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Reproductive Dynamics and Hermaphroditism in the Black-Footed Limpet (*Patella depressa* Pennant, 1777) on an Intertidal Rocky Shore on the Algarve Coast (Southern Portugal)

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Abstract: The present study aimed to describe the reproductive cycle of the black-footed limpet (*Patella depressa* Pennant, 1777) from an intertidal rocky shore on the Algarve coast (southern Portugal). Samples were collected monthly between January 2017 and December 2018, with the species' gametogenic cycle being described based on gonad histology and the mean gonadal index. The presence of both transitional and mosaic hermaphrodites indicates that some individuals are able to change sex (sequential hermaphroditism). Despite the occurrence of hermaphroditism, sex proportions were approximately equal, suggesting the absence of protandric sex change in this species. The population exhibited an extensive occurrence of ripe and spawning gonads throughout almost the whole study period, probably related to consecutive processes of gonadal re-ripening and partial spawning events. The reproductive dynamics of *P. depressa* displayed clear inter-annual differences, with a short resting period recorded in 2017 (June–August) and the absence of resting gonads in 2018. The continued monitoring of this population and collection of environmental data are required to further improve knowledge of the reproductive dynamics of this species. Such information is crucial for proposing additional management measures for the sustainable harvesting of limpets in southern Portugal.

Keywords: reproduction; hermaphroditism; gonad histology; mean gonadal index; spawning season; harvesting management measures

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1. Introduction

Limpets are common inhabitants of and dominant grazers on rocky shores worldwide, playing a key role in structuring intertidal communities and promoting ecosystem balance [1–4]. These marine gastropods are subject to highly variable and unpredictable environmental conditions [5] that can prompt significant changes in general physiological features, including reproductive biology and phenology [6]. In limpets, hermaphroditism might be a strategy to improve success in spawning and recruitment [7,8]. In general, hermaphroditism can be simultaneous (the individual releases both types of gametes) or sequential (individuals of one sex change to another sex later in the life cycle) [9,10]. Sequential hermaphrodites change sex from male to female (protandric species) or from female to male (protogynic species) [7,11]. In addition, in some species, the sex change can be reversed [12]. Protandry hermaphroditism has been reported in several species of the genus *Patella*, namely, *P. vulgata* [13,14], *P. ulyssiponensis* Gmelin, 1791 [14,15], *P. aspera* [16], *P. ferruginea* Gmelin, 1791 [17], *P. rustica* Linnaeus, 1758 [18] and *P. depressa* Pennant, 1777 [19]. Hermaphroditism is a relevant feature for the management of exploited populations of patellid limpets, because the removal of larger individuals might affect population dynamics and decrease reproductive success [17]. In addition, the lack of the predominant sex at a larger size can stimulate the occurrence of a sex change earlier at smaller sizes [12].

The black-footed limpet *P. depressa* is the dominant intertidal limpet in the mid intertidal zone across the Portuguese mainland coast, common in both rocky shores and artificial hard substrata (e.g., within harbors and marinas) [20–22]. Overall, the black-footed limpet is distributed from northern Africa to along the Atlantic coasts of Europe, including southwestern England and Wales [20,23,24]. Diverse limpet species have been traditionally exploited for human consumption in some coastal communities [25,26], such as Mexico and south of the United States [27], Chile [28], Asturias and Canary Islands [29,30]. In Portugal, limpets are collected by professional harvesters and occasionally by recreational harvesters, being a traditional and popular seafood species intensively exploited in the Madeira [31–33] and Azores archipelagos [16,34–37].

The reproductive biology of the black-footed limpet *P. depressa* has been studied throughout its geographic distribution range. This species' reproductive cycle was described for diverse populations, in the UK [23,38,39], on the Basque coast in Spain [40] and also in northern, central and southern Portugal [20,41]. Studies on protandrous sex change were performed in the UK [19] and also in Portugal, in the northern, central and southwestern coastal regions [19,22,41]. A study on the spatial variability in the *P. depressa* reproductive cycle and physiological condition was carried out by Fernández et al. [42] on the Galician coast of Spain. Morais et al. [43] examined gonadal development and fatty acid composition in *P. depressa* populations from exposed and sheltered coastal sites in central Portugal. In addition, studies on the reproduction techniques, larvae development and juvenile growth of the black-footed limpet in a laboratory were performed by Rebouta [44].

