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Reproductive cycle of the non-native Pacific oyster, *Crassostrea gigas*, in the Adriatic Sea

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Abstract

The Pacific oyster *Crassostrea gigas* has been introduced for the aquaculture purposes in many different parts of the world. However, the species has never been officially introduced for commercial farming in the Croatian coast of the Adriatic Sea. Interestingly, in the 1970s, the Pacific oyster was reported in the natural habitats of Lim Bay, Croatia in the northern Adriatic Sea. Although the species was recorded, there is a lack of knowledge about its biology and ecology in this region, including reproductive cycle. Information on the reproductive biology of non-indigenous species in new areas is crucial for prediction of their future survival and possible spread in invaded habitats. In this study, we provide the first data on the reproductive biology of the Pacific oyster in the Adriatic Sea, the northernmost part of the Mediterranean Sea. Quantitative and qualitative methods of gonad tissue analysis were conducted, and effects of environmental conditions on the gametogenesis were evaluated during two reproductive cycles. Our study confirmed that environmental conditions in Lim Bay were favourable for the reproduction of the species. The Pacific oyster reproduced once per year and had a prolonged spawning period. Water temperature was the main factor affecting gonad development and oocyte size, while chlorophyll *a* concentration had an impact on oocyte size.

Keywords: *Magallana gigas*; *Crassostrea gigas;* bivalve; gonad; histology; Pacific oyster; Mediterranean Sea.

Introduction

The Pacific oyster *Crassostrea gigas* (Thunberg, 1793), also known as *Crassostrea (Magallana) gigas* or *Magallana gigas*, is native to the South East Asia and has been introduced for aquaculture purposes in many different parts of the world (see Miossec *et al*. 2009 for a review). Today, the Pacific oyster is one of the leading aquaculture species (Ruesink *et al.*, 2005). In 2016, the world production of the Pacific oyster was 573,616 tonnes and the majority of global production was in China (FAO, 2019). In the Mediterranean Sea, the Pacific oyster was intentionally introduced in the 1960s as a response to the decrease of native oyster production caused by high mortalities associated to parasitic diseases (Gosling, 2003). Introduction of the Pacific oyster was limited to aquaculture sites, but as environmental conditions in those areas were favourable, the species started to reproduce and spread into surrounding areas (see Ruesink *et al.*, 2005 for a review). In Europe, France is the only country that has *C. gigas* production of over 100,000 tonnes per year; other counties, like Ireland, UK, Spain, Germany and Norway cultivate the Pacific oyster in significantly lower quantities (FAO, 2019). In Croatia, *C. gigas* has never been officially introduced in the eastern Adriatic Sea for commercial farming due to the production stability of native *Ostrea edulis* (Linnaeus, 1758) and absence of the abovementioned parasitic diseases. Today in Croatia, only two bivalve species are commercially cultured: the European flat oyster *O. edulis* and black mussel *Mytilus galloprovincialis* Lamarck, 1819.

Interestingly, in the 1970s, wild populations of the Pacific oyster had been reported in the intertidal zone of the rocky shore in Lim Bay, in the northern Adriatic Sea, Croatia (Filić & Krajnović-Ozretić, 1978; Hrs-Brenko, 1982). Official data about *C. gigas* introduction is lacking, but according to several studies (Filić & Krajnović-Ozretić, 1978; Hrs-Brenko, 1982; Stagličić *et al.* 2020), it has been assumed that current presence and high abundance of the Pacific oyster is a consequence of short-term

experimental aquaculture trials conducted in early 1970s. It seems that conditions in the Lim Bay area were favourable and the Pacific oyster started to spread. For several decades, the presence of the Pacific oyster in Croatian waters was neglected; however, the species has recently become a focus of several studies in the eastern Adriatic Sea concerning its presence, distribution and abundance (Šegvić-Bubić *et al.*, 2016; Ezgeta-Balić *et al.*, 2019; Stagličić *et al.*, 2020). Yet, there is a lack of knowledge on *C. gigas* biology and ecology in this region, including its reproductive cycle in environmental conditions of Lim Bay. Successful reproductive cycle and settlement process play a crucial role in the establishment of sustainable populations (McFarland & Hare, 2018); thus, knowledge about the reproductive biology of non-native species is important for the assessment of possible future spread and impact.

The Pacific oyster reproductive cycle is well described along the Atlantic coast of Europe (e.g. Ruiz *et al.*, 1992; Massapina, 1999; Steele & Mulcahy, 1999; Lango-Reynoso *et al.*, 2000, 2006; Dutertre *et al.*, 2009; Enríquez-Díaz *et al.*, 2009; Thomas *et al.*, 2016; Antonio & Camacho, 2019), but in the Mediterranean region, data are generally scarce (Shpigel, 1989; Dridi *et al.*, 2007, 2014; Ubertini *et al.*, 2017), and completely lacking in the Adriatic Sea. The presence of larva and small specimens (~20 mm) in Lim Bay (Ezgeta-Balić *et al.*, 2019; Stagličić *et al.*, 2020) might indicate favourable environmental conditions for successful reproduction of *C. gigas*; however, to confirm this hypothesis, detailed research on its reproductive biology is needed.

