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On the reproduction of the Mediterranean keystone limpet *Patella rustica*: histological overview

IVANA PRUSINA[1], DARIA EZGETA-BALIĆ[2], STIJEPO LJUBIMIR[3], TATJANA DOBROSLAVIĆ[1]
AND BRANKO GLAMUZINA[1]

[1]Department of Aquaculture, University of Dubrovnik, Ćira Carića 4, 20000 Dubrovnik, Croatia, [2]Institute of Oceanography and Fisheries, Šetalište Ivana Meštrovića 63, 21000 Split, Croatia, [3]Institute for Marine and Coastal Research, University of Dubrovnik, Kneza Damjana Jude 12, 20000 Dubrovnik, Croatia

The reproductive cycle of the high shore limpet Patella rustica *is described based on histological analysis as the primary method of staging gonad development. Sex-ratios, shell length at sexual maturity, gonad developmental stages, mean gonad index and oocyte size were investigated. Males and females were found to differ in size distribution, with females becoming more prevalent from ~28 mm onwards. The estimated shell length at which 50% of males were sexually mature was 13.1 mm.* Patella rustica *has only one reproductive cycle per year with a spawning peak between November and December for both sexes, and gonad redevelopment from January. First data on the size–frequency analysis of oocytes for this species are also presented, concurring with the qualitative analysis of the gonad developmental stages. This study presents updated information on the reproductive cycle of this keystone species and provides the first account of the reproductive biology of* P. rustica *in the Adriatic Sea.*

Keywords: *Patella rustica*, reproduction, gonad histology, oocyte size, sexual maturity, Adriatic Sea

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INTRODUCTION

In the Mediterranean Sea the genus *Patella* comprises four species: *Patella rustica* (Linnaeus, 1758); *P. caerulea* (Linnaeus, 1758); *P. ulyssiponensis* (Gmelin, 1791); and *P. ferruginea* (Gmelin, 1971) (Sella *et al.*, 1993; Ridgway *et al.*, 1998). The latter, with its present geographical distribution limited to the western basin, is considered to be at serious risk of extinction (Templado *et al.*, 2004). The other three congeners co-occur on rocky shores but have different vertical zonation; *P. rustica* occurs in the upper intertidal, *P. caerulea* inhabits the lower mid-littoral as well as upper infralittoral, while *P. ulyssiponensis* inhabits the low intertidal shore and infralittoral fringe (Della Santina *et al.*, 1993; Šimunović, 1995; Mauro *et al.*, 2003).

Patella rustica ranges from the Mediterranean to the Atlantic coasts of the Iberian Peninsula and northern Africa, including the Macaronesian Islands (Ridgway *et al.*, 1998; Lima *et al.*, 2006), but recent distributional shifts related to climate change have been recorded in Portugal (Lima *et al.*, 2006; Sousa *et al.*, 2012). Intertidal communities are fundamentally structured by limpet grazing (Hawkins *et al.*, 1992; Paine, 2002; Jenkins *et al.*, 2005; Coleman *et al.*, 2006), hence they are rightfully considered to be a keystone species (*sensu* Power *et al.*, 1996), whose removal or alterations of behaviour may have fundamental ecosystem consequences.

A number of studies have been conducted in order to investigate the reproductive cycles of *Patella* congeners, e.g. *Patella vulgata* (Orton *et al.*, 1956; Thompson, 1980; Bowman & Lewis, 1986; Delany *et al.*, 2002; McCarthy *et al.*, 2008; Ribeiro *et al.*, 2009), *Patella depressa* (Orton & Southward, 1961; Bowman & Lewis, 1986; Brazão *et al.*, 2003; Moore *et al.*, 2007; Ribeiro *et al.*, 2009), *Patella ulyssiponensis* (Evans, 1953; Thompson, 1979; Bowman & Lewis, 1986; Guerra & Gaudêncio, 1986; McCarthy *et al.*, 2008; Ribeiro *et al.*, 2009), *Patella caerulea* (Frenkiel, 1975; Belkhodja *et al.*, 2011) and *Patella ferruginea* (Espinosa *et al.*, 2006; Guallart *et al.*, 2013). The reproductive cycle of *P. rustica* has been previously described on the Algerian coast of the Mediterranean Sea (Frenkiel, 1975), and on the Basque (Othaitz, 1994) and Portuguese coasts of the north-east Atlantic (Ribeiro *et al.*, 2009), based only on macroscopic examination of gonads and the staging scheme developed by Orton *et al.* (1956). Although macroscopic examination based on the size of gonads allows for the description of gonad development, histology provides a more precise and accurate evaluation of gonad developmental stages.

To the authors' best knowledge, no previous research has been performed to describe reproductive cycle of *P. rustica* based on histological analysis, and none of the studies were conducted on limpets from the Adriatic shore. The aim of this study, therefore, was to investigate the reproductive cycle and to develop a staging scheme for gonad development of *P. rustica*, combining qualitative and quantitative

Corresponding author:
I. Prusina
Email: ivana.prusina@unidu.hr

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histological techniques. Sex-ratio, gonad developmental stages, mean gonad index, oocyte size and shell length at sexual maturity were analysed in order to provide new information on the reproductive biology of this species.