The sustainability of harvesting activities (professional or recreational) must be supported by solid knowledge of the reproductive biology of the target species. Therefore, the present study aimed to describe the reproductive cycle (based on gonad histology and mean gonadal index) and compare the main spawning season of *P. depressa* from the Algarve coast (southern Portugal) with that of other populations throughout the species distribution range. In practice, the following hypotheses were tested in this study: (1) the reproductive dynamics in this population of *P. depressa* are influenced by environmental parameters; and (2) this population of *P. depressa* exhibits hermaphroditism. Overall, the present study provides valuable information for proposing additional management measures for the sustainable harvesting of limpets in southern Portugal.

2. Materials and Methods

2.1. Study Area and Field Sampling

Individuals of *P. depressa* were sampled monthly between January 2017 and December 2018 on a rocky shore in Praia da Luz in Lagos (37°5.1′ N, 08°43.8′ W) located on the Algarve coast (southern Portugal) (Figure 1). The sampling area was selected from an extensive rocky area on a predominantly sandy coast as it presents a suitable topography and accessibility of the cliff, with a great local abundance of intertidal rocky fauna including limpet species. Each month, 20 limpets with a broad size range were collected manually and haphazardly along the intertidal area during low tides. In the laboratory, specimens were measured for shell total length (SL—longest distance along the shell dorsal surface) using a digital caliper (precision = 0.01 mm) and weighed for total wet weight (TW) on a top-loading digital balance (precision = 0.01 g).

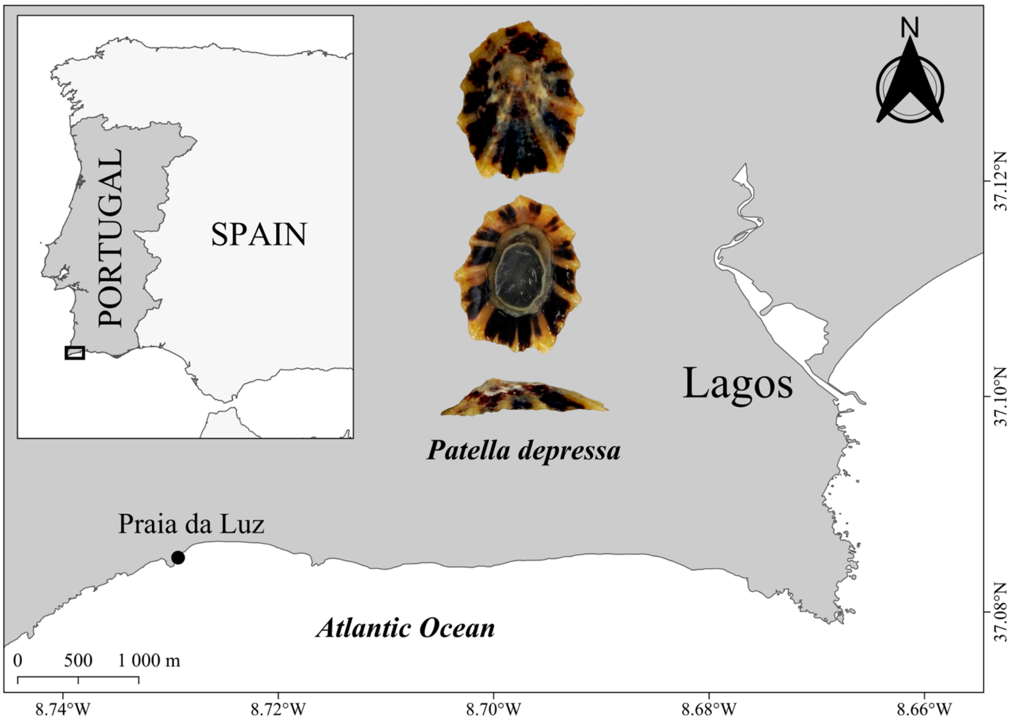

Figure 1. Map showing the location of the collecting site (black box) for the black-footed limpet (*Patella depressa*) on an intertidal rocky shore in Praia da Luz in Lagos (Algarve coast, southern Portugal).

Oceanographic data on surface seawater temperature (SST) and wave significant height (WH), recorded during the study period at the Faro oceanographic buoy (closest buoy to the collecting site: 36°54.3′ N, 07°53.9′ W) of the Portuguese Hydrographic Institute [45], were used to assess their influence on the species' reproductive dynamics. For this purpose, atmospheric data on air temperature (AT) and wind speed (WS) registered in the same period at the Faro airport station (37°02′ N, 07°96′ W) were acquired from Weather Underground [46].