In this study, we provide the first data on the reproductive biology of the Pacific oyster *C. gigas* in the Adriatic Sea. We conducted quantitative and qualitative methods of gonad tissue analysis and evaluated the effects of environmental conditions, including temperature, salinity and chlorophyll *a* concentration, on gametogenesis.

Material and Methods

Study site and specimen collection

The study on the reproductive biology of *C. gigas* was conducted from February 2017 to October 2018 in Lim Bay, Croatia (45.13367° N, 13.72253° E), northern Adriatic Sea (Fig. 1). The Lim Bay was chosen because: (i) non-native *C. gigas* was already described in the area (Filić & Krajnović-Ozretić, 1978; Hrs-Brenko, 1982) and it forms dense reefs (Stagličić *et al.*, 2020); and (ii) this region is one of the main aquaculture areas in the Adriatic Sea where the European flat oysters (*O. edulis*) and black mussel (*M. galloprovincialis*) are cultivated, while the Pacific oyster is often found as fouling organism on aquaculture facilities (Šošić, pers.comm.). During December 2017, large specimens of Pacific oyster were collected from the rocky shore and/or aquaculture facilities (where present as fouling organism) in Lim Bay. Collected specimens were placed in oyster boxes (Intermas, Barcelona, Spain). Approximately 50 oysters were allocated to each box (100 cm x 50 cm), and the boxes were attached to long-lines at 2 m depth. The oyster boxes were cleaned every two months to remove biofouling organisms.

***Fig. 1*:** Map of the sampling location – Lim Bay in the Adriatic Sea.

Environmental variables

Water temperature was measured hourly during the entire sampling period using data loggers (Tinytag, Gemini®) deployed at the same depth as oysters. From March to May 2018, the temperature logger was lost; for temperature analyses during that period, we use the monthly temperature data that was recorded with the YSI probe. Salinity was measured using a YSI probe on a monthly basis.

Water samples for chlorophyll *a* analyses were collected monthly with a Niskin bottle. Every month, 400 mL of water was filtered through Whatman glass microfiber filters GF/F (diam. 2.5 cm; pore size 0.7 µm) and frozen at -20°C until further analysis. Chlorophyll *a* measurements were conducted by Trilogy fluorometer (Turner Designs) following acetone extraction (Strickland & Parsons, 1972).

Gametogenic cycle – qualitative and quantitative approach

From February 2017 to October 2018, at approximately one-month intervals, twenty oyster specimens were collected from the boxes. In total, 420 specimens (mean length ± SD = 97.1 ± 14.1 mm) were processed for histology analysis; 3% of samples were not analysed due to poor quality. The oysters were opened and visceral mass was taken above the pericardial area. Gonad tissue samples were then fixed in 10% formaldehyde and stored for later laboratory analysis. Gonad tissues were then dehydrated in increasing concentration of ethanol, embedded in paraffin, cut at 5 µm and stained with hematoxylin and eosin. Sex and gonad development stages were assessed microscopically, using a modified scale from Steele & Mulcahy (1999): inactive, early active, late

active, ripe, spawning and spent (Table 1 and Figs 2 and 3). Each gonad sample was examined using a Zeiss Axio Lab A1 stereomicroscope under the magnification ranging from 40x to 400x. The numerical value was assigned to each stage (Table 1) and mean gonad index (MGI) was calculated by multiplying the number of individuals from each stage with the numerical ranking of that stage and divided by the number of individuals (Gosling, 2003). The MGI was calculated for all individuals combined and for each sex. For each determinate female specimen, one photograph of a random visual field for each gonad sample was examined under 100x magnification (Axiocam 105 color camera coupled to a Zeiss Axio Lab A1 microscope). Image analysis and processing were carried out using Image J software to obtain diameter of all oocytes with visible nucleus.

Statistical analysis

Spearman's rank correlation was applied to determinate a degree of association between mean gonad indices and oocyte size with temperature, salinity and chlorophyll *a* concentration. Differences in the oocyte size between stages were tested using nonparametric Kruskal–Wallis test. Furthermore, Spearman's rank correlation was calculated between oyster size and oocyte size. Sex ratio was tested using chi-square goodness of fit test. Critical probability value was set at $\alpha = 0.05$.

Results

Environmental conditions

Minimum water temperatures were 9.7°C and 10.8°C in February 2017 and February 2018, respectively (Fig 4A). The maximum temperatures were 28.6°C and 28.7°C in August 2017 and August 2018, respectively. Salinity values ranged from 31.6 to 38.5 with mean value (± SD) of 35.6 ± 2.1 (Fig. 4B). There was no clear seasonal pattern in salinity values. Chlorophyll *a* demonstrated high oscillation during the study period. Values of chlorophyll *a* concentration ranged from 0.18 µg l^{-1} to 9.88 µg l^{-1} and, according to Smith *et al.* (1999), Lim Bay exhibited characteristics from oligotrophic (<1 µg l^{-1}) to hypertrophic (>5 µg l^{-1}) nutrient states. Generally, chlorophyll *a* concentration was higher in the summer/autumn than in the winter/spring (Fig 4C). In 2017, two peaks were recorded in August (5.72 µg l^{-1}) and October (9.88 µg l^{-1}). The peak recorded in October could be a result of increased abundance of species from Class Euglenophyceae observed in the same period (Arapov, personal communication). In 2018, maximal chlorophyll *a* concentration was recorded one month earlier than in the previous year (July chlorophyll *a* = 5.68 µg l^{-1}). We found a moderate, but statically significant correlation between temperature and chlorophyll *a* ($R_s = 0.441$, $p = 0.045$).