MATERIALS AND METHODS

Study site and sampling

The limpet *Patella rustica* was hand collected from the high shores of Zaton Bay in the south-eastern Adriatic (42°41′40″N 18°02′32″E). Sampling was performed monthly, from July 2011 to June 2012. Seawater and air temperatures were obtained from the Croatian Meteorological and Hydrological Service. Additionally, chlorophyll-*a* concentration (chl *a*) at the seawater surface was determined from 500 ml sub-samples filtered through Whatman GF/F glass-fibre filters and stored at −20°C. These were homogenized and extracted in 90% acetone for 24 h at room temperature, analysed fluorometrically with a Turner TD-700 Laboratory Fluorometer (Sunnyvale, CA) calibrated with pure chl *a* (Sigma) and calculated according to the method of Jeffrey & Welschmeyer (1997).

A total of 480 specimens were sampled for histological analysis: (a) 30 individuals were sampled monthly ($N = 355$, mean length ± standard deviation (SD) $= 24.2 \pm 2.9$ mm) except in December 2011, when only 25 individuals were collected due to severe weather conditions; (b) smaller-sized limpets within the range of 10.1–22.4 mm in shell length ($N = 95$, mean length ± SD $= 16.2 \pm 3.0$ mm) were sampled in September–November 2011 and February, March and May 2012 and used for qualitative histological analysis. Out of these 95 smaller-sized limpets, 65 specimens collected in September–November 2011 were used for estimating the smallest size at sexual maturity and for comparison of the sex-ratio with the shell length–frequency distribution of males and females.

Histological analysis

Limpets were dissected and gonad tissue was fixed in 10% buffered formalin. Fixation was followed by standard tissue processing that included dehydration in increasingly concentrated ethanol, clearing with chloroform, embedding in paraffin, sectioning at 5 μm and finally staining using haematoxylin and the eosin method. Sections were examined at 50× 100× and 400× magnifications, sexed and assigned to a developmental stage adopted from McCarthy *et al.* (2008) and Belkhodja *et al.* (2011) and modified for this species (Table 1). Five numerical stages were determined for males (Figure 1): early active (3), late active (4), ripe (5), spawning (2) and spent (1), and seven for females (Figure 2): inactive (0), early active (3), late active (4), ripe (5), atresic (1.5), spawning (2), and spent (1). Individuals whose sex was impossible to determine were classified as undifferentiated. An undifferentiated limpet could be an adult in the resting stage of gonad development or a juvenile that had not yet reached

Table 1. Histological appearance of gonad developmental stages in *Patella rustica* (adopted and modified from McCarthy *et al.*, 2008 and Belkhodja *et al.*, 2011).

Stage no.	Description	Male	Female
0	Inactive		Abundant connective tissue in the gonad. Only a few oogonia are present
3	Early	Islet of small compact acini. One or two layers of spermatogonia line the edges of the testes lobes. Connective tissue still abundant	Acini lumen large and mostly empty. Small oogonia in proliferation adhering to the acini wall. Previtellogenic oocytes of various sizes can also be found. Connective tissue still abundant
4	Late	Layers of reproductive cells completely fill the lumen. These cells are in different stages of maturation forming a regular pattern from the tubular wall to the lumen—spermatogonia, spermatocytes and spermatides. Tails of first spermatozoa are in the tubular lumen, resulting in the pink colour of the lumen	Gonadal acini are enlarged. Reduction of connective tissue. Reproductive cells are in different stages of maturation. Fewer oogonia attached to the acini wall. Large previtellogenic oocytes and few oocytes in the final stages of vitellogenesis are present. Oocytes undergoing different stages of atresia can be present also
5	Ripe	Tubules are enlarged with only one layer of spermatogonia attached to the tubular wall. Free spermatozoa fill the lumen. Light pink connective tissue very thin and stretched between large acini	Gonadal acini large with completely developed free vitellogenic oocytes. Mature oocytes undergoing atresia or complete lysis can be found. Acini with oocytes in advanced vitellogenesis showing signs of atresia are also present
1.5	Atresic		Gonadal tissue is still abundant but majority of oocytes are undergoing high degree of atresia. A few previtellogenic and vitellogenic oocytes may be present also
2	Spawning	The tubules are smaller in size surrounded with loose and abundant connective tissue. Large germinal cells abundant around the edges. Smaller number of spermatozoa inside the tubules	Decrease in free vitellogenic oocytes in the lumen, some of which showing signs of atresia. Symptoms of total voidance of gametes characterized by small acini with no generative activity in their walls. Pedunculated oocytes attached to acini wall can be observed
1	Spent	Thick connective tissue present in the gonad. Haemocytes can be observed in the interstitial and connective tissue surrounding residual spermatozoa	Ovaries showing signs of tissue destruction. Abundant connective tissue present around empty acini. Only a few degenerative oocytes may be present. Haemocytes can be observed in the interstitial and connective tissue