2.2. Gonad Histology and Mean Gonadal Index

Specimens of *P. depressa* were fixed in a Davidson solution (composed of formalin 37%, ethanol 96%, glacial acetic acid and distilled water) for 48 h and then preserved and stored in 70% ethanol. Subsequently, tissues were dehydrated with serial dilutions in ethanol, infiltrated in xylene and embedded in paraffin wax. Afterward, gonads were sectioned (thickness = 7 μm) using an automated rotary microtome (Leica RM2155), stained with hematoxylin and eosin [47] in an automated stainer (Leica ST5010 Autostainer XL) and mounted with DPX using an automated glass cover-slipper (Leica CV5030). Finally, histological sections were examined under magnifications of 40×, 100× and 200× in an optical microscope (Leica DM750) in order to assign the limpets' sex and gonad maturity stage.

Gonad maturity stages were identified and classified using the microscopic maturation scale previously employed by Vasconcelos et al. [14]: inactive (stage 0), early development (stage I), late development (stage II), ripe (stage III), spawning (stage IV) and spent (stage V). Whenever diverse developmental stages were observed within a single gonad, the classification criterion was based on the prevailing maturity stage in the gonad. Limpets with inactive gonads were considered sexually undifferentiated. No maturation stage was assigned to hermaphrodite individuals, i.e., those with both male and female gonadal features.

Afterward, in order to estimate the mean gonadal index (GI), a numerical ranking was assigned to each maturity stage (inactive = 0; early development = 2; late development = 4; ripe = 5; spawning = 3; spent = 1) following the equation proposed by Seed [48]:

$$\mathrm{GI} = \sum (\text{inds. at each stage} \times \text{stage ranking})/\text{total inds. at each month}$$

2.3. Data Treatment and Statistical Analyses

The sex ratio in monthly samples of *P. depressa*, expressed as the proportion of females per male (F:M), was compared with parity (1:1) using the chi-square test (χ^2-test). Cumulative size–frequency distributions were plotted separately for males and females. Specimens' shell length (SL) and total weight (TW) were compared between sexes through an analysis of variance (ANOVA). Whenever ANOVA assumptions (normality of data and homogeneity of variances) were not achieved, the non-parametric Kruskal–Wallis test (ANOVA on ranks) was applied.

A correlation matrix was produced to assess eventual relationships between the mean GI and oceanographic and atmospheric parameters (AT, SST, WS and WH) in the region during the study period. Data normality was verified using the Shapiro–Wilk test, and then, the correlation between variables was evaluated through the Pearson (r) or Spearman (ρ) coefficients, whenever data were normally or non-normally distributed, respectively.

Data treatment and statistical procedures were performed using the software packages Microsoft Excel 2016 and R statistical language [49], with a statistical significance level of $p < 0.05$ considered.

3. Results

3.1. Population Composition and Sex Ratios

The population consisted of a total of 407 individuals of *P. depressa* (mean = 31.0 ± 5.8 mm SL; range = 16.2–45.8 mm SL), comprising 51.1% males, 36.6% females, 6.6% sexually undifferentiated individuals, 4.2% hermaphrodites and 1.5% specimens with gonads affected by parasites or pathologies. Most limpets with pathologies were excluded from further analytical procedures, namely, those with seriously affected gonads, mainly by trematodes (Platyhelminthes) (Figure 2).

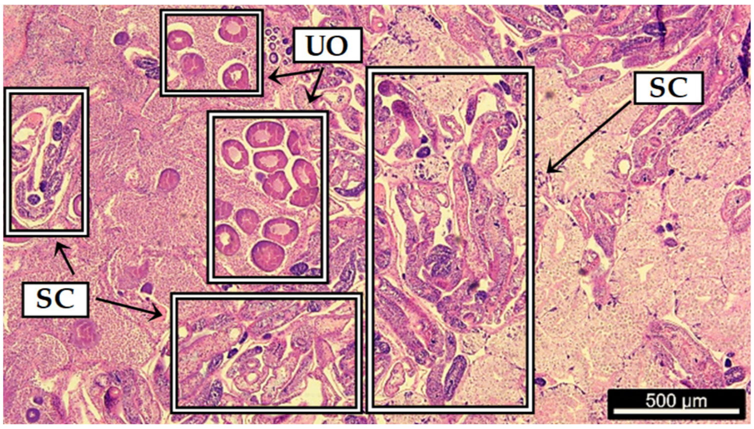

Figure 2. Histological section illustrating trematode infestations in a female gonad of a black-footed limpet (*Patella depressa*). UO: unspawned oocytes; SC: sporocysts with cercariae in different developmental stages.

The main descriptive statistics of the black-footed limpets from the Algarve coast are described in Table 1. In the overall samples examined during the study period, the sex ratio of *P. depressa* was significantly different from parity (1 M:0.72 F: $\chi^2 = 9.75$, $p = 0.002$).