Qualitative gonad analysis

Out of 406 analysed specimens, 160 individuals were females (39.4 %; mean length ± SD = 97.6 ±14.1 mm),

Table 1. Histological appearance of gonad developmental stages in *Crassostrea gigas* (adopted and modified from Steele and Mulcahy, 1999).

Stage no.	Description	Female	Male
0	Inactive (sexual rest)	No evidence of follicle presence or just rare small follicles with stem cells present but sex determination was not possible	
3	Early active	Oogonia arising from the stem cells along the follicles with no free oocytes. Connective tissue is very abundant.	Many of small follicles; spermatogonia and spermatiocytes numerous, no spermatozoa
4	Late active	Free and attached oocytes present with distinct nuclei that stain lighter than cytoplasm	Follicle cells contain predominantly spermatids and spermatozoa; characteristic swirling pattern of spermatozoa with tails toward follicle lumen, in centre of follicle.
5	Ripe	Free vitellogneic oocytes with distinct nucleus and nucleolus.	Inter follicular tissue and germinal epithelium are inconspicuous. Follicles filled with spermatozoa oriented with tail to follicle lumen forming characteristic swirling pattern that completely fills follicle.
2	Partially spawned	Large number of free oocytes appear but not densely compacted, occupy the centre of lumen in the follicle.	Follicles are partially empty, with a large number of spermatozoids but they are not densely compacted.
1	Spent	Follicles walls appear broken and follicles are empty, residual oocytes are in the process of cytolysis.	Most follicles empty or partially so with sperm evident in sperm duct on some individuals.

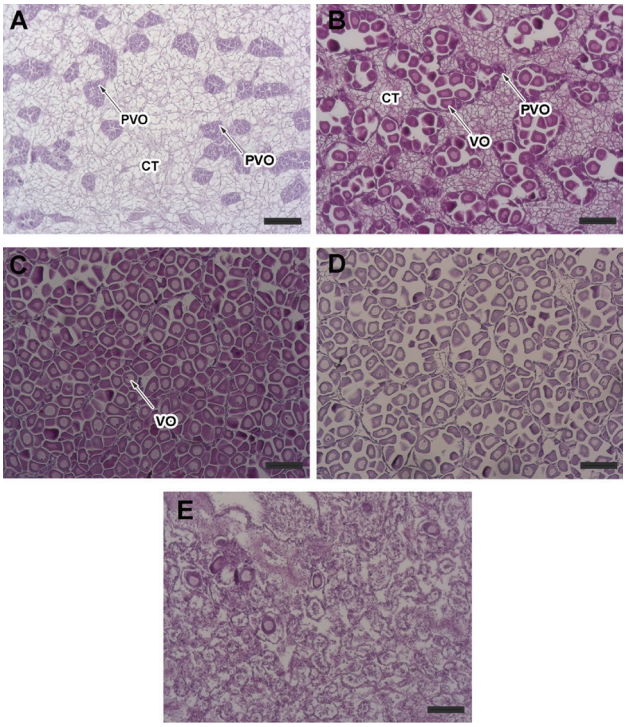

Fig. 2: Histology of gonad development stages of females of *Crassostrea gigas*: (A) early active (B) late active; (C) ripe; (D) spawning; (E) spent. PVO – previtellogenic oocyte; VO – vitellogenic oocyte; CT – connective tissue. Scale bar: 100 µm.