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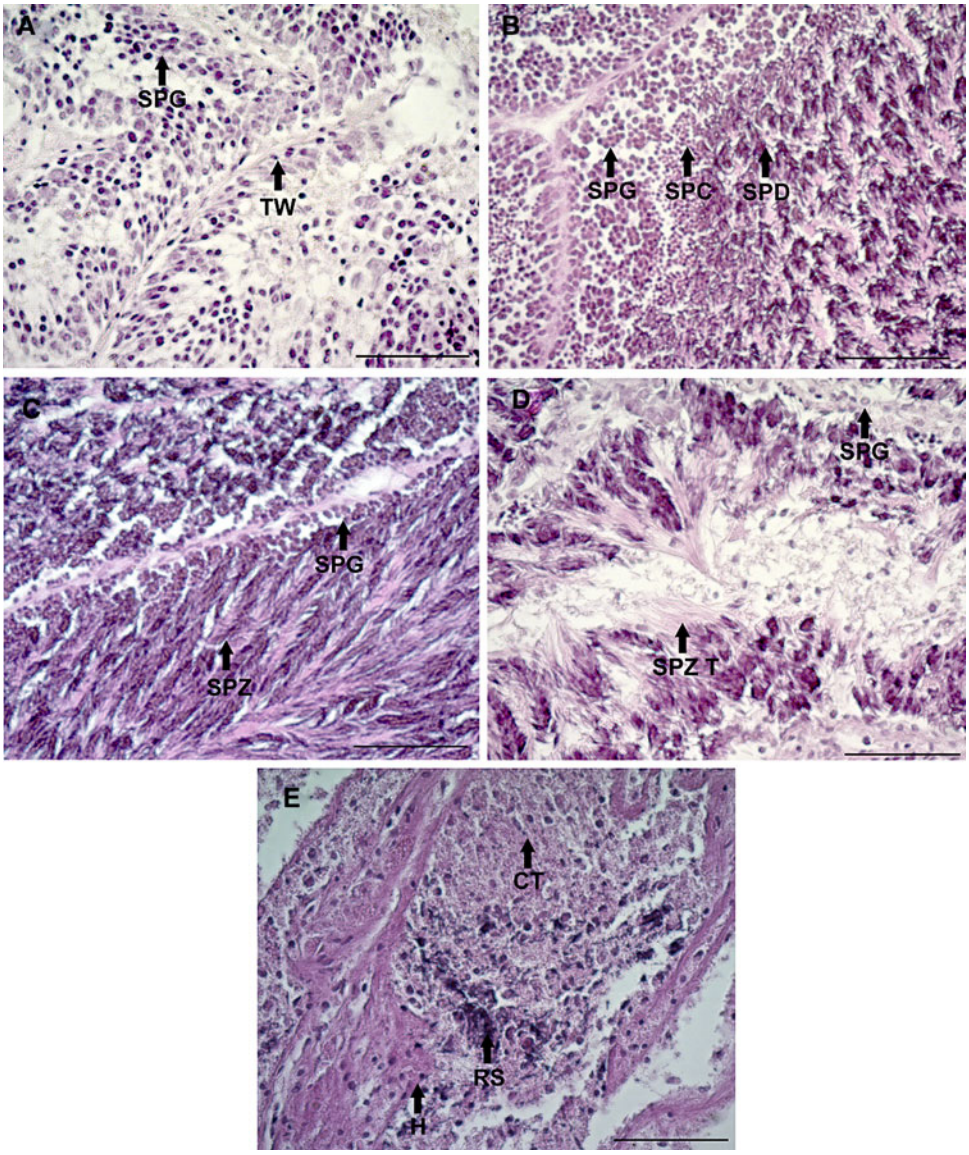

Fig. 1. Photomicrographs of gonad developmental stages in male *Patella rustica*: (A) early active; (B) late active; (C) ripe; (D) spawning; (E) spent. SPG, spermatogonia; TW, tubular wall; TL, tubular lumen; SPG, spermatogonia; SPC, spermatocytes; SPD, spermatids; SPZ, spermatozoa; SPZ T, spermatozoa tails; CT, connective tissue; H, haemocytes; RS, residual spermatozoa. Scale bar: 50 μm.

sexual maturity (see also McCarthy *et al*., 2008). A mean gonad index (MGI) was calculated monthly for males and females to estimate the proportion of individuals in different reproductive stages. Values of MGI were obtained by multiplying the number of individuals at each developmental stage by the numerical ranking of that stage, and dividing the result by the number of individuals of each sex (Gosling, 2003). For quantitative analysis, the size–frequency distribution of maximum oocyte diameter was studied. Oocytes with visible nuclei within one random visual field at 100× magnification ($0.585\ \mathrm{mm}^2$) were measured using Axio Vision Rel 4.8 software in all *P. rustica* females sampled.

Data analysis

Sex-ratios were tested using a chi-square (χ^2) goodness of fit test. To estimate the smallest size at sexual maturity, data were fitted to the equation

$$P = 1/(1 + e^{(a-b\times L)}),$$

where P is the probability that individuals were sexually mature and L is their length. Estimated length at which 50% of analysed individuals were mature was calculated as $L_{50\%} = a/b$ (Sparre & Venema, 1998). Spearman's correlation analysis was applied to the dataset to determine the degree of association between MGI and hydrographic parameters, and between female MGI and oocyte size. In all statistical analysis, critical probability value was set at 0.05.

RESULTS

From a total of 355 specimens collected monthly, 39 individuals (10.9%) were males, 170 individuals (47.9%) were

