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REPRODUCTIVE CYCLE OF THE COMMON LIMPET (*PATELLA VULGATA*) AND ROUGH LIMPET (*PATELLA ULYSSIPONENSIS)* IN SOUTHERN PORTUGAL

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Executive Summary

The common limpet (*Patella vulgata* Linnaeus, 1758) and the rough limpet (*Patella ulyssiponensis* Gmelin, 1791) are common species in intertidal rocky shores along the Atlantic coasts of Europe. Both species are collected by professional harvesters and recreational harvesters in mainland Portugal. These patellid limpets are protandrous hermaphrodites that have been subject to diverse studies on reproductive features throughout their distributional ranges along the coasts of the northeastern Atlantic Ocean. In order to describe the reproductive cycle of *P. vulgata* and *P. ulyssiponensis*, individuals of both species were sampled monthly from intertidal rocky areas from Praia da Luz in Lagos (Algarve coast – southern Portugal) during two consecutive years (January 2017 – December 2018) and subjected to gonad histology and calculation of the mean gonadal index (GI). Overall, were analysed 882 individuals with broad size ranges, 444 *P. vulgata* (19.5 – 43.0 mm SL) and 438 *P. ulyssiponensis* (12.3 – 55.4 mm SL). Both species presented balanced sex ratios (*P. vulgata* = 1M: 0.98F; *P. ulyssiponensis* = 1M: 1.03F), similar size-frequency distribution between sexes and equivalent mean shell length and total weight between males and females. The estimated size at sex change was slightly smaller in *P. vulgata* ($SL_{50} \approx 29$ mm) than in *P. ulyssiponensis* ($SL_{50} \approx 36$ mm). In general, the reproductive cycles of *P. vulgata* and *P. ulyssiponensis* were characterised by a short resting period mainly during summer. In *P. vulgata*, ripe and spawning gonads were mostly recorded from early autumn until early spring, with a main spawning season in early spring – early summer. In *P. ulyssiponensis*, ripe and spawning gonads were scattered almost year-round, with a main spawning season also in early spring – early summer. Both species also displayed subsidiary spawning events scattered throughout the year (variable depending on the sampling year). The reproductive cycles of *P. vulgata* and *P. ulyssiponensis* were influenced by the seasonal variation in surface seawater temperature (SST), with resting periods coincident with warmer SST in summer, gametogenic development and gonadal maturation triggered by decreasing SST during autumn, and main spawning seasons roughly synchronised with increasing SST during early spring – early summer. Populations of *P. vulgata* and *P. ulyssiponensis* from southern Portugal have the later spawning period reported throughout their distributional ranges in the northeastern Atlantic Ocean. Overall, this study provides relevant information for conservation purposes and for the proposal of management measures for the harvesting of *P. vulgata* and *P. ulyssiponensis* in southern Portugal.

Abstract

The present study reports a detailed description of the reproductive cycle of the common limpet (*Patella vulgata*) and rough limpet (*Patella ulyssiponensis*) from Praia da Luz in Lagos (Algarve coast – southern Portugal). Individuals of both species were sampled monthly from intertidal rocky areas during two consecutive years (January 2017 – December 2018) and subjected to gonad histology and calculation of the mean gonadal index (GI). In these protandrous hermaphrodite limpets, the estimated size at sex change was slightly smaller in *P. vulgata* ($SL_{50} \approx$ 29 mm) than in *P. ulyssiponensis* ($SL_{50} \approx$ 36 mm). In general, the reproductive cycles of both species were characterised by a main spawning season in early spring – early summer, sometimes including subsidiary spawning events, followed by a short resting period during summer. The comparison of the main spawning season throughout the species distributional ranges revealed that populations of *P. vulgata* and *P. ulyssiponensis* from southern Portugal have the later spawning period reported for the northeastern Atlantic Ocean. The overall information gathered in this study is relevant for conservation purposes and provides valuable baseline data for the proposal of management measures for the harvesting activity targeting *P. vulgata* and *P. ulyssiponensis* in southern Portugal.

Introduction & Aims

Limpets are keystone gastropod species on rocky shores with a key role in structuring the intertidal communities (e.g. Jenkins *et al.*, 2005; Coleman *et al.*, 2006a; Burgos-Rubio *et al.*, 2015), whose grazing activity influences the algal growth either directly or indirectly (Jenkins *et al.*, 2001; Moore *et al.*, 2007; Firth & Crowe, 2008). Several limpet species have been harvested since ancestral times (Bicho & Haws, 2008; Dean *et al.*, 2012; Fa *et al.*, 2016) and are considered culturally important food source for humans in coastal communities (McCoy, 2008; Mau & Jha, 2018). In mainland Portugal, limpets are collected by professional harvesters and occasionally by recreational harvesters, but are very popular and traditional seafood species intensively exploited in Portuguese archipelagos, namely in the Azores (Santos *et al.*, 1995; Côrte-Real *et al.*, 1996; Martins *et al.*, 2011, 2017; Diogo *et al.*, 2016) and Madeira (Henriques *et al.*, 2012; Sousa *et al.*, 2017, 2019).

The common limpet (*Patella vulgata* Linnaeus, 1758) and the rough limpet (*Patella ulyssiponensis* Gmelin, 1791) are common species in intertidal rocky shores along the Atlantic coasts of Europe. While *P. vulgata* is distributed from Norway, around the British Isles, along the coast of Portugal, extending into the Mediterranean (e.g. Blackmore, 1969; Bowman & Lewis, 1986), i.e. with meridional biogeographic limit in southern Portugal (Guerra & Gaudêncio, 1986; Borges *et al.,* 2015), *P. ulyssiponensis* extends from Norway until North Africa, also occurring throughout the Mediterranean (Weber & Hawkins, 2005).

Both species are protandrous hermaphrodites that have been subject to diverse studies on reproductive features throughout their distributional range, including the size at sex change and the reproductive cycle. For *P. vulgata*, studies on protandrous sex change were performed in the UK (Orton, 1928; Dodd, 1956; Blackmore, 1969; Le Quesne & Hawkins, 2006; Borges *et al.*, 2015, 2016) and in Spain (Ortega, 2018), and studies on the reproductive cycle were carried out in the UK (Orton, 1928; Orton *et al.*, 1956; Blackmore, 1969; Baxter, 1983; Bowman & Lewis, 1986; Jenkins *et al.*, 2001; Moore *et al.*, 2007), Ireland (Thompson, 1980; Delany *et al.*, 1998, 2002; McCarthy *et al.*, 2008) and Spain (Ibanez *et al.*, 1986; Jenkins *et al.*, 2001; Fernández *et al.*, 2015). For *P. ulyssiponensis*, studies are much scarcer and focused only on the species reproductive cycle in Ireland (Delany *et al.*, 1998, 2002; McCarthy *et al.*, 2008) and Spain (Fernández *et al.*, 2016).

In Portugal, previous studies on reproductive aspects of the common limpet and of the rough limpet were performed mainly in the north, central and southwest coasts. Overall, these studies were limited to the relationships between size-frequency distributions and sex ratios of *P. vulgata* (Guerra & Gaudêncio, 1986; Borges *et al.*, 2015) and descriptions of the reproductive cycle based only on macroscopic observations of the gonads and estimation of gonadal indices of *P. vulgata* (Guerra & Gaudêncio, 1986; Jenkins *et al.*, 2001; Ribeiro *et al.*, 2009) and *P. ulyssiponensis* (Ribeiro *et al.*, 2009).

In this context, the present study aimed to estimate the size at sex change and describe the reproductive cycle through histological analyses of the gonads and calculation of the mean gonadal index of *P. vulgata* and *P. ulyssiponensis* from the Algarve coast (southern Portugal). At present, the national legislation that regulates the harvesting of limpets in mainland Portugal stipulates a minimum conservation reference size (MCRS = 20 mm in shell length) for limpet species of the genus *Patella* (DR, 2011). Overall, the present study provides valuable baseline information for the scientifically supported proposal of additional management measures for the sustainable harvesting of *P. vulgata* and *P. ulyssiponensis*.

Material and Methods

Collecting site and biological sampling

Individuals of *P. vulgata* and *ulyssiponensis* were collected during low tide on a sheltered intertidal rocky shore at Praia da Luz in Lagos (37º05'06.5"N, 08º43'45.1"W) located in the Algarve coast (southern Portugal) (Figure 1). The collecting site is a sheltered rocky platform in the south-facing coast, therefore relatively protected from north winds, less exposed to the oceanic circulation, and subjected to milder wave action and weaker swell. In the area of Praia da Luz, limpet harvesting is only performed very sporadically by local recreational harvesters.

Field sampling was performed monthly (≈ 20 individuals per species and month) during two consecutive years (January 2017 – December 2018). In the laboratory, individuals 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), after removing any epibionts and slightly draining on absorbent paper for 5–

10 min. During the study period, data on surface seawater temperature (SST) registered at the Faro oceanographic buoy (closest buoy to the collecting site: 36°54.3'N, 07°53.9'W) was gathered from the Portuguese Hydrographic Institute (IH, 2019), in order to assess eventual relationships between SST and the reproductive dynamics of both limpet species.