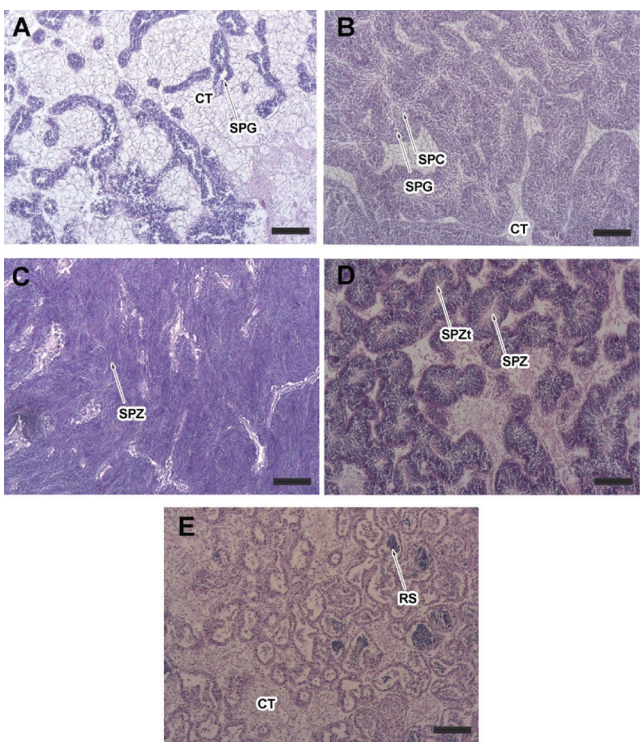

Fig. 3: Histology of gonad development stages of males of *Crassostrea gigas*: (A) early active; (B) late active; (C) ripe; (D) spawning; (E) spent. SPG – spermatogonia; SPC – spermatocytes; SPZ – spermatozoa, SPZ t - spermatozoa tails; RS – residual spermatozoa; CT – connective tissue. Scale bar: 100 µm.

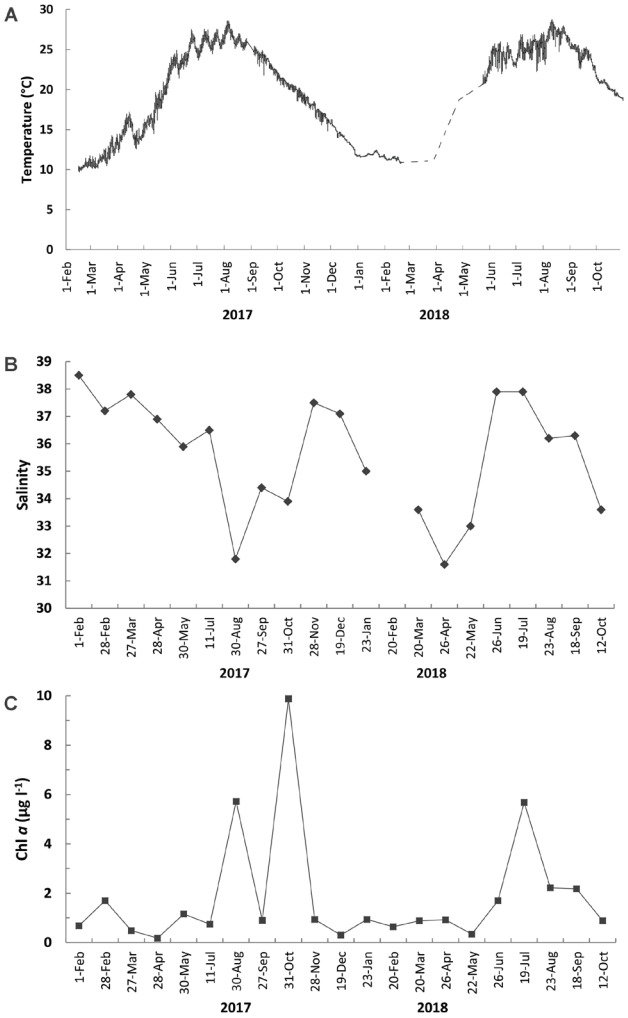

Fig. 4: Seasonal variation in (A) temperature, (B) salinity and (C) concentration of chlorophyll *a* in Lim Bay.

74 were males (18.2 %; 93.1 ± 14.6 mm) and 6 were hermaphrodites (1.5 %; 97.8 ± 12.1 mm; Fig. 5). The other 166 individuals (40.9 %; 97.5 ± 13.7 mm) were sampled during the resting period and sex determination was not possible. Size range of the monthly analysed females and males are presented in the Table 2. During the period of maximal gonad activity, sex ratio (m/f) was significantly dominated by females (m/f = 0.41; χ^2=16.7, df = 1, $p < 0.001$).

The gonad histological analysis confirmed that the Pacific oyster had one reproductive cycle per year. The reproductive cycle demonstrated a period of intensive gametogenic activity, prolonged spawning season, followed by a sexual resting period. The frequency distribution of gametogenic phases observed during the study period is illustrated in Figure 6. The MGI of male and female individuals had the same pattern (Fig. 7), and very strong significant positive correlations ($R_s = 0.875$, $p < 0.001$) was recorded between male and female MGI. Furthermore, a significant strong positive correlation was found between general MGI and temperature ($R_s = 0.674$, $p = 0.001$), confirming that temperature plays an important role in gonad maturation. On the contrary, MGI did not show correlation with either salinity or chlorophyll *a*.