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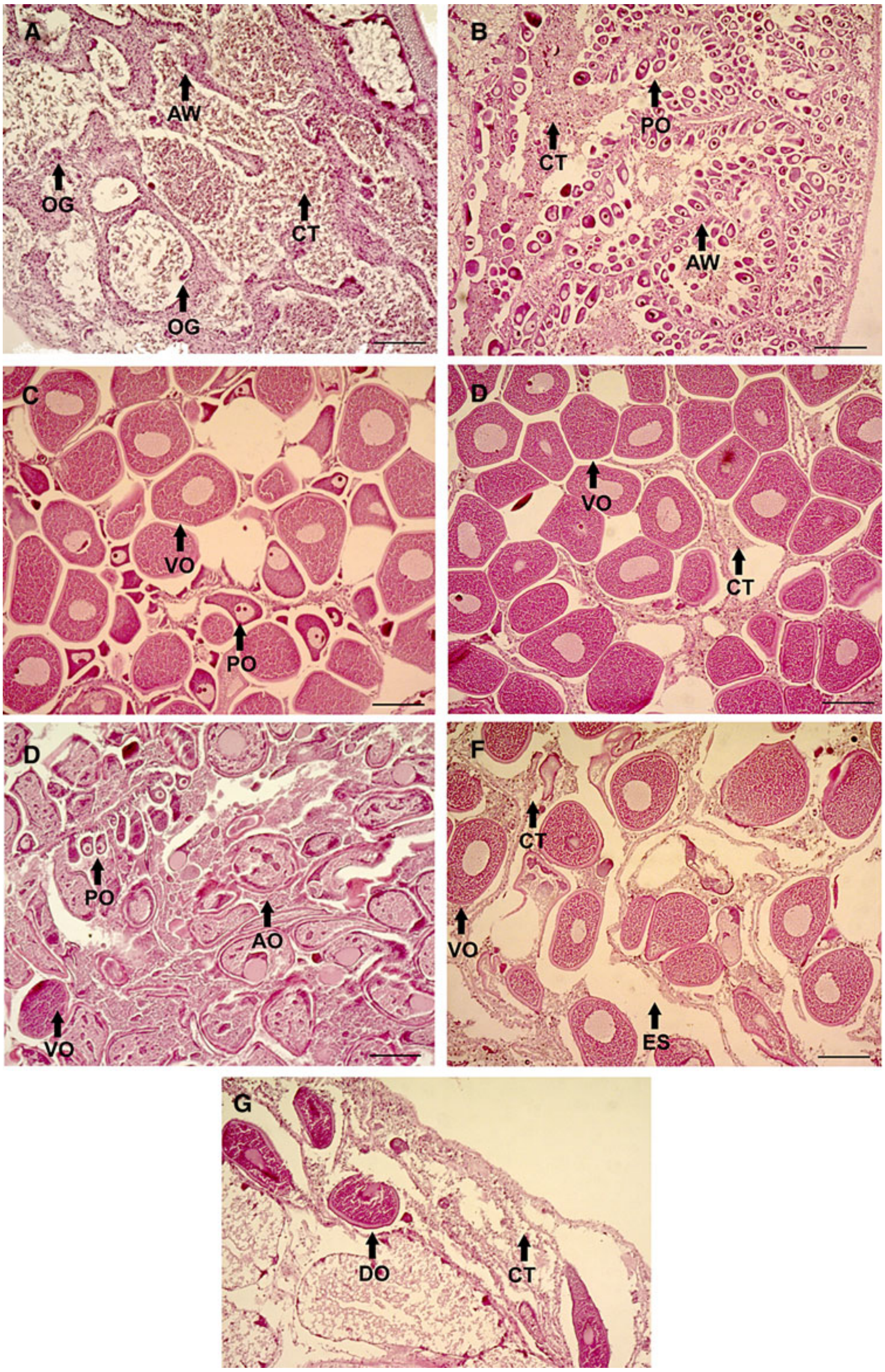

Fig. 2. Photomicrographs of gonad developmental stages in female *Patella rustica*: (A) early active; (B) late active; (C) ripe; (D) atresic; (E) spawning; (F) spent; (G) inactive. AW, acinus wall; OG, oogonia; CT, connective tissue; PO, previtellogenic oocyte; VO, vitellogenic oocyte; AO, atresic oocyte; ES, empty space; DO, degenerative oocyte. Scale bar: 100 μm.

females, three individuals (0.8%) were identified as hermaphrodites, 142 individuals (40.0%) were classified as undifferentiated while one individual (0.3%) was lost in processing (Figure 3). Sex-ratios were calculated only for the specimens with maximum gonadal activity sampled from September to November to avoid sex-biased conclusions. The χ^2 test showed statistically significant differences ($\chi^2 = 7.86$, $P =$ 0.005) in the proportion of females (N = 56) and males (N = 30) with a female:male ratio of 1.9:1. In addition, gonad samples were collected from the 95 smaller-sized individuals, out of which 40 were males (42.1%), 9 were females (9.5%) and 46 were classified as undifferentiated (48.4%). The χ^2 test calculated for the specimens with maximal gonad activity showed statistically significant differences

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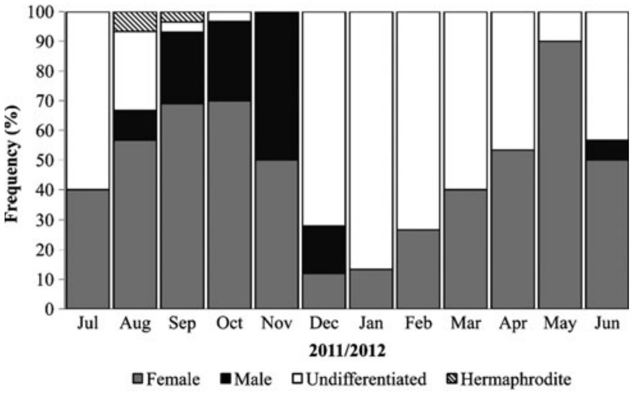

Fig. 3. Frequency of *Patella rustica* females, males, undifferentiated individuals and hermaphrodites, according to sampling month (mean shell length ± standard deviation = 24.2 ± 2.9 mm).

($\chi^2 = 23.17$, $P < 0.001$) between the number of females ($N = 7$) and males ($N = 40$) with a female:male ratio of 1:5.7. The estimated shell length at which 50% of analysed males were sexually mature was 13.1 mm. Comparing the sex-ratio and the shell length–frequency distribution of males and females, it can be seen that males dominate the smaller-sized class while females become prevalent as size increases, from ~28 mm onwards (Figure 4).