Gonad histology and mean gonadal index

After removing the soft body from the shell, the tissues were fixed in a Davidson solution for 48 h and then stored in 70% ethanol. The preserved tissues were dehydrated with serial dilutions in ethanol, infiltrated and embedded in the paraffin wax. Subsequently, gonads were cut into thin sections (7 µm) using an automatic microtome, stained with haematoxylin and eosin and mounted between a coverslip and glass slide using synthetic resin. Finally, the histological sections were examined under a light microscope (magnifications of 4x, 10x and 20x) for analysing and describing the main features of the reproductive cycle of both limpet species.

The maturation stage of each individual was identified following a microscopic maturation scale (briefly described in Table 1) adapted and modified from McCarthy *et al.* (2008) and Prusina *et al.* (2014). Whenever multiple developmental stages were observed within a single histological section, the classification criterion was based on the prevailing maturity stage in the gonad. Individuals with inactive gonads were considered sexually undifferentiated. Individuals displaying both male and female gonadal features were classified as hermaphrodites and no maturation stage was assigned.

In addition, the mean gonadal index (GI) in each monthly sample was calculated through the following equation (Seed, 1976):

$$\mathrm{GI} = \frac{\sum \text{ind. each stage x stage ranking}}{\text{total ind. each month}}$$

For each stage of gonadal development, a numerical ranking value was assigned: inactive = 0; early development = 2; late development = 3; ripe = 5; spawning = 4; spent = 1. Accordingly, the GI ranged from 0 (only inactive individuals in the monthly sample) to 5 (only ripe individuals in the monthly sample).

Data treatment and statistical analysis

The sex ratio (males: females) in the samples of *P. vulgata* and *P. ulyssiponensis* was compared with parity (1M: 1F) using the chi-square test (χ^2). Eventual differences in the size-frequency distribution between sexes were analysed in both limpet species through the Kolmogorov-Smirnov (K–S) goodness of fit test for two samples [H_0: F_A (x) = F_B (x)]. In both *P. vulgata* and *P. ulyssiponensis*, specimen mean size (SL) and weight (TW) were compared between sexes through 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 adopted. All statistical analyses were performed following (Zar, 1996) with significance level considered for $P < 0.05$.

Results

Population structure and size-specific sex ratios

The main descriptive statistics of the samples of common limpets and rough limpets from Praia da Luz are compiled in Table 2. Overall, were histologically analysed 882 individuals, comprising 444 *P. vulgata* and 438 *P. ulyssiponensis*. Both limpet species presented some sexually undifferentiated individuals (17 in *P. vulgata* and 27 in *P. ulyssiponensis*) and a few hermaphrodites (2 in *P. vulgata* and 8 in *P. ulyssiponensis*). In the overall samples collected during the two years study period, the sex-ratio was fairly balanced and not significantly different from parity in both *P. vulgata* (1M: 0.98F: $\chi^2 = 0.04$, $P > 0.05$) and *P. ulyssiponensis* (1M: 1.03F: $\chi^2 = 0.04$, $P > 0.05$). In addition, the comparison of mean size and weight between sexes did not detect any statistically significant differences between males and females of both *P. vulgata* (SL: $F = 0.006$, $P = 0.939$; TW: $H = 0.002$, $P = 0.962$) and *P. ulyssiponensis* (SL: $H = 0.101$, $P = 0.751$; TW: $H = 2.518$, $P = 0.113$) (Table 2).

The comparison of the size-frequency distribution between males and females of *P. vulgata* and *P. ulyssiponensis* is illustrated in Figure 2. The common limpet presented a slightly narrower size range (*P. vulgata*: 19.5 – 43.0 mm SL) compared to the rough limpet (*P. ulyssiponensis*: 12.3 – 55.4 mm SL). Overall, the population size-frequency distribution was not significantly different between sexes in both *P. vulgata* (K-S: $D = 0.026$, $P > 0.05$) and *P. ulyssiponensis* (K-S: $D = 0.052$, $P > 0.05$). Nevertheless, due to protandrous sex change in both species, some size-specific sex ratios reflected slightly outnumbering males in smaller size classes and females in larger size classes. In *P. vulgata*, there were only males below 20 mm SL and males (83.7%) slightly prevailed over females (81.4%) until the class 30 – 35 mm SL, whereas females (18.6%) slightly predominated over males (16.3%) in the larger classes (e.g. > 40 mm SL = 1M: 2.00F). In *P. ulyssiponensis*, males (90.5%) slightly prevailed over females (85.3%) until the class 40 – 45 mm SL, while females (14.7%) slightly predominated over males (9.5%) in the larger classes (e.g. 45 – 50 mm SL = 1M: 1.32F) and there were only females above 50 mm SL. Accordingly, these data provide an initial indication that protandric sex change occurs at smaller size in *P. vulgata* compared to *P. ulyssiponensis* (Figure 2).

Size at sex change

Subsequently, the cumulative proportion of females as a function of specimen size (grouped into 5 mm SL classes) was employed to estimate the size at sex change (SL_{50}) in *P. vulgata* and *P. ulyssiponensis*, i.e. the shell length at which 50% of the non-neuter individuals are females (Figure 3). Corroborating the previous assumption that protandric sex change occurs at smaller size in the common limpet than in the rough limpet, the estimated size at sex change was lower in

P. vulgata ($SL_{50} \approx 29$ mm) compared to *P. ulyssiponensis* ($SL_{50} \approx 36$ mm) (Figure 3). In agreement, protandrous hermaphrodites in the samples of both species (i.e. transitional stage between males and females) displayed a mean size of 30.6 ± 3.8 mm SL in *P. vulgata* and 38.2 ± 5.8 mm SL in *P. ulyssiponensis* (Table 2). In addition, the minimum size recorded for the "second" sex (i.e. smallest female) was 20.7 mm SL in *P. vulgata* and 23.8 mm SL in *P. ulyssiponensis* (Table 2). The relative size at sex change, i.e. the ratio between the size at sex change and the maximum size in the samples (SL_{50} / SL_{max}), revealed that SL_{50} was reached at fairly comparable size in *P. vulgata* (67% SL_{max}) and *P. ulyssiponensis* (65% SL_{max}). Unfortunately, the approach of estimating the SL_{50} through logistic regression was unsuccessful in the present study, probably because the lower representativeness of the samples in the smallest and largest size classes induced slightly biased sex ratios. Overall, the correlation coefficients were relatively low (*P. vulgata*: $r = 0.687$; *P. ulyssiponensis*: $r = 0.765$), revealing flawed fittings of the logistic equation and inaccurate regression slopes (b) that resulted in unrealistic estimates of SL_{50}.

Gonad maturation stages and reproductive cycle

The most relevant features of the gametogenesis in the gonad maturation stages of *P. vulgata* and *P. ulyssiponensis* are illustrated in Figures 4 and 5, whereas the monthly frequencies of males and females at each maturation stage are presented in Figures 6 and 7, respectively. In general, *P. vulgata* with inactive gonads were quite scarce and occurred mainly during summer, revealing a quite short resting period in the reproductive cycle (especially in 2018) (Figure 6). Following rapid gonad maturation (early + late development) in late summer – early autumn, both sexes were ripe during autumn and winter, but during a slightly shorter period in males compared to females (especially in 2017). Although ripe and spawning gonads co-occurred from early autumn until early spring, *P. vulgata* displayed a spawning season mainly in early winter – late spring. Apparently, while gametes of both sexes were emitted almost every month (sometimes were just partial emissions during minor spawning events), spawning was slightly asynchronous between sexes, with longer periods of male gametic emission preceding shorter periods of female spawning. For instance, at the beginning of 2017, while virtually all males were already emitting gametes, a fraction of females was still ripe. During the two years study period, the least represented maturation stages (inactive, early and late development) confirmed the short resting period and rapid gonad maturation, while the higher annual proportion of spawning males (58.2%) compared to spawning females (39.4%) highlighted the slightly different duration of the gametic emission between sexes of *P. vulgata* (Figure 6).

Regarding *P. ulyssiponensis*, individuals with inactive gonads also occurred mainly during summer, in higher proportions in males than in females and with a longer resting period in both sexes in 2017 compared to 2018 (Figure 7). Once again after rapid gonad maturation, ripe males and females were recorded during autumn and winter, frequently accompanied by spawning individuals especially during winter. The main ripening and spawning periods of *P. ulyssiponensis* also displayed some asynchrony between sexes, lasting longer in males compared to females, although without a clearly evident spawning peak in both sexes (i.e. variable proportions of spawning individuals from autumn until spring). In fact, ripe and spawning individuals were scattered almost through the entire study period (although much less during the short resting phase in summer), eventually indicating consecutive processes of gonadal re-ripening and partial spawning events throughout the reproductive cycle (particularly in 2018). During the two years study period, ripe and spawning gonads clearly prevailed over less-developed maturation stages, further confirming the extended period of partial or total spawning throughout the year, whereas different proportions of spawning individuals between sexes (males = 48.9%; females = 39.1%) also reflected the slightly longer gametic emission in males than in females (Figure 7).

