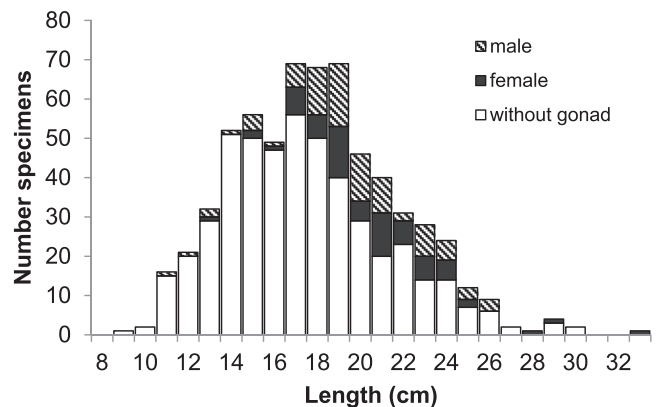
for females (F: 7.159, p = 0.003) (Table 3B). A Tukey's post-hoc test showed differences between spring and the remaining two seasons (summer and winter). Female tubules were wider in spring, with mean values of 800 ± 273 μm, compared to summer (446 ± 126 μm) and winter (563 ± 150 μm).

Differences in gonad weight by sex and season were tested using a two-way ANOVA. There were significant differences in gonad weight among seasons (Table 4), specifically between spring and winter (Tukey's post-hoc, p = 0.029). Gonads weighed more in spring than in winter, both for males and females (Fig. 9). However, there were no differences between sex or an interaction effect (sex × season).
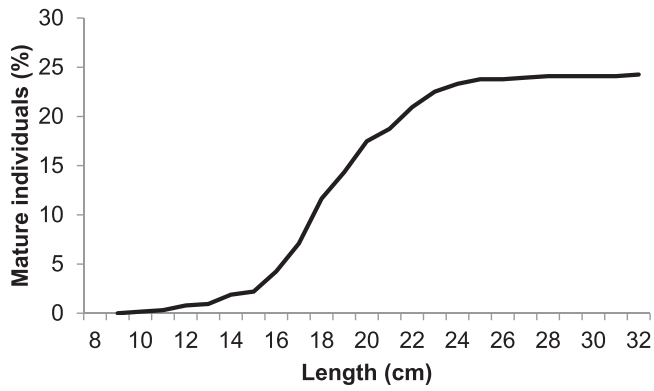
Fig. 6. Maturity percentage by size class throughout the study period.

3.3. Microscopic analysis of gonads

The reproductive cycle could be characterized by five stages in both males and females (Fig. 10), in addition to an undifferentiated stage.

(0) Undifferentiated stage. Translucent gonad not well developed, where sex cannot be defined.

3.3.1. Oogenesis

1) Development. Along the surface of the germinal epithelium, many small oocytes and some pre-vitellogenic oocytes are present. Production of previtelogenic spherical oogonia joined to the epithelium by a peduncle. Oocyte nucleus has a visible peripheral nucleolus. Large amount of connective tissue is present. Cell growth is observed towards the lumen and there is the presence of acini.
2) Growth. Lumen of tubules begins to fill with small oocytes and abundant previtellogenic oocytes are present along the surface of the germinal epithelium. In the lumen of tubules, large previtellogenic and vitellogenic oocytes are observed.
3) Mature. Tubules have thin walls and are dilated, almost completely filled with mature oocytes surrounded by their vitelline membrane. Each oocyte contains a well-defined germinal vesicle and a single nucleolus; a few immature oocytes may be seen.
4) Spawning: Oocytes are released during this stage; those that remain decrease in size and lose their shape. The lumen then has abundant empty spaces.
5) Post-spawning. Thin or broken gonadal wall with follicles collapsed, nearly empty, undergoing reabsorption. There is a large amount of connective tissue and the remaining follicles have irregular shapes with isolated and amorphous oocytes.

3.3.2. Spermiogenesis

1) Development. Tubule wall is thick and shows many deep invaginations. Spermatids heavily concentrate on the follicular walls. The tubules contain a few spermatozoa and nutritive phagocytes are common.
2) Growth. The tubule wall is thinner; its invaginations reach their maximum thickness and form a well-defined maze-like pattern in the tubular lumen.
3) Mature. Lumen reaches maximum diameter and is filled with mature spermatozoids leaving no empty spaces. The tubule wall is very smooth and stretched to its greatest extent.
4) Spawning. The lumen begins to evacuate the spermatozoa, resulting in a lower concentration of sperm in the tubules, including the appearance of empty areas along the length of the tubules. The tubule wall shows some invaginations.
5) Post-spawning. Tubule lumen is empty, with some residual spermatozoa present.