Histological analysis showed that *P. rustica* has one reproductive cycle per year. The frequency distribution of different gametogenic stages observed in females and males throughout the study period is illustrated in Figure 5. Female gametogenesis started in February and by May 96% of females were in early developmental stage. From February to May no males were recorded, while all males sampled in June and August had gonads in early developmental stage. Females went through ripening from September, when 70% of sampled females were in late developmental stage, to October when 67% of females had ripe gonads. During ripening, 5% of female gonads were in the atresic stage, characterized by a majority of previtellogenic oocytes and mature oocytes undergoing atresia or even complete citolysis. All males sampled in September were in the late developmental stage, and in November 60% of males had ripe gonads. The spawning peak occurred between November and December for both females and males. In December and January, all individuals had either spent gonads or were in the inactive stage.

The frequency distribution of oocyte diameter according to sampling month is presented in Figure 6. Monthly mean oocyte diameter ranged from 8.1 μm (January) to 118.7 μm (October). Oocytes increased in size from September (63.8 ± 40.1 μm) to November (104.5 ± 49.6 μm). After April, the frequency distribution of oocyte size had a relatively uniform pattern until October, illustrating a continuous process of maturation and release of gametes in November. There was a significant positive correlation between oocyte diameter and female MGI (Spearman $r = 0.685$, $P = 0.014$).

Analysis of environmental parameters at the sampling site (Figure 7) showed that the mean seawater surface temperature ranged from 12.5°C in February to 25.8°C in September, while the mean daily air temperatures ranged from 7.5°C in February 2012 to 27.1°C in August 2011. Chl *a* values ranged from 0.03 $\mu g\, l^{-1}$ in September to 1.68 $\mu g\, l^{-1}$ in April.

DISCUSSION

The present study investigated the annual reproductive cycle of *Patella rustica* using standard qualitative and quantitative histological techniques as the primary method for staging gonad development. The same method has been applied to several other limpet species, such as *Patella vulgata*, *Patella ulyssiponensis* (McCarthy *et al*., 2008) and *Patella caerulea* (Belkhodja *et al*., 2011), but not to *P. rustica*. Histology allows a more precise evaluation of gonad developmental stages, especially early developmental stages, that might be overlooked by other methods (McCarthy *et al*., 2008), such as macroscopic examination of gonads according to the scale established by Orton *et al*. (1956).

Results showed that *P. rustica* gonads have only one reproductive cycle per year. The reproductive cycle includes a longer period of gamete development from February to

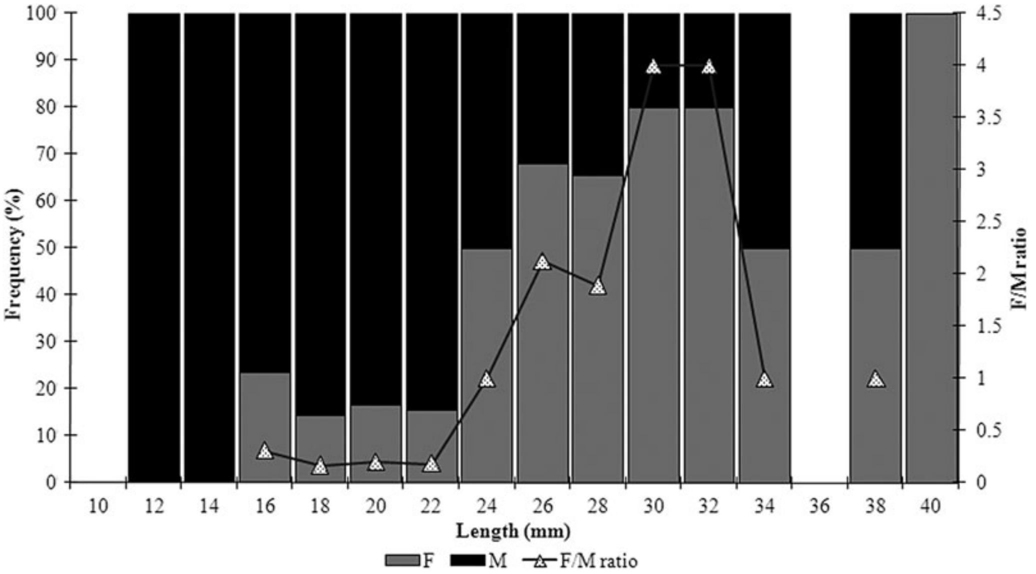

Fig. 4. Female:male ratio and frequency distribution of male (M) and female (F) *Patella rustica* with respect to shell length.