Mean gonadal index and seawater temperature

The monthly variation of the mean gonadal index (GI) in males and females of *P. vulgata* and *P. ulyssiponensis* as a function of surface seawater temperature (SST) are presented in Figure 8. In general, males and females of both species displayed similar temporal variation in GI throughout the study period, although with clear inter-annual differences probably associated with thermal oscillation between consecutive years. Indeed, while SST in 2017 was fairly stable throughout a long period during summer (June – October), the seasonal fluctuation of SST in 2018 was clearly distinct, with a much shorter period of warmer temperatures (August – October) and an abnormally late peak of SST in September. These inter-annual differences in SST were reflected in the reproductive cycle of both species, as revealed by the monthly variation in GI throughout the two years study period (Figure 8).

In general, lowest GI values coincided with warmer summer temperatures (especially in 2017), corresponding to less developed maturation stages (inactive and spent gonads) during the relatively short resting periods of both *P. vulgata* and *P. ulyssiponensis* (Figure 8). Gametogenic development and gonadal maturation, reflected by the sharp and simultaneous increase in GI of males and females of both species, were apparently triggered by the gradual decrease in SST during autumn. Subsequently, higher GI values corresponding mostly to ripe and spawning individuals were roughly distributed between autumn and spring. This trend was followed by abrupt decreases in GI of both species, more evident in 2017 than in 2018, denoting the main spawning season during early spring – early summer (*P. vulgata*: March – June/July; *P. ulyssiponensis*: March – July). Once again, inter-annual differences in SST were apparently responsible for changes in the reproductive cycle of both species, with a single spawning peak at more stable SST in 2017 against successive episodes of gonadal re-ripening and partial spawning events at more atypical SST in 2018 (Figure 8).

Discussion

Population structure and size-specific sex ratios

The present populations of common limpet and rough limpet from southern Portugal showed highly balanced and almost parity sex ratios (*P. vulgata* = 1M: 0.98F; *P. ulyssiponensis* = 1M: 1.03F), similar size-frequency distribution between sexes and equivalent mean shell length and total weight between males and females. In protandrous hermaphrodite limpets, maturing as males and changing sex to females during ontogeny, *a priori* it would be expected size-biased sex ratios, population size structures left-skewed towards male dominance and right-skewed towards female dominance, as well as smaller and lighter males against larger and heavier females. In fact, the theory predicts that sex changing organisms should have a sex ratio biased towards the sex that individuals first reach reproductive maturity (termed "first" sex) (Allsop & West, 2004), in practice meaning that protandrous (male first) *P. vulgata* and *P. ulyssiponensis* should display male-biased sex ratios.

Accordingly, several studies noticed males outnumbering females and/or reported male-biased sex ratios in both species, mostly in *P. vulgata* (e.g. Orton, 1928; Orton *et al.*, 1956; Blackmore, 1969; Baxter, 1983; Guerra & Gaudêncio, 1986; McCarthy *et al.*, 2008; Fernández *et al.*, 2015), but also in *P. ulyssiponensis* (McCarthy *et al.*, 2008; Ciancimino *et al.*, 2014; Fernández *et al.*, 2016). However, scientific literature describes a wide variety of sex ratios in populations of *P. vulgata* (Fernández *et al.*, 2015) and contrasting trends in sex ratios were also reported for populations of *P. ulyssiponensis*. For instance, in the northern and central coasts of Portugal, the sex ratios showed an overall predominance of females in both *P. vulgata* (0.6M: 1F to 1.0M: 1F) and *P. ulyssiponensis* (0.6M: 1F to 1.1M: 1F) (Ribeiro *et al.*, 2009). In addition, populations of *P. vulgata* from the north, central and southwest coasts of Portugal presented less male-biased sex ratios, similar size distributions between sexes and equal or very similar median sizes between males and females (Borges *et al.*, 2015), whereas in a field study with *P. vulgata* in the UK, males (30.1 ± 2.3 mm SL) were significantly smaller than females (36.2 ± 2.4 mm SL) (Borges *et al.*, 2016).

Previous studies showed that the degree of exposure influences the sex ratio of *P. vulgata*, with a general predominance of males in sheltered or moderately exposed areas and balanced sex-ratios or predominance of females in highly exposed areas (Fernández *et al.*, 2015 and references therein), which does not match with the fairly balanced sex ratio in a sheltered collecting site reported in the present study. Alternatively, since *P. vulgata* is a northern species with biogeographic limit in southern Portugal and that becomes increasingly rare progressing southwards (Guerra & Gaudêncio, 1986; Borges *et al.*, 2015), the present balanced sex ratio could be simply explained by lower recruitment in southern Portugal, producing populations with less smaller males and more larger females (Borges *et al.*, 2015). Indeed, variation in the sex ratio of *P. vulgata* as a function of latitude, with a decreasing likelihood of being male towards southern Portugal, might indicate that sex ratio is involved in determining the species distributional range (Borges *et al.*, 2015). The present balanced sex ratios in *P. vulgata* and *P.*

ulyssiponensis further confirm that both populations are locally subjected to low exploitation level (sporadic recreational harvesting). In fact, hand collection is expected to generate size-biased catches towards larger sizes, because larger limpets are more conspicuous and more easily detected than smaller limpets (especially in species sometimes hidden inside intertidal crevices). In protandrous hermaphrodite limpets such as *P. vulgata* and *P. ulyssiponensis*, size-selective harvesting might impact the species reproductive efficiency by selectively removing mostly larger females and male-biasing the sex ratios (e.g. Espinosa *et al.*, 2006; Fenberg & Roy, 2012; López *et al.*, 2012; Ciancimino *et al.*, 2014; Marra *et al.*, 2017; Casal *et al.*, 2018), making some limpet species particularly vulnerable to overexploitation and recruitment overfishing (e.g. Santos *et al.*, 1995; Guerra-García *et al.*, 2004; Henriques *et al.*, 2012; Riera *et al.*, 2016).

Size at sex change

In the present study, the size at sex change (SL_{50}) was estimated at approximately 29 mm for *P. vulgata* and 36 mm for *P. ulyssiponensis*. In the earlier studies focusing on this subject, the size at sex change in *P. vulgata* was basically supported by the occurrence of significantly higher percentages of males in young individuals, roughly balanced sex-ratios in medium-sized limpets and significantly higher percentages of females in older specimens, sometimes accompanied by a few transient hermaphrodites in the process of changing from male to female in intermediate size classes (e.g. Orton, 1928; Dodd, 1956; Orton *et al.*, 1956; Blackmore, 1969). In practice, the sexual transition occurs during the resting phase of the gonads between spawning (Branch, 1981) and hermaphrodites represent an arrest of developmental change during sex-reversal in *P. vulgata* (Dodd, 1956). However, the process of sex change might be even more complex because *P. vulgata* is apparently capable of performing two-way sex change (Le Quesne & Hawkins, 2006), a sexual strategy that requires further research on the factors and mechanisms prompting sex change and its effects on population dynamics (Le Quesne & Hawkins, 2006; Guallart *et al.*, 2013). In general, balanced sex ratios indicative of the change-over in gender proportions from males to females occurred at approximately 25 – 35 mm SL (Orton, 1928) or 40 mm SL (Orton *et al.*, 1956). More recently, based on the size class overlap between males and females, it was predicted that sex change in *P. vulgata* might occur within the size range 15 – 25 mm SL (Le Quesne & Hawkins 2006).

In addition, a recent field experiment to assess the effects of simulated human exploitation on the protandry of *P. vulgata* revealed that sex change is size-dependent, with the SL_{50} extracted from the logistic regression decreasing with increasing exploitation level (control: $SL_{50} = 50.5 \pm 6.4$ mm; low: $SL_{50} = 42.6 \pm 1.5$ mm; high: $SL_{50} = 32.2 \pm 0.2$ mm SL) (Borges *et al.*, 2016). This trend of harvesting pressure and exploitation level inducing phenotypic plasticity in the size at sex change, with smaller males compensating the extraction of larger females by undergoing sex change at smaller sizes and promoting more balanced sex ratios, has also been reported for other protandrous limpet species (e.g. Collin, 2006; Rivera-Ingraham *et al.*, 2011; Fenberg & Roy, 2012; Martins *et al.*, 2017). However, this anthropogenic impact apparently does not greatly affect the present populations of *P. vulgata* and *P. ulyssiponensis* only sporadically subjected to low/moderate exploitation level by local recreational harvesters. On the other hand, similarly to other protandrous limpet species with environmentally-mediated and socially-controlled sex change (e.g. Wright, 1989; Warner *et al.*, 1996; Rivera-Ingraham *et al.*, 2011), sex change in *P. vulgata* is also density-dependent (Borges *et al.*, 2015). In this context, low intraspecific competition would prompt a higher incidence of males changing sex at smaller sizes (producing populations with greater proportion of females at low densities) (Borges *et al.*, 2015). Moreover, under weak recruitment and subsequent low densities, the scarcity of larger individuals would promote sex change from male to female at smaller sizes (generating populations with less smaller males and females) (Borges *et al.*, 2015), which might be particularly relevant in the case of *P. vulgata* that becomes increasingly rare towards southern Portugal.