3.4. Reproductive cycle

There are minor differences in the reproductive cycle between years
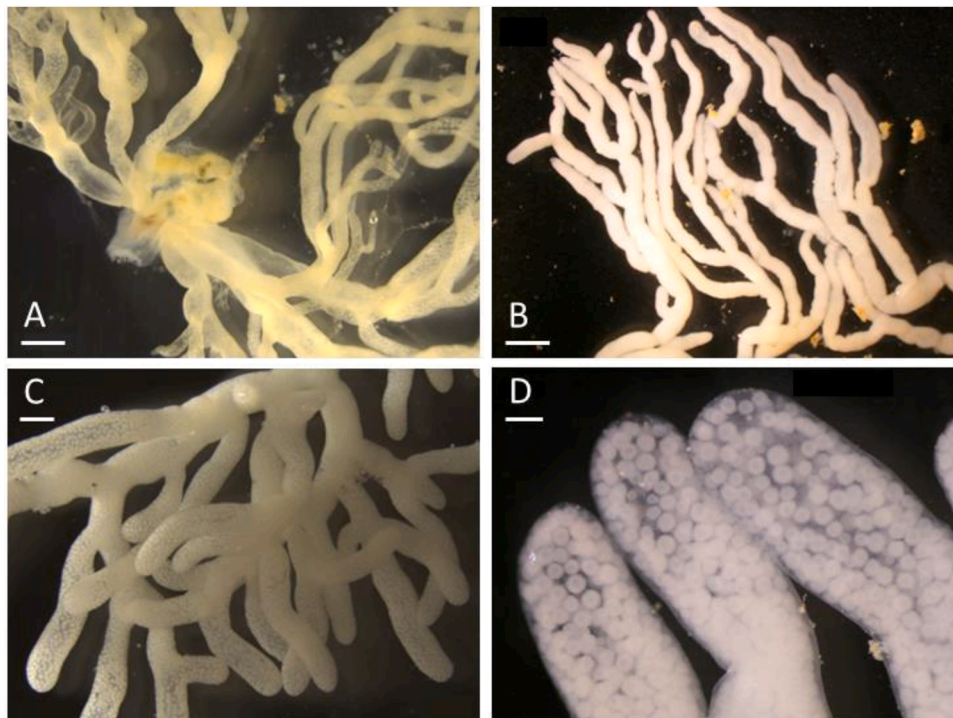

Fig. 7. Gonad morphology of *P. regalis*. A: Mature ovary showing the dichotomous tufts of tubules arising from a gonad base. B and C: Several tubules with bifurcations resulting in branches of variable lengths. D: Ripe ovary tubules with mature oocytes inside. Bar indicates 1 mm in A and C, 2 mm in B and 250 μm in D.
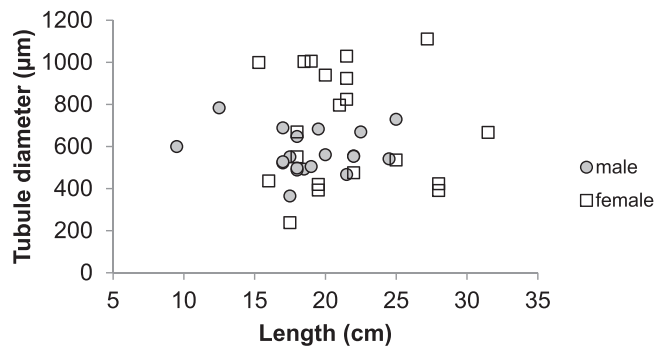
Fig. 8. Monthly mean diameter of *P. regalis* gonad tubules.

Table 2

Two-way ANOVA results for differences in the tubule diameter of *P. regalis* between sex and seasons. Asterisks indicate significant differences.

Factor	S. S	df	M. S	F	P
Season	0.152	2	0.076	6.150	0.004*
Sex	0.001	1	0.001	1.21	0.729
Season × sex	0.084	2	0.042	3.398	0.042*
Error	0.542	44	0.012		
Total corrected	0.834	49			

Table 3

One-way ANOVA results for differences in the tubule diameter of *P. regalis* among season for males (A) and females (B). Asterisks indicate significant differences.