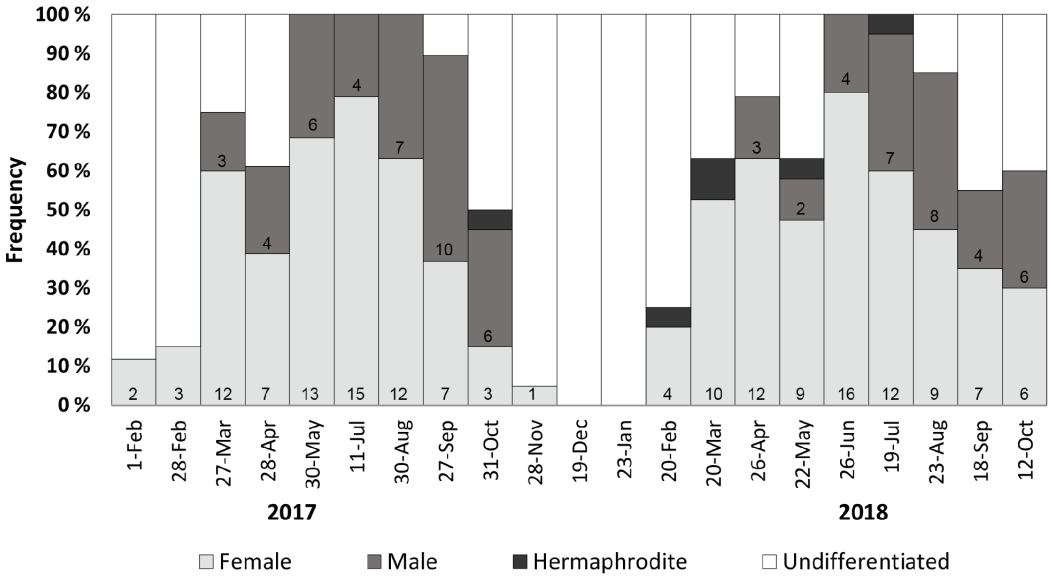

Fig. 5: Frequency of *Crassostrea gigas* females, males, hermaphrodites and undifferentiated individuals. Numbers of analysed females and males are indicated on the graph.

Table 2. Size range of the analysed female and male *Crassostrea gigas*.

	Sampling date	Female	Male
2017	01-Feb	101 -111 mm	
	28-Feb	103 - 125 mm	
	27-Mar	90 - 129 mm	96 - 122 mm
	28-Apr	85 - 120 mm	80 - 100 mm
	30-May	90 - 125 mm	98 - 125 mm
	11-Jul	85 - 125 mm	95 - 130 mm
	30-Aug	80 - 101 mm	81 - 101 mm
	27-Sep	84 - 105 mm	80 - 111 mm
	31-Oct	110 - 115 mm	92 - 120 mm
	28-Nov	94 mm	-
	19-Dec	-	-
	23-Jan	-	-
2018	20-Feb	95 - 126 mm	-
	20-Mar	90 - 118 mm	-
	26-Apr	89 - 110 mm	93 -109 mm
	22-May	78 - 90 mm	78 - 80 mm
	26-Jun	80 - 107 mm	80 - 110 mm
	19-Jul	80 - 109 mm	79 - 97 mm
	23-Aug	75 - 90 mm	75 - 88 mm
	18-Sep	78 - 110 mm	76 - 88 mm
	12-Oct	80 - 95 mm	75 - 86 mm

Gonad maturation process of females was longer than males, as early active females were observed in February in both years (Fig. 6A). As oocyte maturation continued, late active females were recorded in May 2017, while in 2018 they occurred one month earlier, in April. Consequently, ripe females occurred earlier in 2018 than in 2017; 44.4% female specimens were in the ripe phase in May 2018, while there were no ripe females at that time in 2017. Spawning period of females started in June/July and lasted until the beginning of autumn. From December to January, there were no visible oocytes, and females were in the gametogenic resting period. In 2017, gametogenesis of males started in March; in 2018, the first males in an early active phase were identified in April (Fig. 6B). Ripe males were recorded in May in both years, and spawning started during summer (i.e., in August 2017 and July 2018). Resting period of the male part of population was three months longer than that of females, and lasted from November 2017 to March 2018.

Oocyte size

A total of 17,181 oocytes diameters were measured. The mean oocyte size for each month and each development phase are presented in Figure 8. Statistically significant differences in the oocyte size were recorded ($p < 0.001$), and the results of post hoc pairwise tests on oocyte size between stages were: ripe > partially spawned > spent = late active > early active. In general, oocyte size increases with gonad maturation (Table 3). In 2017, mean oocyte size increased slowly through late winter and early spring (mean size 14.3 µm) and then showed an abrupt increase in May (mean size 33.4 µm). In 2018, oocytes did not have so sharp increase in average size, but they increased gradually from 11.5 µm in March to 22.7 µm in April, being on average 33.1 µm in May (Fig. 8B). Mean oocyte size showed significant positive correlation with temperature ($R_s = 0.711$, $p = 0.001$) and significant moderate correlation with chlorophyll *a* ($R_s = 0.557$, $p = 0.013$). There was no correlation between oocyte size and oyster size (R_s=-0.109; p=0.471), but we emphasise that only large specimens were analysed.