In addition, three monthly samples also displayed significant deviations from parity (1:1), both with male-biased sex ratios, namely, November 2017 (1 M:0.13 F: $\chi^2 = 5.44$, $p = 0.020$), January 2018 (1 M:0.33 F: $\chi^2 = 5.00$, $p = 0.025$) and October 2018 (1 M:0.36 F: $\chi^2 = 4.26$, $p = 0.039$).

Table 1. Main descriptive statistics of the samples of black-footed limpets (*Patella depressa*) from the Algarve coast (southern Portugal). N: number of individuals; M: male; F: female; SL: shell length (mm); TW: total weight (g); SD: standard deviation.

Patella depressa	**N**	**Sex Ratio (M:F)**	**Mean SL ± SD (min–max)**	**Mean TW ± SD (min–max)**
Males	208	1 M:0.72 F	32.0 ± 5.0 (17.2–43.6)	4.3 ± 2.2 (0.6–11.7)
Females	149		31.6 ± 5.8 (16.2–45.8)	4.3 ± 2.8 (0.4–13.6)
Undifferentiated	27		22.3 ± 3.6 (11.7–30.0)	1.2 ± 0.6 (0.1–2.9)
Parasites/pathologies	6		28.4 ± 5.8 (23.5–39.3)	3.6 ± 2.9 (1.4–8.9)
Hermaphrodites (transitional)	11		32.4 ± 7.5 (22.2–43.2)	5.1 ± 4.2 (0.7–13.4)
Hermaphrodites (mosaic)	6		24.4 ± 2.2 (21.3–27.7)	1.6 ± 0.5 (0.9–2.3)
Total	407		31.0 ± 5.8 (11.7–45.8)	4.1 ± 2.6 (0.1–13.6)

In addition, the comparison of shell length and weight between sexes did not reveal any statistically significant differences between males and females of *P. depressa* (SL: K-W = 0.54, $p = 0.464$; TW: K-W = 0.45, $p = 0.504$).

In a protandric hermaphrodite species, it is expected that males would be predominant in smaller sizes, while females would prevail in larger classes. However, females outnumbered males in scattered size classes (20–25; 35–40; ≥45 mm SL), males prevailed in the 30–35 mm SL size class, and the percentage of males and females was very similar in the SL size classes of 25–30 and 40–45 mm (Figure 3). Moreover, the cumulative size frequencies of male and female black-footed limpets exhibited both genders scattered through most size classes, as well as very similar median sizes between males and females, suggesting a gonochoristic life history (Figure 4).

Figure 3. Comparison of size–frequency distributions between male and female black-footed limpets (*Patella depressa*) from the Algarve coast (southern Portugal). Numbers above bars indicate sample sizes.

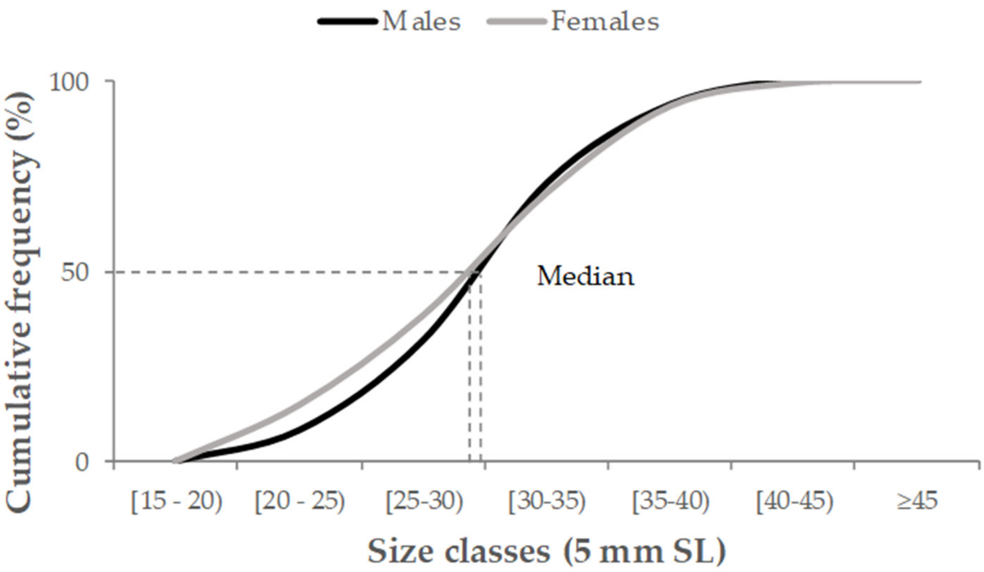

Figure 4. Cumulative size frequencies for male (black line) and female (gray line) black-footed limpets (*Patella depressa*) from the Algarve coast (southern Portugal).