A						
Factor	S.S	df	M.S.	F	P	
Season	0.019	2	0.009	1.795	0.193	
Error	0.098	19	0.005			
Total corrected	0.117	21				
B						
Factor	S.S	df	M.S.	F	P	Differences
Season	0.254	2	0.127	7.159	0.003*	Spring > summer
						Spring > winter
Error	0.444	25	0.018			
Total corr.	0.698	27				

for both females and males. In females, spawn and resting in 2013 was advanced by one month compared to 2012 whereas the start of the new cycle was delayed to January 14 instead of starting in December 13. In males, the spawning period in 2012 was very short compared to 2013.

The reproductive cycle is described seasonally. In autumn, there were no gonads in the specimens examined. Ripe females (Stage 3) were observed at the end of winter and throughout spring (Fig. 11A). Spawning (stage 4) occurred from April to June, whereas post-spawning (stage 5) extended from May to July in females. In December the cycle began again with the initial development of the gonad (stage 1), and its growth (stage 2) until early spring.

Early in the winter, there were either no gonads or no males found, depending on the sampling year. Later in the winter, males had primitive gonads in developmental stages (stage 1) and growth (stage 2) (Fig. 11B). In spring, mature males (stage 3) were already found. Stages of spawning (stage 4) and post-spawning (stage 5) were observed in males from the end of spring and during summer until July–August.

In most months more than one gonadal stage co-occurred, both for males and females. The gonadal cycle of females seemed to be somewhat advanced compared to males, with a longer spawning period. Undifferentiated individuals, where sex cannot be identified, were found in low numbers in May, August and December 2012 (1, 3, and 1 specimens, respectively).

Table 4

Two-way ANOVA results for differences in *P. regalis* gonad weight between sex and seasons. Asterisks indicate significant differences.

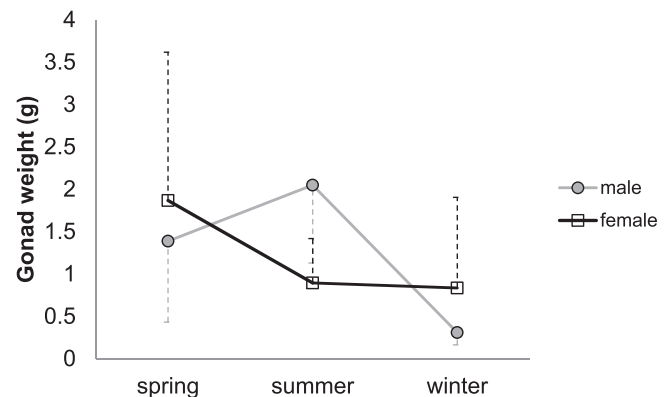|	S. S	df	M. S	F	P	Differences
Seasons	0.293	2	0.147	3.950	0.027*	Spring > winter
Sex	0.005	1	0.005	0.133	0.717	
Interaction	0.109	2	0.054	1.465	0.243	
Error	1.523	41	0.037			
Total corr.	1.90	46				

Fig. 9. Seasonal evolution of the gonad weight by sex. Discontinuous lines indicate SD.