Reproductive cycle

In general, the reproductive cycles of *P. vulgata* and *P. ulyssiponensis* were characterised by a short resting period mainly during summer. In *P. vulgata*, ripe and spawning gonads were mostly recorded from early autumn until early spring, with a main spawning season in early spring – early summer. In *P. ulyssiponensis*, ripe and spawning gonads were scattered almost year-round, with a main spawning season also in early spring – early summer. In both species, spawning duration was slightly asynchronous between sexes, being a bit longer in males than in females. Moreover, ripe and spawning *P. vulgata* and *P. ulyssiponensis* were recorded almost throughout the year, apparently denoting sequential gonadal re-ripening and partial spawning events. Overall, both species displayed a considerable inter-annual variation in their reproductive dynamics, namely in terms of spawning duration and intensity (clear spawning peak *vs*. partial spawning events).

Similarly, some previous studies suggested that individuals within each population were poorly synchronised (i.e. asynchronous gamete release) in both *P. vulgata* (e.g. Blackmore, 1969; McCarthy *et al.*, 2008; Fernández *et al.*, 2015) and *P. ulyssiponensis* (e.g. Ribeiro *et al.*, 2009), with most stages of gonadal development present almost year-round and spawning occurring through the emission of ripe gametes before all gametes are fully-developed and ready for spawning (Bowman & Lewis, 1986). In practice, this is reflected by the occurrence of additional subsidiary spawning events dispersed throughout the year, which have been reported to occur in both *P. vulgata* (Orton *et al.*, 1956; Bowman & Lewis, 1986; Ribeiro *et al.*, 2009; Fernández *et al.*, 2015) and *P. ulyssiponensis* (Ribeiro *et al.*, 2009; Fernández *et al.*, 2016). These partial spawning episodes are followed by an outbreak of gametogenesis (re-development) or a continuous development of unripe gametes (re-ripening) prior a later spawning (Bowman & Lewis, 1986), already recorded in *P. vulgata* (Bowman & Lewis, 1986; Guerra & Gaudêncio, 1986; Ibanez *et al.*, 1986) and *P. ulyssiponensis* (Ribeiro *et al.*, 2009).

The comparison of the spawning season and peak in *P. vulgata* and *P. ulyssiponensis* throughout their distributional ranges in European waters (northeastern Atlantic Ocean) are compiled in Table 3. First of all, it is worth recognising that such comparisons are fairly difficult and should be carefully interpreted, namely because different studies involved diverse sampling periods and methodologies with distinct detail and accuracy (macroscopic observation *vs.* gonadal indices *vs.* histology). Additionally, as confirmed in the present study, different years can be characterised by distinct patterns of spawning (Orton *et al.*, 1956; Thompson, 1980), although this inter-annual variation might be highly variable among species. For instance, depending on the population location, *P. vulgata* presented considerable annual differences in the timing, extent and frequency of spawning in the UK (Bowman & Lewis, 1986) against synchronised seasonal breeding cycles and minimal year to year differences in northern Portugal (Ribeiro *et al.*, 2009). On the opposite, *P. ulyssiponensis* displayed substantial variation in the basic reproductive pattern among consecutive years in northern Portugal (Ribeiro *et al.*, 2009).

Nevertheless, the comprehensive comparison of the main spawning season and peak in several populations of *P. vulgata* and *P. ulyssiponensis* (Table 3), allowed highlighting a general geographical gradient and latitudinal cline in the reproductive pattern and spawning features of both species along their distributional ranges. Overall, the breeding cycle of both species displayed a latitudinal trend, with reproductive seasons increasing southwards, earlier spawning in northern populations and later spawning in southern populations, as already recognised in previous studies with *P. vulgata* (e.g. Bowman & Lewis, 1986; Guerra & Gaudêncio, 1986; Ribeiro *et al.*, 2009) and *P. ulyssiponensis* (e.g. Ribeiro *et al.*, 2009; Fernández *et al.*, 2016). In addition, both species apparently exhibited more flexible breeding cycles towards their southern distributional limit, with an increasing trend of multiple spawning events in *P. vulgata* from northwestern Spain (Fernández *et al.*, 2015), as well as several spawning episodes in *P. ulyssiponensis* from northwestern Spain (Fernández *et al.*, 2016) and virtually continuous breeding and loose spawning synchrony in *P. ulyssiponensis* from northern Portugal (Ribeiro *et al.*, 2009). Altogether, the present study revealed that populations from southern Portugal have the later spawning period reported for *P. vulgata* (season: early spring – early summer; peak: March – June / July) and *P. ulyssiponensis* (season: early spring – early summer; peak: March – July) (Table 3), further reinforced in certain years by the occurrence of multiple spawning events throughout the reproductive cycle.

Influence of seawater temperature on the reproductive cycle

The present study confirmed the influence of SST on the reproductive cycle of both *P. vulgata* and *P. ulyssiponensis*, with resting period coincident with warmer SST in summer, gametogenic development and gonadal maturation triggered by decreasing SST during autumn, and main spawning season roughly synchronised with increasing SST throughout early spring – early summer. In general, most previous studies performed in the UK suggested that spawning activity in *P. vulgata* is apparently associated to rough weather conditions, strong onshore winds and high wave action (e.g. Orton *et al.*, 1956; Bowman & Lewis, 1977, 1986; Thompson, 1980; Baxter, 1983), further contributing for spatial (place to place) and temporal (year to year) variation in the main spawning period (Orton *et al.*, 1956).

Nevertheless, there is general consensus that water temperature is the main environmental factor influencing the reproductive cycle of patellid limpets (Ribeiro *et al.*, 2009). In fact, studies performed in northern populations of *P. vulgata*, suggested that the duration of the resting phase was apparently related with local temperature (Orton *et al.*, 1956) and the main spawning period was associated with a drop in SST below 12ºC (e.g. Bowman & Lewis, 1977, 1986; Delany *et al.*, 2002). Regarding this temperature threshold, SST only falls to ≈12ºC during mid-winter in northern Portugal (Guerra & Gaudêncio, 1986) and such low SST was never reached during the present study period in southern Portugal (minimum SST: 14.2ºC in 2017 and 13.8ºC in 2018). On the opposite, higher seawater temperature seems to stimulate spawning in Portuguese limpet populations (Ribeiro *et al.*, 2009), which appears to be exactly the case of *P. vulgata* and *P. ulyssiponensis* from sheltered areas in southern Portugal. This reproductive strategy with spawning season in early spring–early summer might eventually enhance larval development and settlement, profiting from suitable and stable weather conditions, seawater temperature and food availability.

Conclusion

Despite several previous studies on this subject, the present work is the second detailed description of the reproductive cycle of *P. vulgata* and *P. ulyssiponensis* based on gonad histological analyses. Besides improving the knowledge on diverse aspects of the reproduction in both species (e.g. size-specific sex ratios, size at sex change and reproductive cycle), this study is relevant for conservation purposes and provides baseline information for the eventual proposal of additional management measures for the professional / recreational harvesting of *P. vulgata* and *P. ulyssiponensis*.

As previously mentioned, the current regulations concerning the collection of patellid limpets in mainland Portugal only stipulate the minimum conservation reference size (MCRS = 20 mm SL), which only protects the smaller individuals and mostly the male fraction of the populations. In this context, taking into account the size at sex change estimated for *P. vulgata* ($SL_{50} \approx 29$ mm) and *P. ulyssiponensis* ($SL_{50} \approx 36$ mm), the establishment of a slightly larger MCRS (30 mm SL as compromise solution for both species that are sympatric and hardly distinguishable by the harvesters) would enable catches with more balanced sex ratios. In addition, as already implemented for other exploited patellid limpets, a maximum harvesting size could also be established to protect the larger individuals and mostly the female fraction of the populations (i.e. comprising the larger spawning females with higher reproductive output), although recognising its more difficult enforcement by the fishery inspectors and compliance by the harvesters.

Furthermore, in the hypothetical scenario of a significant increase in the harvesting effort targeting these species, the information gathered in this study might support the proposal of a closed season in the harvesting activity during the spawning peak of *P. vulgata* and *P. ulyssiponensis* (April – May as compromise solution for both species that are jointly harvested). Ultimately, this management measure would help protecting the most crucial phase of the species reproductive cycle and promoting the long-term sustainable exploitation of this resource in southern Portugal.

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Data policy

The raw data analysed in the present thesis are not publicly available, but are duly stored and available following reasonable and justified request to the Head of IPMA–Olhão (Miguel B. Gaspar).