3.2. Reproductive Cycle, Gonadal Index and Environmental Parameters

The histological examination of the gonadal tissues allowed the identification of hermaphrodite gonads, displaying simultaneous male and female gonadal characteristics (Figure 5). Two levels of hermaphroditism were disclosed: transitional and mosaic hermaphroditism. Predominantly male gonads containing some immature oocytes were considered transitional hermaphrodites (Figure 5A). In the present study, 11 transitional hermaphrodites (32.4 ± 7.5 mm SL) were detected in May, July and August of 2017 and also in June and July of 2018. Limpets whose gonad comprised almost equal portions of male and female tissue were designated as mosaic hermaphrodites (24.4 ± 2.2 mm SL) (Figure 5B) and were observed in July and August of 2017 and then in June and July of 2018. Although no maturation stage was assigned to hermaphrodite individuals, all mosaic hermaphrodites observed in the present study had gonads with typical features of stage I or V (early development and spent, respectively).

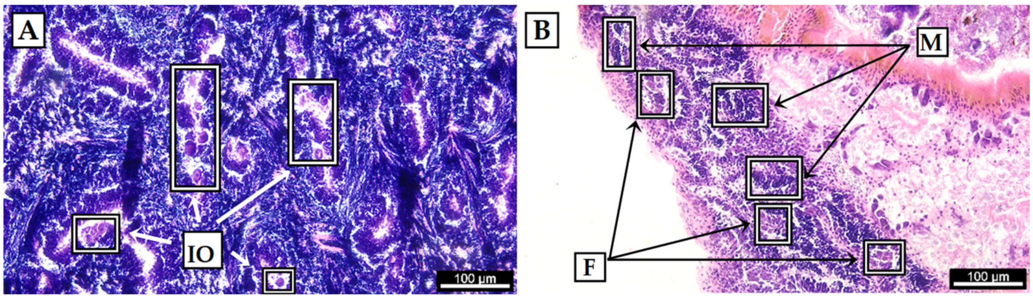

Figure 5. Histological sections presenting hermaphrodite gonads of black-footed limpets (*Patella depressa*): (**A**) transitional hermaphroditism in a male gonad containing some immature oocytes (IO) and (**B**) mosaic hermaphroditism in a gonad comprising similar portions of male (M) and female (F) gonadal features (inside the rectangles).

Histological sections displaying the diverse gonad development stages in male and female black-footed limpets are illustrated in Figure 6. The monthly variation in gonad maturity stages and the mean gonadal index (GI) is presented in Figure 7. Clear inter-annual differences were detected during the study period, with resting gonads only being observed in 2017, mainly between June and August. Individuals at an early development stage were recorded between July and September of 2017, whereas in 2018, later development gonads occurred more frequently and during a longer period (February–July; November–December). Ripe and spawning individuals were scattered throughout almost the whole study period (excluding June–August 2017). In general, ripe gonads were observed between September 2017 and April 2018, and then from July 2018 until the end of the year. Spawning individuals occurred between January and May of 2017, and from October 2017 until the end of 2018. This extensive occurrence of ripe and spawning gonads, prevailing over less-developed maturation stages, is probably due to consecutive processes of gonadal re-ripening and partial spawning events throughout the reproductive cycle.