4. Discussion

The gonad of *P. regalis* is similar to that of other members of the family Stichopodidae, consisting of two tufts of elongated tubules, one on each side of the dorsal mesentery (Conand, 1993; Koehler, 1921). Sea cucumbers show a well-defined annual reproductive cycle and many Aspidochirotida species spawn during the summer months (Foglietta et al., 2004; Smiley et al., 1991; Tanaka, 1958), as observed in this study. Seasonal reproductive cycles are characteristic of temperate shallow water invertebrates, where temperature is an important timing factor (Giese, 1959). For instance, the holothuriiadae *Holothuria tubulosa* spawns during the warm season, from July to September in the Adriatic Sea, when the surface water temperature ranges from about 22–26 °C (Despalatovic et al., 2004). Similarly, *Apostichopus mollis* has an annual reproductive cycle, spawning in the austral summer of New Zealand (Sewell, 1992). However, other holothurians spawn as a response to an increase in light intensity and the consequent phytoplankton bloom, such as *Apostichopus californicus* (Cameron and Fankboner, 1986). Another environmental factor that can influence the reproductive cycle in holothurians is pluvial precipitation, which explained 29% of the total variance of the gonadal index in the shallow species *Holothuria fuscocinerea*, in Mexico (Benitez Villalobos et al., 2013). Accordingly, the breeding season of *H. spinifera* (November–March) coincided with a period of rainfall, low salinity and temperature (Asha and Muthiah, 2008), which can promote high phytoplankton productivity. *Parastichopus regalis* has an annual reproductive cycle in the northwestern Mediterranean Sea, with the cycle starting in winter, gonad maturation occurring during spring, spawning in summer and complete gonad resorption in autumn. Temperature is not likely to influence the gonadal cycle of *P. regalis* because this parameter is quite constant at the depths in which the species was collected. Temperature values at 60–150 m depth are about 13–14 °C in winter, spring and summer when the reproductive cycle develops from initial phases to spawning (Fig. 7 in Vargas-Yáñez et al., 2017). In autumn, when temperatures decline with depth from 18 °C at 50 m to 13 °C at 150 m, no gonads were found in any *P. regalis* studied. Salinity could be another factor influencing the
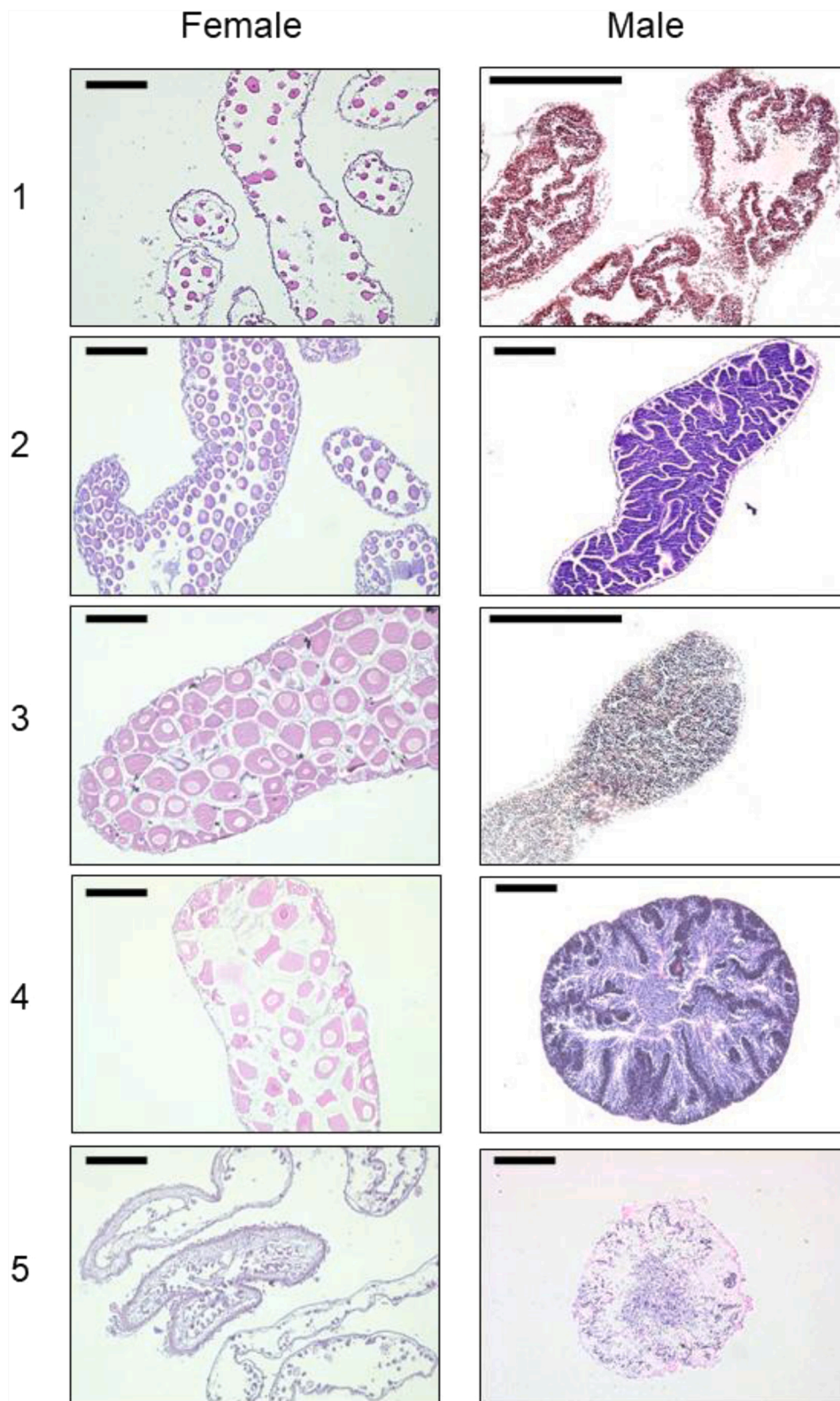
Fig. 10. Micrographs of sections of female gonad in the left column and male gonad in the right column. 1. Development, the gonad begins its development and spermatogonia appear; 2. Growth, lumen of tubules begins to fill with small oocytes in females and sperm in males; 3. Mature, the gonads are fully developed and ripe; 4. Spawning, the sexual cells are released and lumen fills with empty spaces; 5. Post-spawning; thin or broken gonadal wall, the lumen is nearly empty, undergoing reabsorption. Black bar is 200 μm.