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Figures legends:

Figure 1. Map showing the location of the collecting site of *Patella vulgata* and *Patella ulyssiponensis* on intertidal rocky shores of Praia da Luz in Lagos (Algarve coast – southern Portugal).

Figure 2. Comparison of the size-frequency distribution between males and females of **(a)** *Patella vulgata* and **(b)** *Patella ulyssiponensis* (grouped into 5 mm SL classes).

Figure 3. Size at sex change (SL_{50}) in *Patella vulgata* and *Patella ulyssiponensis* (grouped into 5 mm SL classes). SL_{50} of each species highlighted with dashed lines.

Figure 4. Photomicrographs of histological sections showing the gonad maturation stages in males (left) and females (right) of *Patella vulgata*: **(a)** Inactive; **(b)** Early development; **(c)** Late development; **(d)** Ripe; **(e)** Spawning; **(f)** Spent.

Figure 5. Photomicrographs of histological sections showing the gonad maturation stages in males (left) and females (right) of *Patella ulyssiponensis*: **(a)** Inactive; **(b)** Early development; **(c)** Late development; **(d)** Ripe; **(e)** Spawning; **(f)** Spent.

Figure 6. Monthly variation (bar charts) and annual proportion (pie charts) of gonad maturation stages in **(a)** males and **(b)** females of *Patella vulgata* during the study period (January 2017 – December 2018).

Figure 7. Monthly variation (bar charts) and annual proportion (pie charts) of gonad maturation stages in **(a)** males and **(b)** females of *Patella ulyssiponensis* during the study period (January 2017 – December 2018).

Figure 8. Monthly variation of the mean gonadal index (GI) in males and females of **(a)** *Patella vulgata* and **(b)** *Patella ulyssiponensis* as a function of surface seawater temperature (SST) throughout the study period (January 2017 – December 2018). SST presented as mean and range (dashed and dotted lines).

Tables captions:

Table 1. Brief description of the microscopic maturation scale employed to classify the gonad maturation stages in both sexes of *Patella vulgata* and *Patella ulyssiponensis* [adapted and modified from McCarthy et al. (2008) and Prusina et al. (2014)].

Table 2. Main descriptive statistics of the samples of *Patella vulgata* and *Patella ulyssiponensis* from intertidal rocky shores of Praia da Luz in Lagos (Algarve coast – southern Portugal).

Table 3. Comparison of the spawning season and peak in *Patella vulgata* and *Patella ulyssiponensis* throughout their distributional ranges in European waters (northeastern Atlantic Ocean).

Figure 1.

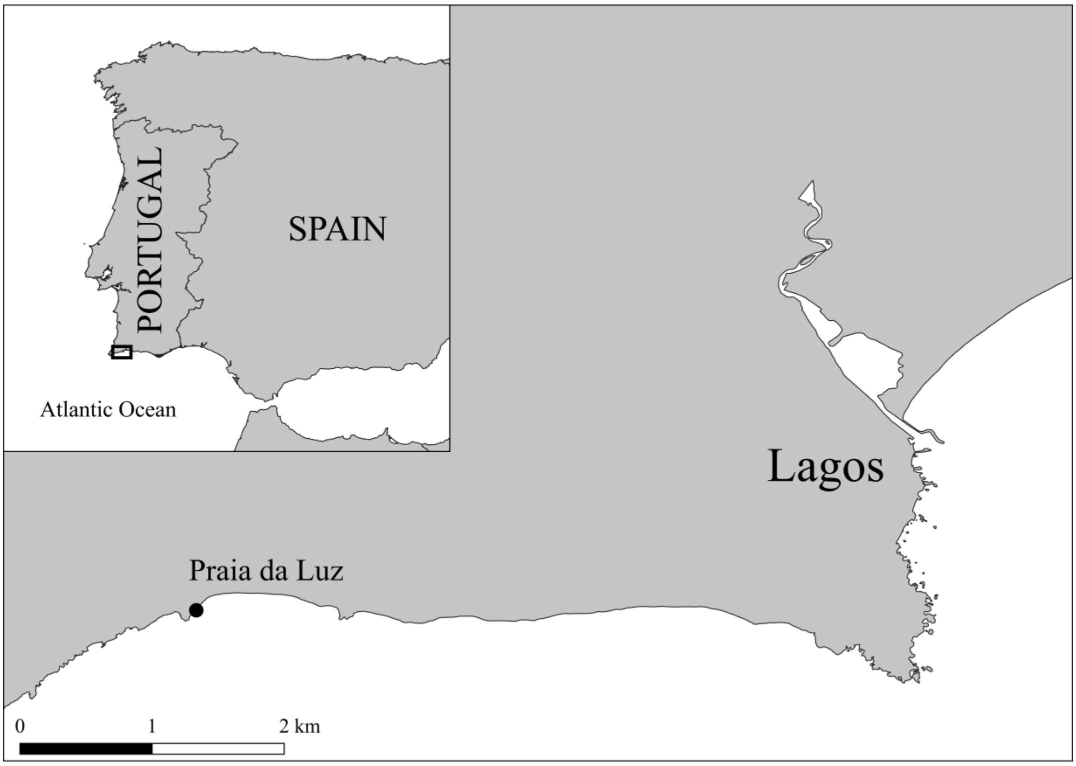
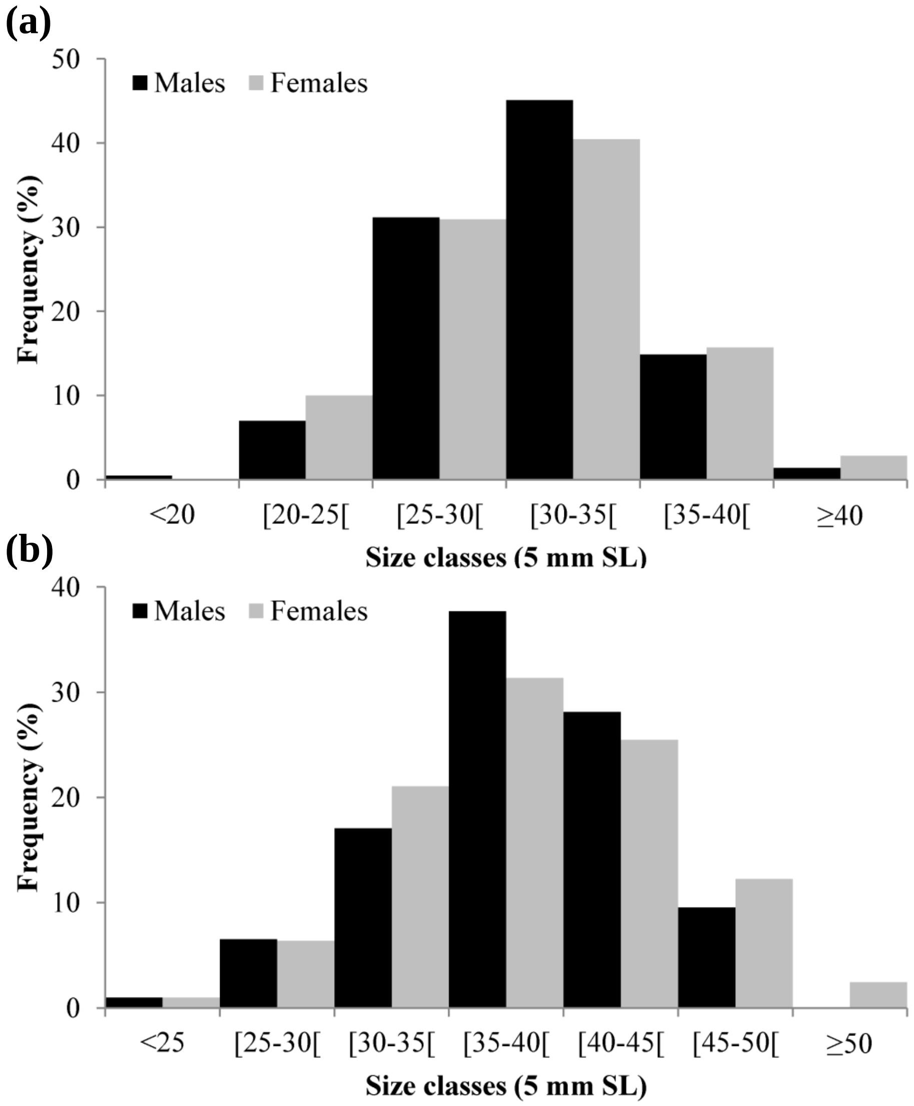