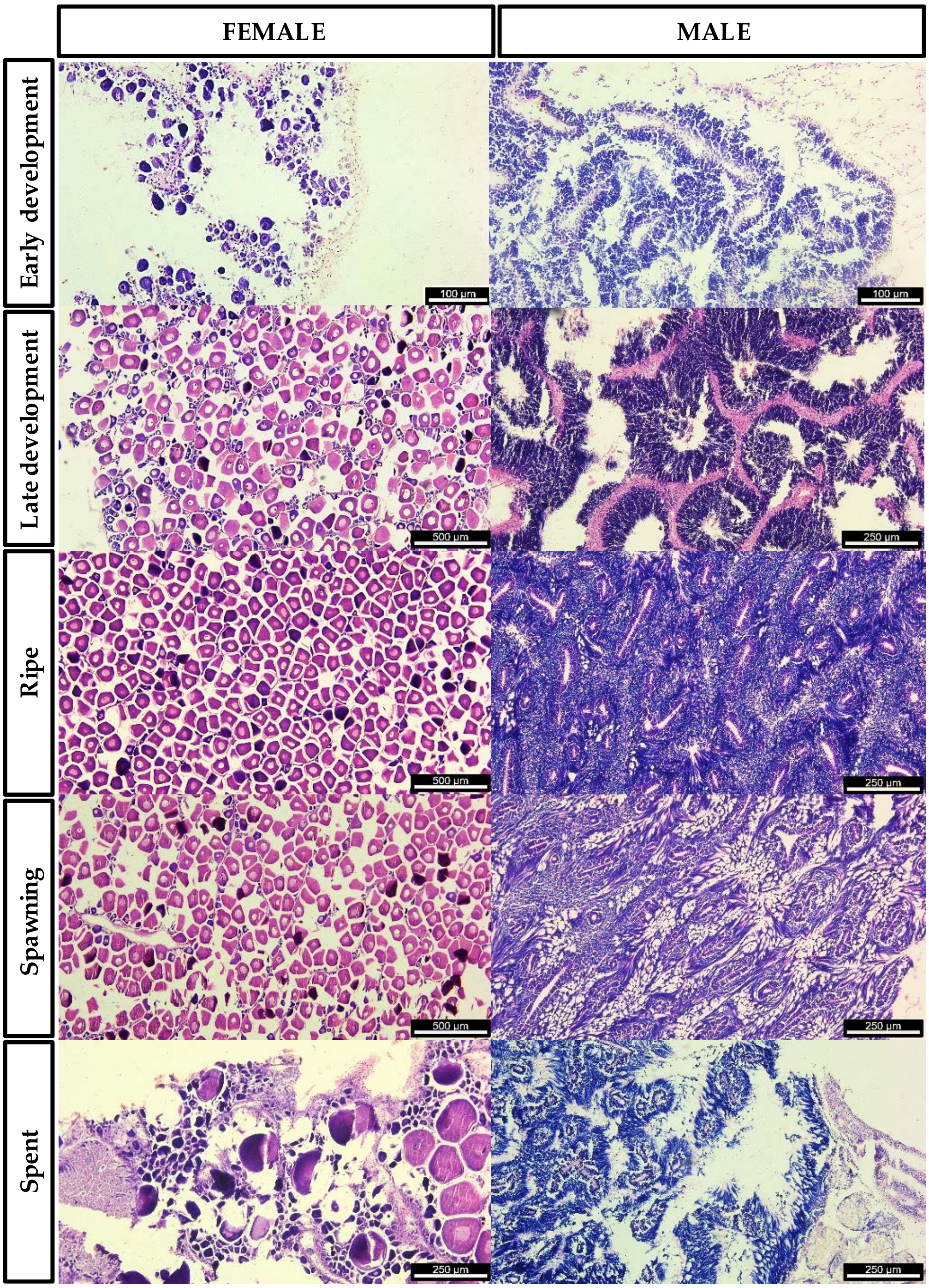

Figure 6. Histological sections presenting gonad maturity stages of female (left) and male (right) black-footed limpets (*Patella depressa*) from the Algarve coast (southern Portugal).

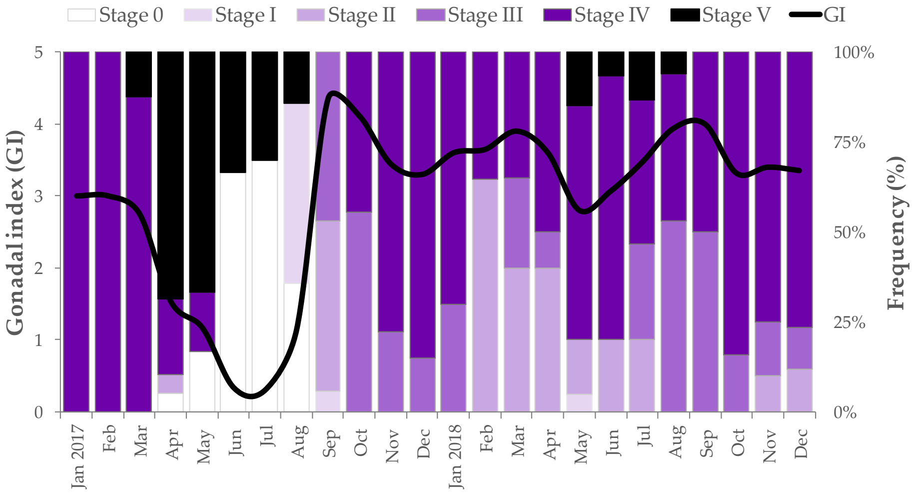

Figure 7. Monthly variation in the mean gonadal index (GI) and gonad maturity stages in the black-footed limpet (*Patella depressa*) from the Algarve coast (southern Portugal) during the 2-year study period (2017–2018). Stage 0 = inactive; stage I = early development; stage II = late development; stage III = ripe; stage IV = spawning; and stage V = spent.