reproductive cycle because salinity ranged from 38.3 psu in winter to 38.4 psu in spring and summer at 100 m depth (Vargas-Yáñez et al., 2017). Therefore, it does not seem to be a determinant factor. We could not gather information on food availability at the sampling location. Thus, the parameters triggering seasonality of gametogenesis and spawning of *P. regalis* are yet to be described.

The greatest monthly percentage of *P. regalis* specimens with gonads was 73% although most values were below 30%. It is not common to find, for so many months, such a low proportion of specimens with gonads (in addition to the months with a total absence of gonads). A study by Christophersen et al. (2020) on the gonadal development of *P. tremulus*, the closest species phylogenetically (currently the genus
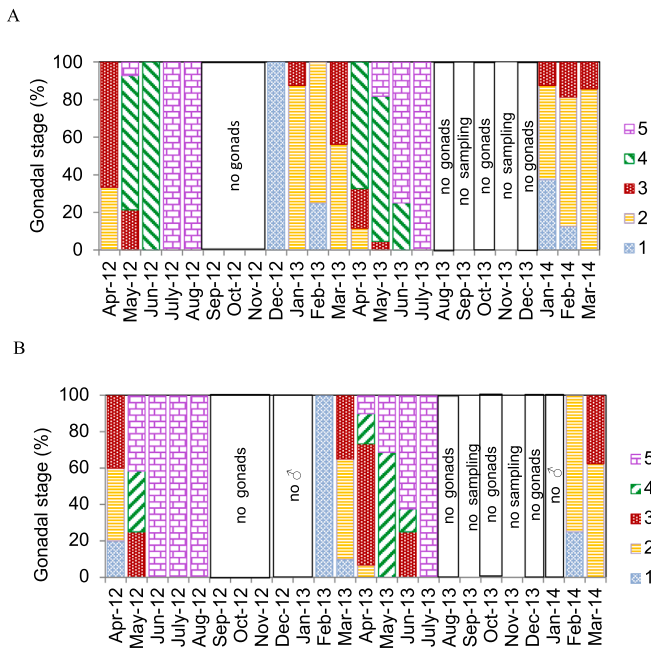
Fig. 11. Relative frequency of the different gonad development stages throughout the period of study for females (A) and males (B). Stage 1. development; Stage 2. growth;.
Stage 3. ripe; Stage 4. spawning; Stage 5. post-spawning.

Parastichopus is considered to have only two species; WoRMS, 2021), showed that 67–100% of the specimens have gonads every month of the year. These values are much greater that those reported here, which could be explained either by the fact that no selection of sizes was performed in our study (i.e., juveniles were sampled together with adults), or because some specimens had gonads but had expelled them. The latter explanation seems more feasible, for several reasons. On the one hand, sea cucumbers that did not possess gonads also had other organs missing (intestine, respiratory tree) and the likelihood of evisceration in the trawl was about 47%. On the other hand, the maximum percentage of mature *P. regalis* specimens per 1 cm size class did not exceed 24%, even for the larger specimens. Holothurians have the capacity to undergo evisceration and eject some of their viscera, which can be regenerated later (Bertolini, 1930). Some Aspidochirota showed a seasonal absence of viscera (Byrne, 1985). The manner of evisceration, which organs are expelled, and the order of subsequent regeneration, differ between holothurian orders and species (García-Arrarás and Greenberg, 2001). *P. regalis* lose the respiratory trees, digestive tube, hemal system, and gonads when eviscerating organs in stressful conditions (Bertolini, 1930). In fact, evisceration may not only explain the low percentage of *P. regalis* specimens with a gonad but also the lack of a relationship between body length/weight and gonad weight.