Figure 2.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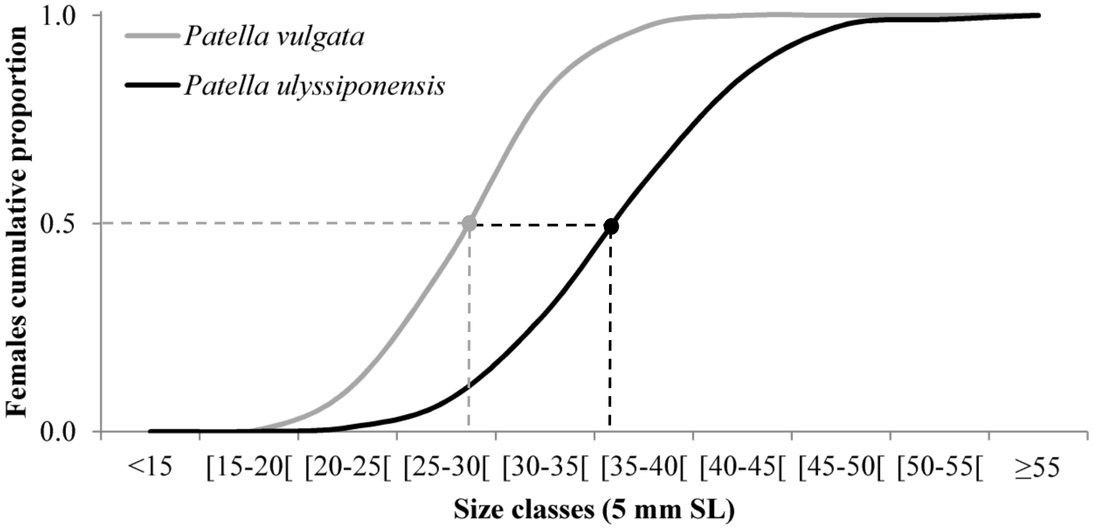
Size classes (5 mm SL)

Figure 3.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Size classes (5 mm SL)

Figure 4.

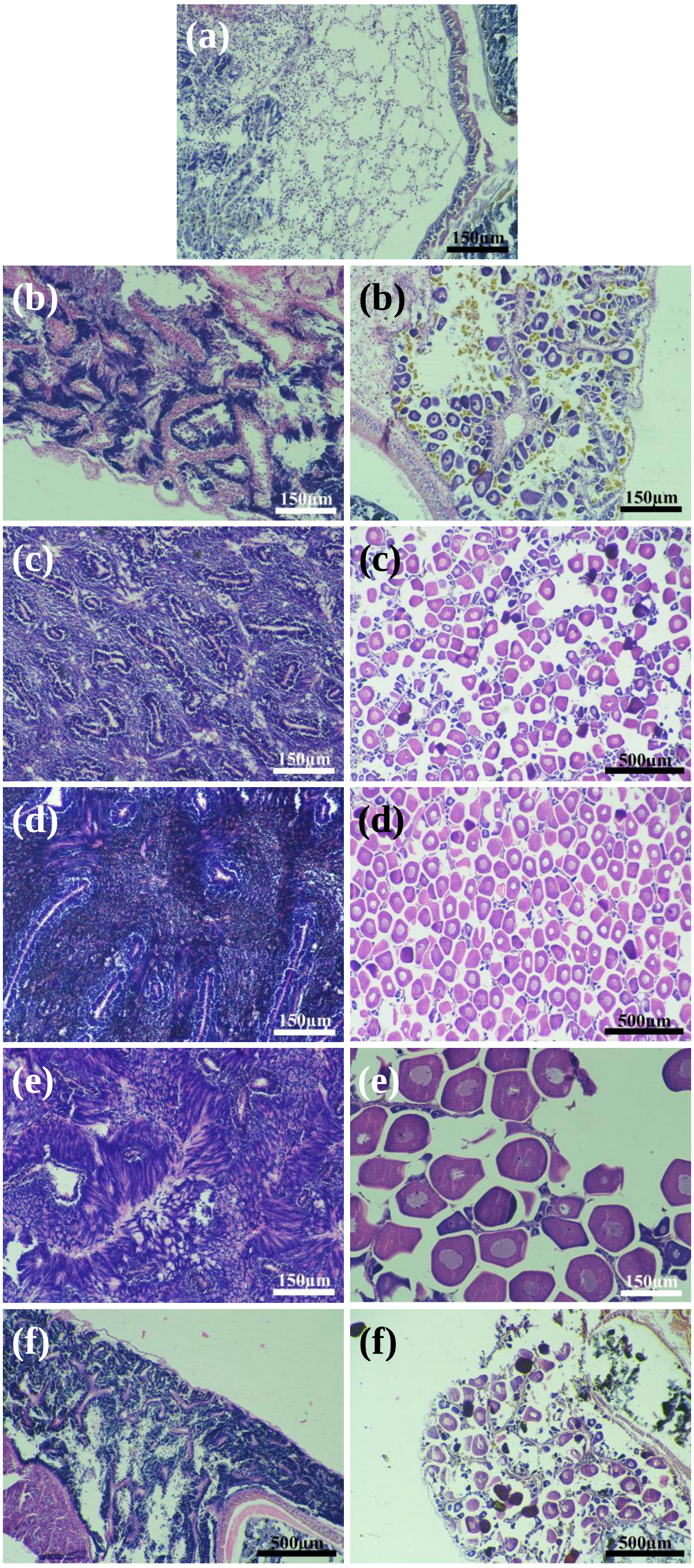
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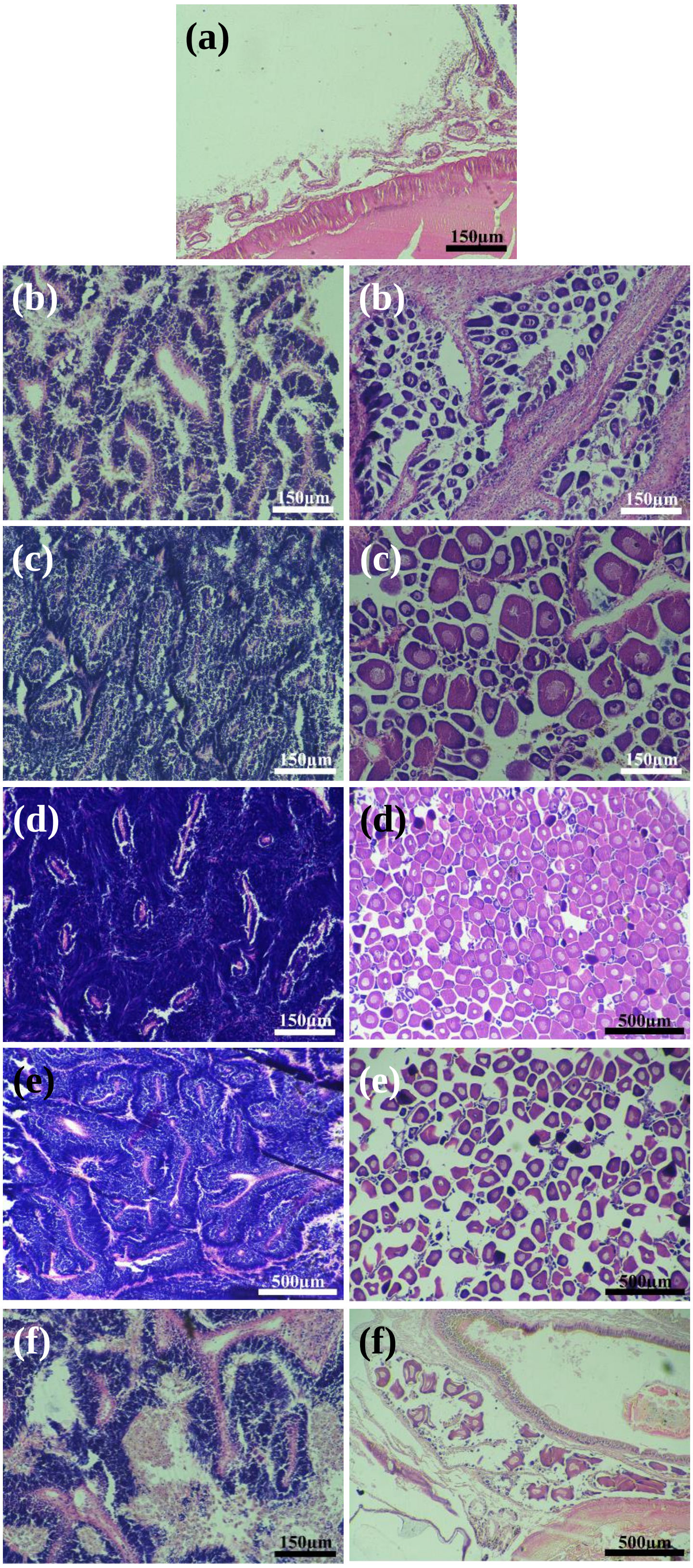
Figure 5.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150μm
(d)
500μm
(e)
500μm
(e)
500μm
(f)
150μm
(f)
500μm

Figure 6.