The monthly variation in the GI reflected the reproductive dynamics of *P. depressa*, displaying clear inter-annual differences throughout the study period. In the 2-year study period, the lowest GI (0.30) was recorded in July 2017 and the highest (4.35) in September 2017. In 2018, GI values never dropped below 2.80, reflecting the presence of late development, ripe and spawning gonads during the above-mentioned consecutive processes of gonadal re-ripening and partial spawning events. Values of the GI below 2.00, corresponding to inactive, early development and spent gonads, were observed only between April and August of 2017, coinciding with the resting period mentioned earlier.

The inter-annual differences observed in GI fluctuation, mirroring the monthly variation in gonad maturity stages, were probably due to oscillation in atmospheric and oceanographic parameters. Indeed, the mean GIs appear to be negatively influenced by WS ($\rho = -0.528$, $p < 0.05$) and WH ($\rho = -0.461$, $p < 0.05$). However, other parameters, namely, AT and SST, did not seem to affect the mean GI ($\rho = 0.031$, $p > 0.05$ and $\rho = 0.077$, $p > 0.05$, respectively) and, consequently, the reproductive dynamics of *P. depressa* (Table 2).

Table 2. Spearman (ρ) correlations established between the mean gonadal index (GI) of *Patella depressa* and atmospheric/oceanographic parameters (air temperature—AT; surface seawater temperature—SST; wind speed—WS; and wave significant height—WH) recorded on the Algarve coast (southern Portugal).

	AT	SST	WS	WH
Patella depressa GI	$\rho = 0.031$ $p = 0.888$	$\rho = 0.077$ $p = 0.722$	$\boldsymbol{\rho = -0.528}$ $\boldsymbol{p = 0.008}$	$\boldsymbol{\rho = -0.461}$ $\boldsymbol{p = 0.023}$

Note: statistically significant correlations ($p < 0.05$) are highlighted in bold.

4. Discussion

The present study recorded a male-biased sex ratio (1M:0.72F) in the black-footed limpet population from the Algarve coast. Previous studies also reported the prevalence of males over females in other populations of *P. depressa* along the mainland coast of Portugal [20,41,50]. On the contrary, although males prevailed over females, the proportion between sexes did not differ significantly in two populations of *P. depressa* from the central coast of Portugal [22].

The assumption of protandry in patellid limpets is usually based on sexually unbalanced size–frequency distributions and/or on the occurrence of individuals displaying both male and female gonads in intermediate size classes [7]. In fact, the present study detected several specimens with gonads displaying both male and female features. Nevertheless, the proportion of hermaphrodites observed in the present study (4.2%) was much higher than that recorded in British populations of *P. depressa* (0.021%) [51]. Branch [52] distinguished between two forms of hermaphrodites, transitional and mosaic. Transitional hermaphrodites have a morphologically uniform gonad which is predominantly male, with small oocytes scattered throughout the gonadal tissue. Subsequently, all the sperm in the gonad is either shed or resorbed, and the limpet becomes entirely female. The transitional form can be interpreted as an intermediate stage in the transformation from a male gonad to a female gonad, i.e., sequential hermaphroditism [53]. Mosaic hermaphrodites contain some patches of gonad that are functionally female and others that are functionally male, also being designated as simultaneous hermaphrodites [52].

Mosaic hermaphroditism had low and sporadic incidence in several patellid species from England [51] and South Africa [52], which is not necessarily related to sex change [7]. In addition, Dodd [51] believed that due to the rarity of this form and its wide occurrence amongst gonochoristic species, mosaic hermaphrodites could be considered an aberration. In the present study, about 2.7% of the total analyzed individuals presented transitional hermaphroditism spread over a wide size range (22.2–43.2 mm SL), whereas mosaic hermaphroditism was detected in a lower proportion of limpets (1.5%) and only in smaller specimens (21.3–27.7 mm SL). In the present study, all mosaic hermaphrodites had gonads with typical features of maturation stage I or V, suggesting that they were in a transitional stage of sequential sex change [33]. Accordingly, the occurrence of both transitional and mosaic hermaphrodites in the studied population indicates that some individuals are able to change sex (sequential hermaphroditism).