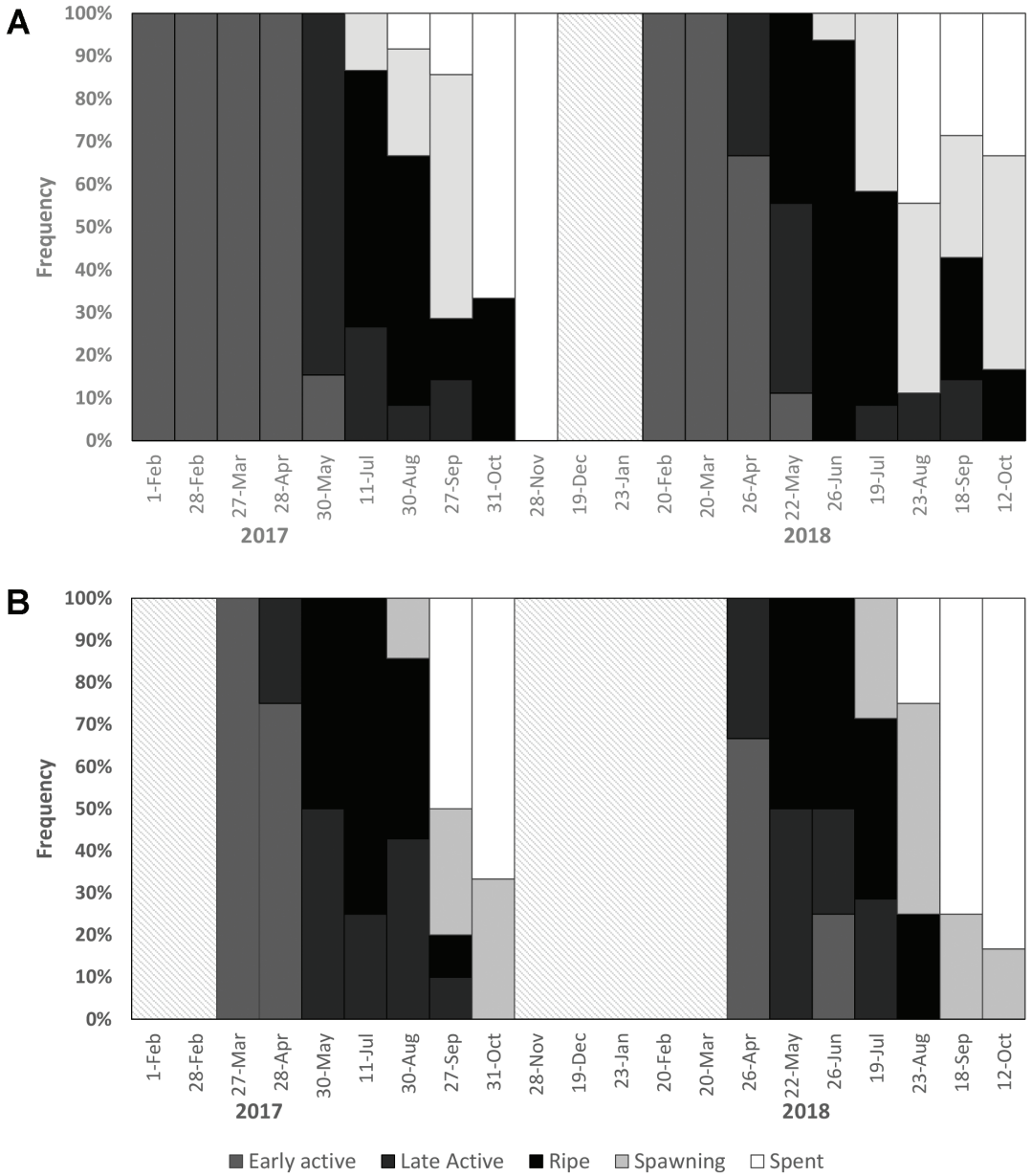

Fig. 6: Monthly variation in the frequency of different gametogenic stages of (A) females and (B) males of *Crassostrea gigas*.