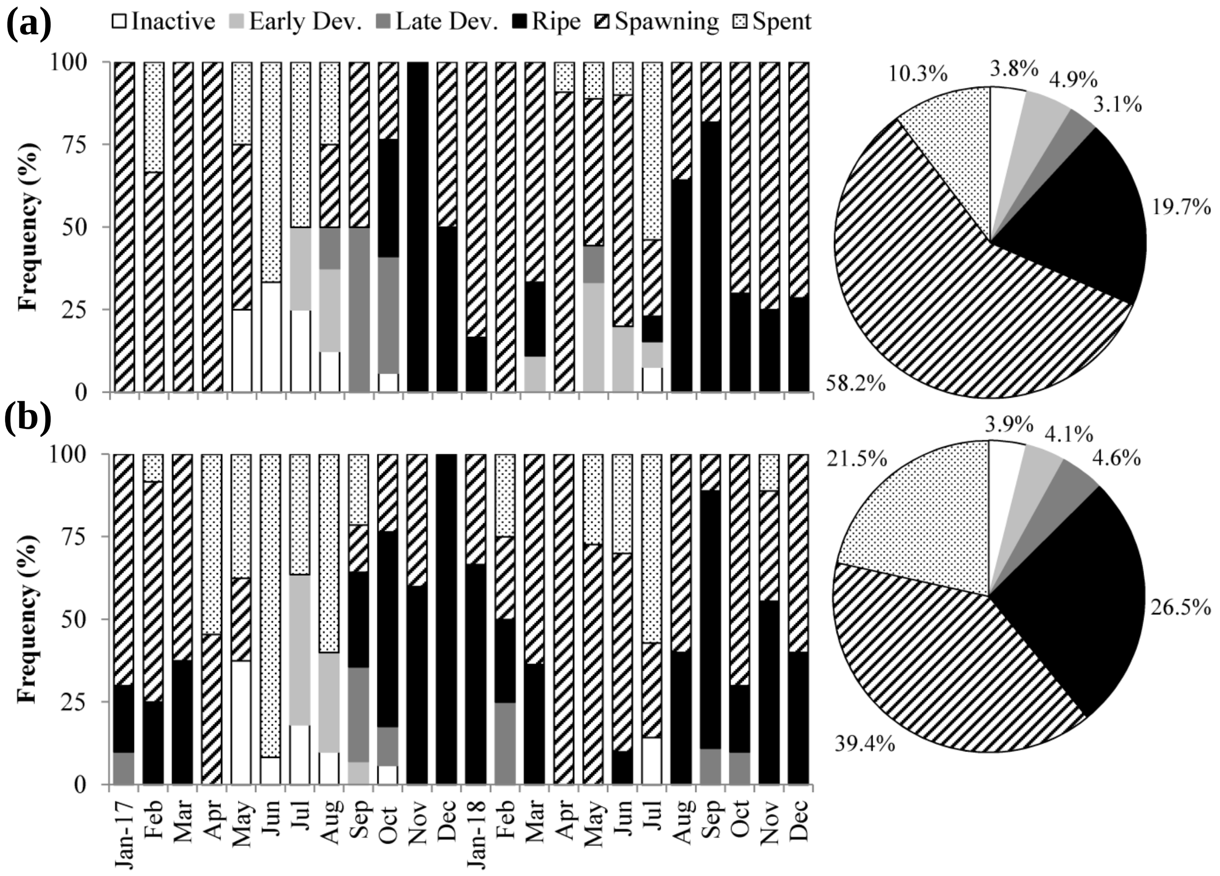
(a)
☐ Inactive
Early Dev.
Late Dev.
■ Ripe
▨ Spawning
⊡ Spent
Frequency (%)
100
75
50
25
0
10.3%
3.8%
4.9%
3.1%
19.7%
58.2%
(b)
Frequency (%)
100
75
50
25
0
21.5%
3.9%
4.1%
4.6%
26.5%
39.4%
Jan-17
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan-18
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec

Figure 7.

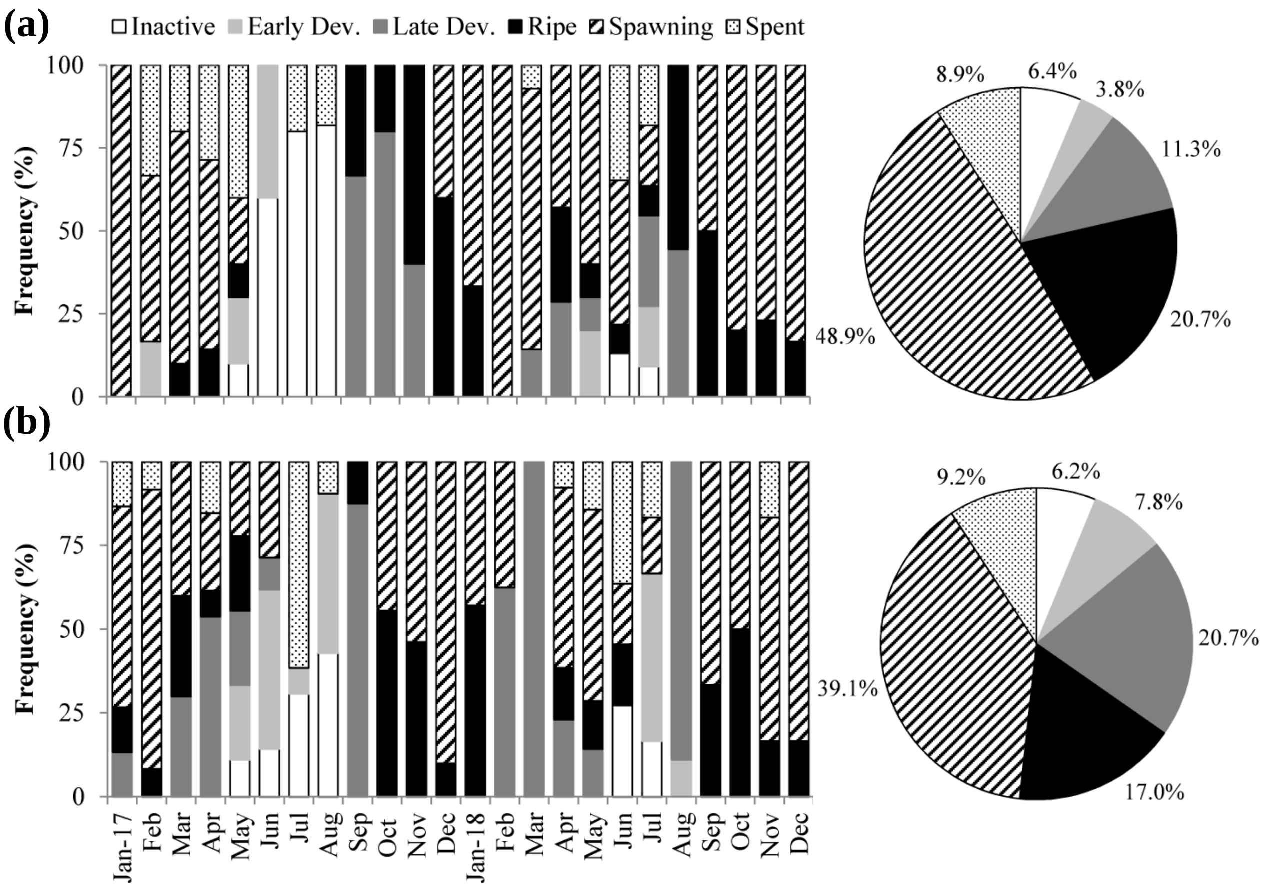
(a)
☐ Inactive
Early Dev.
Late Dev.
■ Ripe
▨ Spawning
▨ Spent
Frequency (%)
100
75
50
25
0
8.9%
6.4%
3.8%
11.3%
20.7%
48.9%
(b)
Frequency (%)
100
75
50
25
0
9.2%
6.2%
7.8%
20.7%
17.0%
39.1%
Jan-17
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan-18
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec

Figure 8.