The gonadal index (G.I.) has been used to document the pattern of gonadal growth in most holothurian species such as *P. tremulus* (Christophersen et al., 2020), *Isostichopus badionotus* (Foglietta et al., 2004), and *Stichopus chloronotus* (Conand et al., 2002). Cameron and Fankboner (1986) found a high standard deviation on mean G.I. monthly values in *Apostichopus californicus*, especially when maximum gonad size occurred during summer. Our results showed a high dispersion between individuals of the G. I. monthly values, in both *P. regalis* males and females, and thus we do not recommend its use to obtain information on the reproductive cycle.

In December and January, when few *P. regalis* specimens had gonads, they were all females, which most likely indicate that females begin the gonadal cycle somewhat earlier than males. We found only five indeterminate specimens in the two studied years, which suggests that the transition from gonadal state 0–1 develops rapidly. In contrast, other

species such as *Stichopus horrens* (formerly known as *S. variegatus*) had undetermined individuals practically every month of the year (Conand, 1993).

The tubular diameter did not show significant differences between sexes in *P. regalis* This result contrasts with a common pattern found for many holothurians, in which female tubules are wider than male tubules, i.e. *Roweia stephensoni* and *Neostichopus grammatus* (Foster and Hodgson, 1995), *Holothuria sanctori* (Navarro et al., 2012), and *H. spinifera* (Asha and Muthiah, 2008). The mean values of tubule diameter found in *P. regalis* were comparatively much lower than in other Stichopodidae such as *Isostichopus fuscus* (Toral-Granda and Martínez, 2007), a shallow water species. The diameter of the tubules in females of *P. regalis* increased as the gonad developed from winter to spring, but not in males. In species such *H. spinifera* the tubules were present throughout the year and were not absorbed after spawning (Asha and Muthiah, 2008), unlike that observed in *P. regalis* (this study) and *Neostichopus grammatus* (Foster and Hodgson, 1995).

Minimum legal size limits for sea cucumber fisheries have been typically based on the size at first sexual maturity (Bruckner, 2006). First sexual maturity is defined as the size (LT50) or gutted body weight (GBW50) at which the gonads of 50% of the individuals are mature. The aim of this measure is to protect juveniles and recently matured adults from being fished, allowing these individuals to spawn before they can be marketed (Purcell et al., 2009). In *P. regalis*, the maximum percentage for each 1 cm length class of specimens with gonads found in this study did not exceed 24%, so the estimation of LT50 was not possible. The high percentage of specimens without gonads found may be related to the fact that *P. regalis* lives too deep to be collected by divers or artisanal fisheries, with trawls being the only fishing vessels able to catch them. Trawl fisheries favor evisceration as a result of harvest and handling but we cannot rule out that the high percentage of gutting could be a characteristic trait of this species, which differs from other holothurians where organ loss is rare or seasonal (Dimock, 1977; García-Arrarás and Greenberg, 2001). Therefore, some mature *P. regalis* could not be differentiated from non-mature individuals, preventing the calculation of LT50.

In conclusion, we have shown for the first time that *Parastichopus regalis* in the Mediterranean has an equal sex ratio and an annual reproductive cycle, with gonad maturation during spring and spawning in summer. Along with the reproduction cycle we aimed to study and estimate useful parameters to help the implementation of a sustainable exploitation. One of these parameters was size at first sexual maturity but it could not be estimated due to evisceration. Therefore, scientific management of this target species should be based on other biological aspects such as the establishment of a harvest season and a closure during spawning. In accordance, we propose one closed season from April to June. The influence of evisceration in the calculations of basic fishery parameters that are based on the presence or weight of the gonad (size at first maturity, gonadal index) further emphasize the interest in delving into unknown aspects of the biology of *P. regalis* and its implications for fishery.

CRediT authorship contribution statement

M. Ramón: Conceptualization, Formal analysis, Lab. sampling, Investigation, Writing – original draft, Visualization, Funding acquisition. **M. J. Amor:** Lab. work, Investigation. **E. Galimany:** Field and lab sampling, Investigation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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