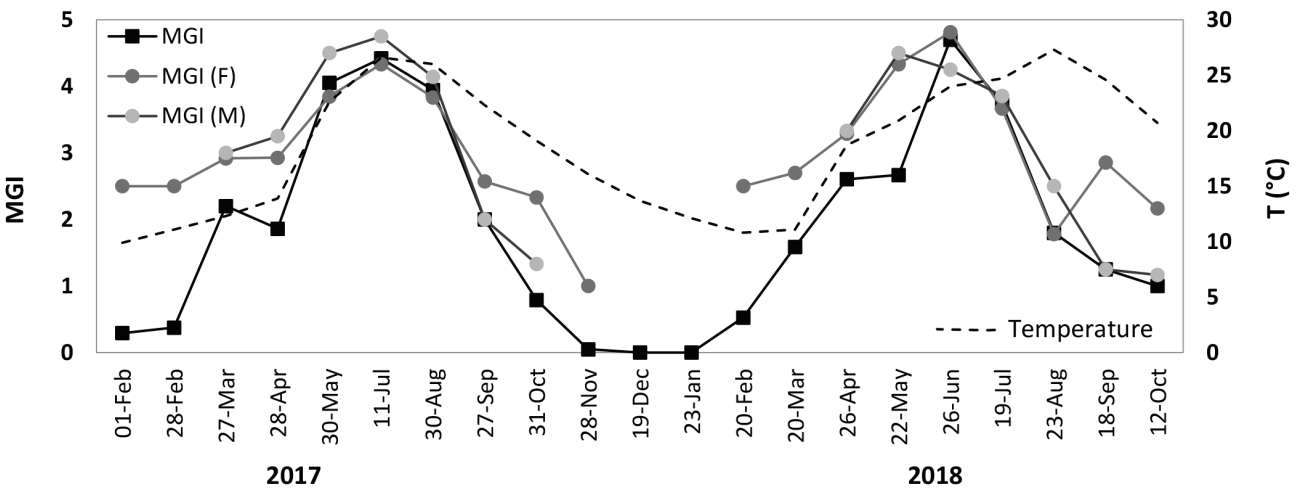

Fig. 7: Monthly variation in the mean gonad indices of *Crassostrea gigas* and mean daily temperatures values. MGI – Mean Gonad Index; MGI (F) – Female Mean Gonad Index; MGI (M) – Male Mean Gonad Index.

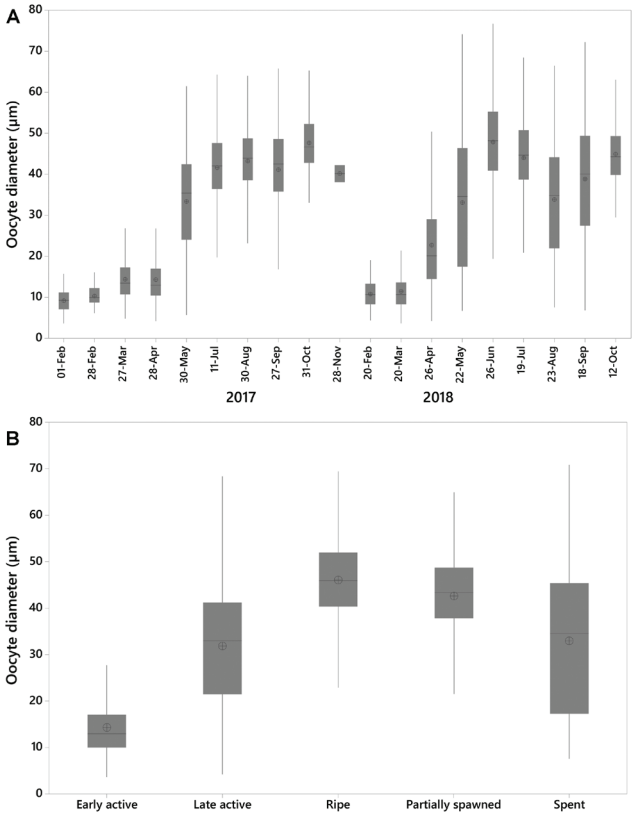

Fig. 8: Changes in oocyte size in regards to sampling month (A) and developmental phase (B). Graphs present mean size and interquartile range.

Table 3. Diameter of *Crassostrea gigas* oocyte according to gametogenic phases.

Stage	N	Mean size ± SD
Early active	4049	14.2 ± 6.6µm
Late active	4105	31.8 ± 11.9 µm
Ripe	6335	46.0 ± 9.7 µm
Spawning	2500	42.6 ± 9.7 µm
Spent	192	32.9 ± 16.2 µm

N – number of measured oocyte

Discussion

The Pacific oyster *C. gigas* is an asynchronous hermaphrodite that most commonly first mature as male (Gosling, 2003; FAO, 2019). In Lim bay, hermaphrodite individuals occurred sporadically, which is in accordance with previous studies (Steele & Mulcahy, 1999; Dridi *et al.*, 2014; Antonio & Camacho, 2019). In our study, we observed a female-biased sex ratio. Although males in our study reached sizes up to 130 mm (Table 2), the observed ratio could be due to sampling of larger specimens that resulted in collection of predominantly females, but an effect of food availability could not be excluded. According to FAO (2019), in areas with good food supply, such as Lim Bay, the sex ratio of adult oysters shows a predominance of females, while in limited food areas it is male dominated. From an ecological point of view, the predominance of females is desirable as the reproductive potential of any species is determined by the number of matured females and their capacity to produce viable eggs and larvae (Murua *et al.*, 2003). However, more detailed studies taking in account different size classes needs to be performed to provide information on the sex change strategy of the Pacific oyster in this region.

Nowadays, the Pacific oyster is present in many parts of the world, either as cultured or wild populations. The broad geographical range that the Pacific oyster inhabits (e.g. Escapa *et al.*, 2004; Robinson *et al.*, 2005; Carrasco & Barón, 2010; Troost, 2010; Wrange *et al.*, 2010)1793 points to a wide environmental tolerance of the species. However, the presence of the Pacific oyster in an area does not automatically imply a complete life cycle; it has pelagic larvae that could have been delivered to a new area suitable for settlement and growth by transport vectors, such as ship ballast water. Water temperature and food availability are considered to be the main factors that influence the reproductive cycle in bivalves (Gosling, 2003; Pouvreau *et al.*, 2006; Bernard *et al.*, 2011). Nevertheless, many other factors such as turbidity (Dutertre *et al.*, 2010), contaminants (Sussarellu *et al.*, 2016) and ocean acidification (Boulais *et al.*, 2017) may also have an effect on reproduction. In areas with unfavourable environmental conditions for spawning, the gametogenic cycle of the Pacific oyster can end with gonad reabsorption or atresia (Steele & Mulcahy, 1999; Dutertre *et al.*, 2010).