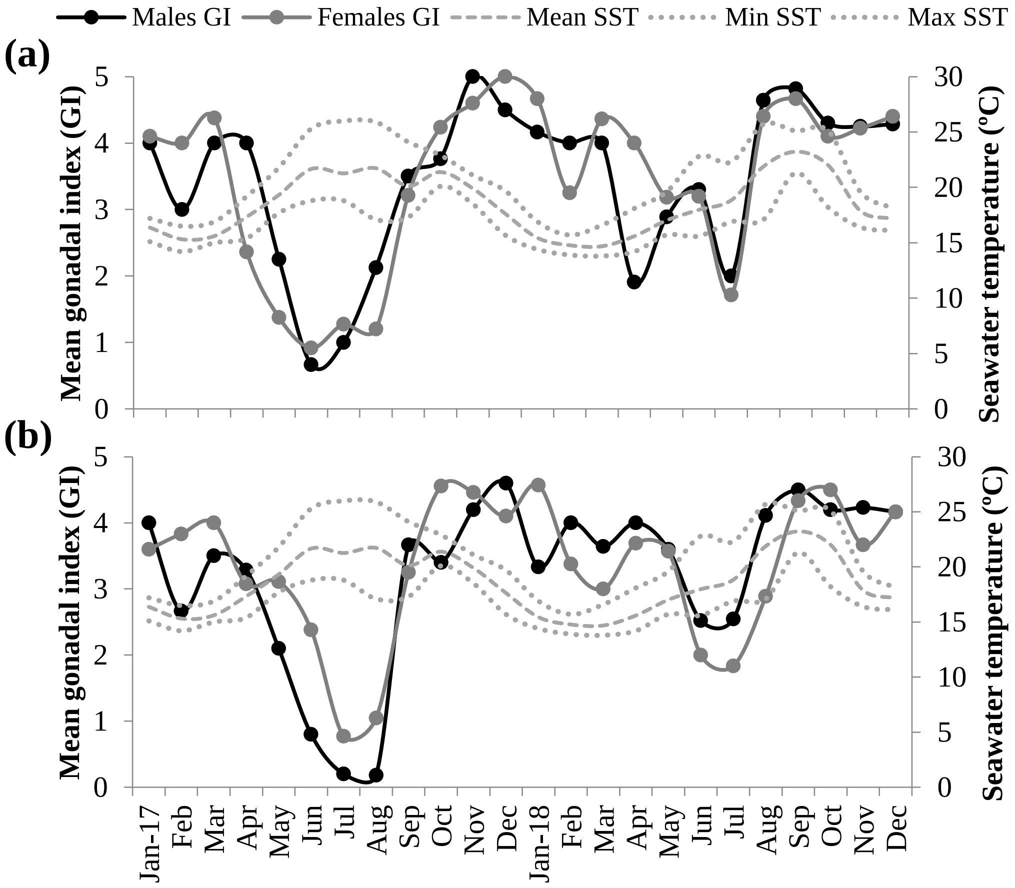
Males GI
Females GI
Mean SST
Min SST
Max SST
(a)
(b)
Mean gonadal index (GI)
Seawater temperature (ºC)
0
1
2
3
4
5
0
5
10
15
20
25
30
Jan-17
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan-18
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec

Table 1. Brief description of the microscopic maturation scale employed to classify the gonad maturation stages in both sexes of *Patella vulgata* and *Patella ulyssiponensis* [adapted and modified from McCarthy *et al.* (2008) and Prusina *et al.* (2014)].

Stage	Males	Females
Inactive	Very few undifferentiated gametes; abundant connective tissue in the gonad	
Early development	Few spermatogonia at the edges of the testes lobes; still abundant connective tissue	Small oogonia emerging from the acini wall; early oocytes of varied sizes
Late development	Layers of reproductive cells (spermatogonia, spermatocytes and spermatids) distributed from the tubular wall to the lumen	Enlarged acini; large previtellogenic oocytes and few oocytes in late vitellogenic stages; reduced connective tissue
Ripe	Tubules filled with mature reproductive cells; free spermatozoa fill the lumen	Large acini with free and mature oocytes; mature oocytes undergoing atresia can be also observed
Spawning	Smaller tubules surrounded by abundant connective tissue; star-shaped lumina with spermatozoa tails in the centre of the tubules	Large ovaries but becoming slack; small acini without generative activity in the walls; mature oocytes observed in the connective tissue
Spent	Haemocytes can be observed in the interstitial and connective tissue surrounding residual spermatozoa	Slack ovaries with signs of tissue degeneration; haemocytes can be observed in the interstitial and connective tissue; few degenerative oocytes may be present

Table 2. Main descriptive statistics of the samples of *Patella vulgata* and *Patella ulyssiponensis* from intertidal rocky shores of Praia da Luz in Lagos (Algarve coast – southern Portugal).

	N	**Sex ratio (M: F)**	**Mean SL ± S.D. (min. – max.)**	**Mean TW ± S.D. (min. – max.)**
Patella vulgata				
Males	215	1M: 0.98F	31.1 ± 4.1 (19.5 – 42.7)	6.3 ± 2.5 (1.6 – 16.0)
Females	210		31.1 ± 4.6 (20.7 – 43.0)	6.4 ± 2.8 (1.6 – 15.9)
Undifferentiated	17		28.1 ± 3.3 (20.2 – 35.6)	4.9 ± 2.1 (1.8 – 9.2)
Hermaphrodites	2		30.6 ± 3.8 (27.9 – 33.3)	4.7 ± 1.1 (3.9 – 5.5)
Total	**444**		**31.0 ± 4.3 (19.5 – 43.0)**	**6.3 ± 2.7 (1.6 – 16.0)**
Patella ulyssiponensis				
Males	199	1M: 1.03F	38.1 ± 5.4 (12.3 – 49.8)	12.3 ± 6.1 (0.3 – 38.7)
Females	204		38.5 ± 5.8 (23.8 – 55.4)	11.8 ± 6.6 (2.2 – 36.7)
Undifferentiated	27		33.0 ± 3.7 (26.7 – 41.3)	7.2 ± 3.5 (2.6 – 13.1)
Hermaphrodites	8		38.2 ± 5.8 (32.0 – 50.3)	14.4 ± 10.0 (5.7 – 34.8)
Total	**438**		**38.0 ± 5.7 (12.3 – 55.4)**	**11.8 ±6.4 (0.3 – 38.7)**

Table 3. Comparison of the spawning season and peak in *Patella vulgata* and *Patella ulyssiponensis* throughout their distributional ranges in European waters (northeastern Atlantic Ocean).

Species	**Country**	**Location**	**Study period**	**Methods**	**Spawning season**	**Spawning peak**	**References**
P. vulgata	UK	Plymouth and Cornwall	n/a	MO	August–March	January–February	Orton (1928)
	UK	Six locations	1945–1952*	MO	October–March	October–December	Orton *et al.* (1956)
	UK	Robin Hood’s Bay, North Yorkshire	Sep 64–Dec 66	MO, GI	September/November–March	Autumn	Blackmore (1969)
	Ireland	Bocarnagh Bay, Bantry Bay	Mar 72–Jul 73	MO, GI	September–January/February	December–February	Thompson (1980)
	UK	Sandwick Bay, Orkney Islands	Aug 79–Mar 82	MO, GI		December–February	Baxter (1983)
	UK	Robin Hood’s Bay, North Yorkshire	1968–1985	MO, GI	August–December	September–October	Bowman & Lewis (1986)
	UK	Ten locations	1973–1984*	MO, GI	August–January	September–November	Bowman & Lewis (1986)
	Portugal	Moledo do Minho	Mar 81–Mar 84	MO, GI	September–January	November–January	Guerra & Gaudêncio (1986)
	Spain	Fuenterrabia, San Sebastian and Zumaya	Feb 83–Aug 85	GI	December–May		Ibanez *et al.* (1986)
	Ireland	Clare Island	n/a	JR	Winter		Delany *et al.* (1998)
	UK	Isle of Man	Apr 97–Jul 98	MO, GI	October–December	October–November	Jenkins *et al.* (2001)
	Ireland	Clare Island	1993–1994	n/a	October–November		Delany *et al.* (2002)
	UK	Wembur and Heybrook Bay	Aug 03–Dec 03	MO	November–January	December	Coleman *et al.* (2006b)
	UK	Kingsand and Andurn Point	Aug 03–Mar 05	MO	October–January	November	Moore *et al.* (2007)
	Ireland	Garrettstown	Sep 03–Oct 04	MO, GH	September–December	December	McCarthy *et al.* (2008)
	Portugal	Moledo do Minho, Viana do Castelo and Homem do Leme	Jan 99–Mar 01	MO		November–January	Ribeiro *et al.* (2009)

	Spain	Galician coast (five locations) $^{\mathrm{CH}}$	Jul 10–Jun 11	MO	Late autumn–Early winter	October–January	Fernández *et al.* (2015)
	Portugal	Praia da Luz, Algarve coast $^{\mathrm{RH}}$	Jan 17–Dec 18	GH, GI	Early spring–Early summer	March–June/July	Present study
P. ulyssiponensis	Ireland	Clare Island	n/a	JR	Winter		Delany *et al.* (1998)
	Ireland	Clare Island	1993–1994	n/a	September–October		Delany *et al.* (2002)
	Ireland	Garrettstown	Sep 03–Oct 04	MO, GH	September–December	December	McCarthy *et al.* (2008)
	Portugal	Moledo do Minho, Cabo do Mundo and Aguda	Dec 97–Jan 01	MO	September–January / March–June		Ribeiro *et al.* (2009)
	Spain	Galician coast (five locations) $^{\mathrm{RH}}$	Jul 10–Jun 11	MO	Several spawning events throughout the year		Fernández *et al.* (2016)
	Portugal	Praia da Luz, Algarve coast $^{\mathrm{RH}}$	Jan 17–Dec 18	GH, GI	Early spring–Early summer	March–July	Present study

Abbreviations: CH, commercial harvesting; RH, recreational harvesting; n/a, not available; *, variable depending on the sampling site; MO, macroscopic observation of the gonads; GI, gonadal indices; JR, juvenile recruitment; GH, gonad histology.