Contrary to gonochoristic species that usually have similar proportions of males and females [19], protandric hermaphrodite species are expected to present male-biased sex ratios, in which younger and smaller individuals are males and then the proportion of females gradually increases with age and size [9,51,54,55]. Despite the occurrence of hermaphrodites and the male-biased sex ratio recorded in the present study, black-footed limpets from the Algarve coast presented both sexes scattered through most of the size classes, suggesting a gonochoristic life history [19]. Indeed, the comparison of cumulative size–frequency distributions among sexes further confirmed quite similar median sizes between males and females. Corroborating these findings, populations of *P. depressa* from the UK [20,23,51] and Portugal [22] were also considered non-protandric. In fact, contrary to *P. vulgata* [13,14,19], this population of black-footed limpets from the Algarve coast does not seem to be protandric hermaphrodites, in which sex changes with age/size. However, Borges et al. [19] considered the possibility of a slight occurrence of protandry in *P. depressa* from Portugal because individual size differed faintly between sexes in this population. In the present study, no significant differences were detected in shell length or weight between sexes; therefore, the occurrence of hermaphroditism might relate to the species' variable life history depending on environmental and demographic constraints [19]. In addition, hermaphroditism is interpreted as an advantageous strategy for increasing reproductive success, based on the assumption that sex-changing individuals will improve their reproductive efficiency [56,57]. Moreover, the direction of sex change is supposed to be determined by the relative fitness return for both sexes throughout limpets' lifetime and not by their size [8,58]. In some species, sex change is triggered by specific environmental conditions, whereas in other species, it appears to be determined genetically and occurs mainly after the first reproductive period [59,60].

The black-footed limpets from the Algarve coast exhibited an extensive occurrence of ripe and spawning gonads throughout almost 2 years (excluding June–August 2017), probably related to consecutive processes of gonadal re-ripening and partial spawning events. The spawning of *P. depressa* seems to be negatively influenced by wind speed and wave height, and its reproductive cycle exhibited clear inter-annual differences, namely, a short resting period only recorded in 2017 (June–August). Similar reproductive dynamics were observed in diverse populations of *P. depressa*. Brazão et al. [22] reported that two populations from the central coast of Portugal spawned in the summer, but earlier partial emissions occurred in January. These populations exhibited a resting phase in a period quite similar to that observed in the present study (June to August 2017). Guerra and Gaudêncio [20] recorded diverse patterns in the reproductive cycles of *P. depressa* populations from the north to south coasts of mainland Portugal. These authors considered the populations from the north and central coasts very asynchronous, exhibiting gonads in different maturity stages scattered year-round, suggesting frequent spawning events and gonad re-ripening, as in the southern population in the present study. Orton and Southward [23] also reported a trend of multiple spawning events in *P. depressa* from the UK and, similarly to the present study, detected considerable inter-annual variability in the species' reproductive activity that was attributed to differences in temperature throughout the study. On the central coast of Portugal, *P. depressa* spawning appears to be induced by high wind speed under a favorable air temperature [22].

However, in the present study, air and seawater temperatures did not seem to significantly influence *P. depressa* spawning, whereas wind speed and wave height were negatively correlated with the mean GI. Near its northern limit of occurrence (SW England), the spawning of *P. depressa* seems to occur under maximum air temperature and wave action, with a resting period during the winter months [23,24]. Since the Portuguese coast is the southern distributional limit, the resting phase coincided with increasing air and seawater temperatures (i.e., summer), reflecting the adverse conditions of excessive heat near the southern limits, as suggested by Lewis et al. [61]. In addition, as in the present study, the southern populations analyzed by Guerra and Gaudêncio [20] showed rapid gonad development during the late summer, with spawning individuals increasing progressively through autumn until early spring.

Previous studies reported that high wind speed, associated with wave action stimulation, might induce spawning in patellid limpets, because strong onshore winds act as a mechanical trigger [13,19,20,22,23]. Although according to Lewis [61], spawning in patellid limpets involves an environmental trigger, the conditions controlling *P. depressa* spawning on the Algarve coast were not fully identified. The inter-annual differences revealed in the present study might be related to complex and specific combinations of environmental parameters acting together. Nevertheless, contrary to previous studies, the spawning of black-footed limpets appears to be negatively influenced by wind speed and wave height on the Algarve coast.

5. Conclusions

Despite the occurrence of hermaphroditism, the Algarve coast black-footed limpet population presented both sexes scattered through most size classes, suggesting the absence of protandric hermaphroditism, in which sex changes with age/size. The reproductive cycle of *P. depressa* displayed clear inter-annual differences, and the influence of environmental parameters in its reproductive dynamics still remains slightly unclear. In particular, maybe the use of environmental data collected directly at the sampling site might eventually contribute to clarifying the relationship between these parameters and the species' reproductive dynamics. Future studies should continue monitoring this limpet population, aiming to further improve knowledge of the *P. depressa* reproductive cycle and dynamics. Such information is crucial to proposing management measures for promoting the sustainability of limpet harvesting in southern Portugal.

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