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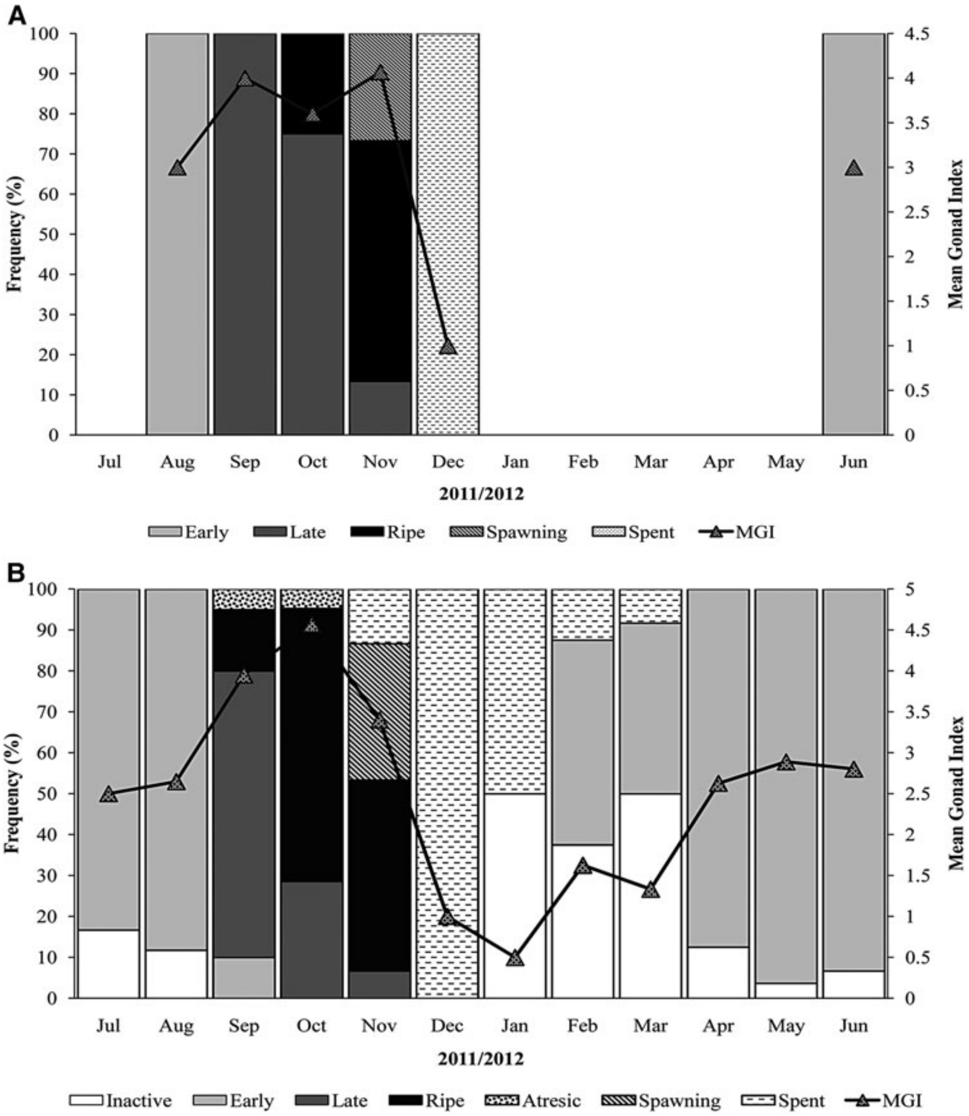

Fig. 5. Monthly variation in the frequency of different gametogenic stages and mean gonad index in *Patella rustica* (mean shell length ± standard deviation = 24.2 ± 2.9 mm): (A) males; (B) females.

October for females and from June to November for males, with a spawning peak in November/December for both sexes and gonad redevelopment from January. During gonad redevelopment, most limpets had undifferentiated gonads, probably in the post-spawning and inactive or resting periods. While for females it is possible to histologically detect inactive gonadal stage due to the presence of oogonia, this is not the case for male gonads where only connective tissues and undifferentiated gametes are present. Consequently, this would explain the lack of males in the samples from February to May and in July (see Figure 5). Undifferentiated limpets, with a higher percentage in the post-spawning period than with gonads maturing, have been reported in previous studies on *P. vulgata* (53% of specimens in the post-spawning period, McCarthy *et al.*, 2008), *P. ulyssiponensis* (80% of specimens in the post-spawning and early development periods, McCarthy *et al.*, 2008) and *P. caerulea* (51% of specimens in the spawning and post-spawning periods, Belkhodja *et al.*, 2011). In addition, the gap between early development of females and males could be due to the fact that female gonads usually develop more slowly than male gonads of similar size (Bowman, 1985; McCarthy *et al.*, 2008; Belkhodja *et al.*, 2011).

Findings from this study confirm the general reproductive pattern of *P. rustica* described previously on Algerian (Frenkiel, 1975), Basque (Othaitz, 1994) and Portuguese coasts (Ribeiro *et al.*, 2009), but also showed a few marked differences. This study suggests that *P. rustica* in the Adriatic Sea has a longer gametogenic period, suggested also by Frenkiel (1975) and Othaitz (1994), as opposed to the shorter period reported by Ribeiro *et al.* (2009). The longer gametogenic period could result from no inhibitory effect of extreme low temperatures on gonad development, as suggested by Ribeiro *et al.* (2009) for *P. depressa* and *P. ulyssiponensis*, since the mean seawater surface winter temperature (December–March) in the Adriatic Sea was 14.9°C. There was, however, no evidence of multiple spawning events between August and November, as reported by Othaitz (1994). The seasonality of spawning varies between species and with seawater temperature, causing the timing of spawning to vary depending on latitude (Guerra & Gaudêncio, 1986).

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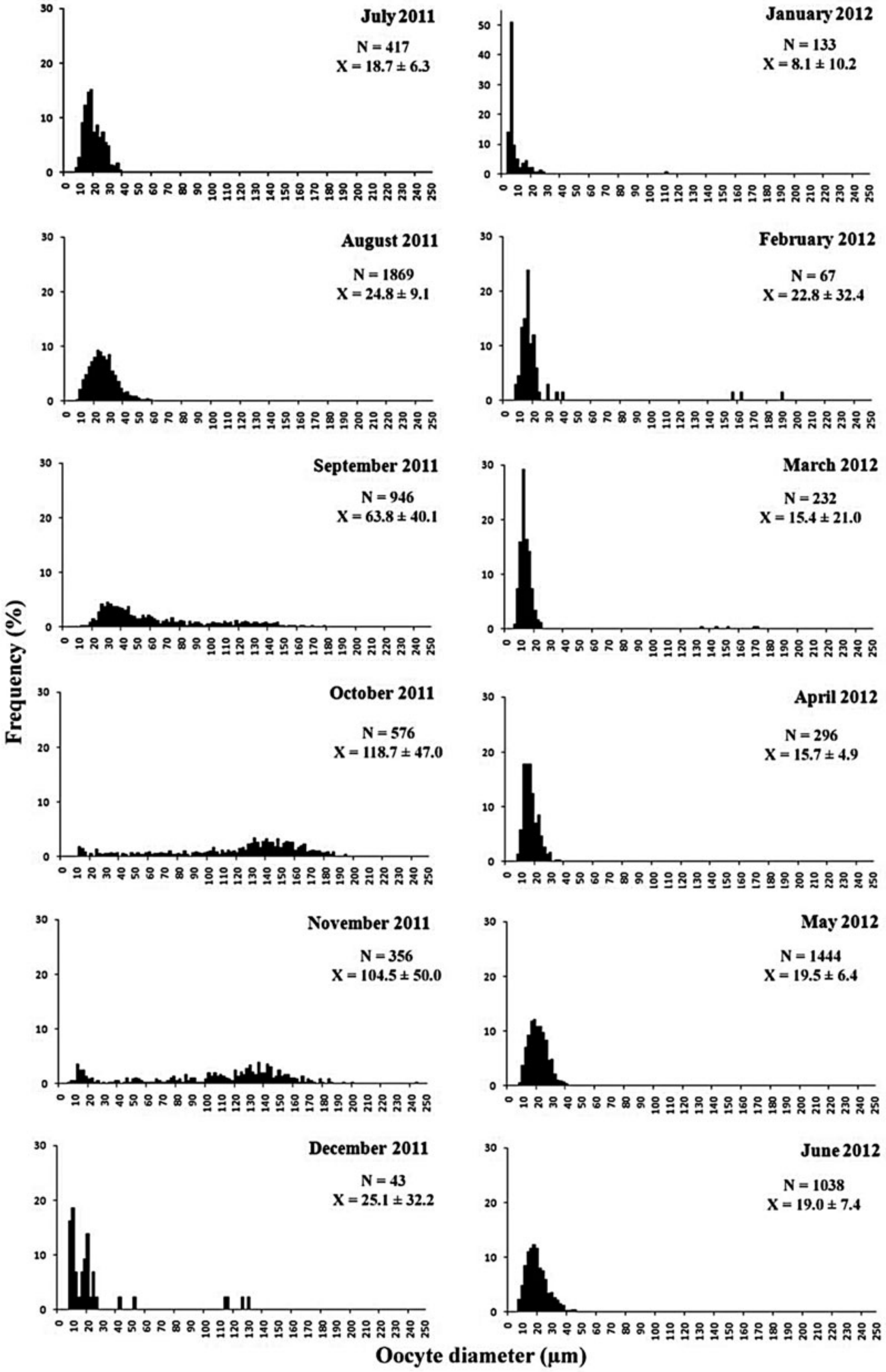

Fig. 6. Monthly variation in the frequency of oocyte diameter in *Patella rustica*. N, number of oocytes; X, mean oocyte diameter ± standard deviation.

Temperature has often been linked to the reproductive cycles of *Patella* species (Orton *et al.*, 1956; Fretter & Graham, 1976). Thus, *P. rustica* has only one spawning peak near its northern range (Ribeiro *et al.*, 2009) and in the present study, as opposed to multiple spawning events on the warmer Mediterranean coasts (Frenkiel, 1975; Othaitz, 1994). Spawning is often associated with strong wave action (Orton *et al.*, 1956; Orton & Southward, 1961; Branch, 1974) and a drop in seawater temperature (Bowman, 1985; Delany *et al.*, 2002). Sea roughness as a trigger for spawning can be extremely important for high shore species (Branch, 1981; Shanks, 1998), such as *P. rustica*. Maturation and

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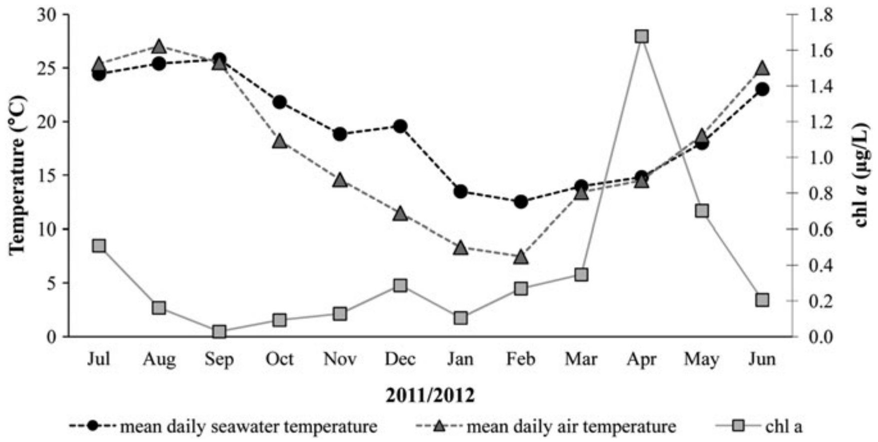

Fig. 7. Monthly variation of mean daily surface seawater temperature, air temperature and chlorophyll-*a* (chl *a*) concentration values from July 2011 to June 2012.

spawning can be correlated with food abundance: maturation when there is enough food supply for the necessary higher energy input, while spawning just prior to the phytoplankton bloom ensures food for planktotrophic larvae (Branch, 1974, 1981). However, site-specific variations are likely to contribute to the rate of gonad development, as well as the timing of spawning (McCarthy *et al.*, 2008). Findings from this study showed no concurrence between the reproductive cycle of *P. rustica* and environmental parameters, although the reproductive patterns generally fit the latitudinal trend previously described for *Patella* species, where spawning occurs later in the year, possibly triggered by low temperatures and/or sea roughness.

A number of limpet species are thought to be hermaphrodites, and most are considered to be protandrous, including *P. caerulea* (Bacci, 1947; Belkhodja *et al.*, 2011), *P. ulyssiponensis* (Thompson, 1979; McCarthy *et al.*, 2008), *Helcion pectunculus* (Gray & Hodgson, 2003), *Lotia gigantea* (Kido & Murray, 2003) and *Patella ferruginea* (Rivera-Ingraham *et al.*, 2011a). The sex-ratio and shell length–frequency distribution of male and female *P. rustica* showed that males dominated the smaller-sized class while females became prevalent with increasing size, from ~28 mm onwards. The preponderance of larger females could suggest that *P. rustica* is a protandrous hermaphrodite, although assumptions based only on size–frequency distribution provide no direct evidence of protandry (Le Quesne & Hawkins, 2006). The hermaphroditic phenomenon is most likely regulated by other factors, such as environmentally and genetically determined mechanisms (Lindberg & Wright, 1985; Rivera-Ingraham *et al.*, 2011a). A male-biased sex-ratio, frequent for protandric patellids, was observed only in smaller-sized *P. rustica*. Estimated length at which 50% of males were mature was 13.1 mm, and according to the age–length key for this species these males were younger than 2 years (Prusina, 2013). Similar findings were demonstrated for other protandrous limpet species (Blackmore, 1969; Gray & Hodgson, 2003). However, a considerable percentage of males found among the larger-sized classes indicate the possibility that some specimens never change sex (Espinosa *et al.*, 2009) or are capable of two-way sex change (Le Quesne & Hawkins, 2006), a common phenomenon found in *P. ferruginea* (Guallart *et al.*, 2013). This reverse sequential hermaphroditism could help regulate the population dynamics by controlling the sex-ratio and maximizing the reproductive fitness under different environmental conditions (Guallart *et al.*, 2013). What causes change of sex and what is the adaptive significance of protandry is still not completely explained. Generally, protandry is considered to be beneficial, especially to broadcast spawners and species relying on an r-strategy, such as *P. rustica*, since larger females would be able to produce a greater number of energetically more expensive eggs (Hoagland, 1978).

Since qualitative histological methods of determining the reproductive stages may be to some extent subjective (Gosling, 2003), a quantitative method of measuring the size of oocytes (expressed as oocyte diameter) was also employed. In this study, quantitative analysis followed the pattern observed using qualitative histological methods. Part of the oocytes undergo lysis and different stages of atresia during the whole period of development, and particularly at the beginning of the spawning season. Oocytary atresia is not unusual and has been reported for other limpet species (see Morriconi, 1999; Belkhodja *et al.*, 2011). Although it is known that oocyte size can be influenced by life strategy, location or environmental factors, comparisons can be made only with other limpet species, since this is the first assessment of oocyte size for *P. rustica*. Similar oocyte size and bimodal pattern of oocyte distribution was reported for other limpet species, such as *Patella granularis* or *H. pectunculus* (Vat, 2000; Gray & Hodgson, 2003). Many limpet species are known to have high fecundity, with oocyte diameter <180 μm, suggesting these species produce planktotrophic larvae (Branch, 1974). Spawning in such species is considered to be correlated and/or triggered with the peak of primary production (Brêthes *et al.*, 1994; Morriconi, 1999), although some studies suggested that veligers are non-feeding, so that spawning and the phytoplankton bloom are not necessarily correlated (Hadfield *et al.*, 1997). The latter may explain the absence of correlation between spawning of *P. rustica* and chl *a* concentration in the study area.

Coastal natural resources are under unsustainable pressures nowadays, the harvesting of intertidal macroinvertebrate resources being one of them (Katsanevakis *et al.*, 2008). Uncontrolled harvesting and poor management measures have led to the destructive decline of several limpet species, such as *Patella candei candei* in the Canary Islands (Hawkins *et al.*, 2000; Navarro *et al.*, 2004), *P. ulyssiponensis* in the Azores, and even to the risk of extinction, such as *P. ferruginea*, whose distribution has been extremely reduced during the 20th Century (Templado *et al.*, 2004;

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Espinosa & Ozawa, 2006; Rivera-Ingraham *et al.*, 2011b). One of the reasons for their increased vulnerability is their protandric life history, since larger individuals have greater probability of being collected, resulting in recruitment failure due to decreased reproductive output (Hawkins *et al.*, 2000). Although limpets in Croatia are currently not commercially exploited, they are either systematically or occasionally collected for human consumption or as fishing bait (Zavodnik & Kovačić, 2000).

The present study is vital for the rational management of stocks, since *P. rustica* is considered to be a keystone species on Mediterranean rocky shores, with potential for commercial exploitation. Additionally, it is the first histological description of gonad developmental stages and the first description of oocyte size and distribution in *P. rustica*. Data gathered give a more detailed insight into the reproductive biology of *P. rustica* and contribute to our understanding of the biology of *Patella* species. Reported findings can serve as a good baseline and point of comparison for studying intraspecific differences across a latitudinal gradient throughout the Mediterranean Sea.

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Correspondence should be addressed to:
I. Prusina
Department of Aquaculture, University of Dubrovnik, Ćira Carića 4, 20000 Dubrovnik, Croatia
email: ivana.prusina@unidu.hr

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