This study confirmed that environmental conditions in Lim Bay were favourable for the reproduction of *C. gigas*. The Pacific oyster reproduced once per year and had a prolonged spawning period. In general, *C. gigas* has a seasonal reproductive cycle related to temperature, and its gametogenesis begins during winter, continuing more intensely throughout spring, and reaches its maximal activity in summer when water temperature is the highest, followed by spawning (Fabioux *et al.*, 2005; Chávez-Villalba *et al.*, 2007). In our study, the Pacific oyster exhibited the same reproductive pattern, with some minor differences in the maturation process of males and females. Duration of sexual resting period lasted longer for males than for females. In females, gametogenesis was initiated when the temperature was approximately 10°C, while males preferred a higher temperature (~12°C). In other regions of the Mediterranean Sea, initiation of gametogenesis was recorded at higher temperatures, such as 12°C in the Gulf of Lilat, Israel (Shpigel, 1989) and 14-15°C in the Bizerte lagoon, Tunisia (Dridi *et al.*, 2006). However, in general, the species follows the same reproductive pattern as in our study. Furthermore, in our study, ripe individuals of both sexes occurred at ~21°C, while spawning started at ~24°C. The temperatures of ripening and spawning were within the range of temperatures recorded for this stage in other parts of the Mediterranean

Sea (Shpigel, 1989; Dridi *et al.*, 2006, 2014). Considering that Pacific oyster larvae need water temperature of 22°C and above for optimal development (Rico-Villa *et al.*, 2009), the temperatures in our study were favourable for larval development.

Water temperature seemed to be the main factor affecting gonad development and oocyte size, while chlorophyll *a*, which is a proxy for food (phytoplankton) availability, had a more pronounced impact on the size of oocytes during development than on gonad development stages. This is in concordance with previous studies that also highlighted temperature as the primary factor for gametogenesis. However, in different geographical areas, studies have found a broad range of temperature characteristics for different stages of gametogenesis (Table 4 and references therein), pointing to high adaptability of the species. The temperature range in Lim Bay was similar to other Mediterranean regions where reproduction of the Pacific oyster was studied (Shpigel, 1989; Dridi *et al.*, 2007, 2014; Ubertini *et al.*, 2017) and to the temperature range of its native area (Kang *et al.*, 2010); thus, Lim Bay presented a good precondition for successful reproduction of the species.

Female gonad maturation, in terms of oocyte size, also followed a temperature pattern. In 2017, water temperature sharply increased from 13.9°C to 22.6°C between April and May, with oocyte size of oysters increasing sharply. In 2018, temperature increased more gradually from 11.1°C in March, 18.7°C in April to 20.9 °C in May, with oocyte size also increasing gradually. A detailed study on oocyte size proposed a reproductive scale based on the oocyte diameter where mean oocyte size is 8.47 ± 4.6 µm in 'early gametogenesis', 21.4 ± 8.4 µm in the 'growing stage', 36.0 ± 4.4 µm in the 'mature stage' and 46.0 ± 7.3 µm in the 'degenerative stage' (Lango-Reynoso *et al.*, 2000). On average, mean oocyte size for given stages were approximately 10 µm higher in Lim Bay (Table 3) than along the French Atlantic coast. Temperatures in these two areas also differed; Lim Bay exhibited higher temperatures (9.7 - 28.7°C) than the west coast of France (7.1 - 21.5°C; Lango-Reynoso *et al.*, 2006) suggesting that temperature could also be an important factor that besides gonad maturation affects oocyte size. In experimental conditions, Chávez-Villalba *et al.* (2002) determined that at lower temperatures (16°C), the size of mature oocytes was below 50 µm, while at higher temperatures (22°C), mature oocyte size exceeded 50 µm. However, oocytes in Lim Bay were larger than those in the Gulf of

Table 4. Environmental conditions and gamogenetic stages of *Crassostrea gigas* at different geographic locations.

Region	Country	Location	Temperature (°C)				Chlorophyll *a* range	Source
			Temperature range	Beginning of gametogenesis	Gonad maturation	Spawning		
NE Atlantic	France	Anse du Roz & Marennes Oléron	-	~7.1-17.0°C	~16.8-21.5°C	-	-	Lango-Reynoso *et al.*, 2006
NE Atlantic	France	Bourgneuf Bay	-	8-10°C	-	>18°C	-	Dutertre *et al.*, 2009
NE Atlantic	Ireland	Dungarvan	2.7-18.45°C	-	-	-	2.5-26.3 µg l^{-1}	Steel & Mulcahy, 1999
NE Atlantic	Spain	El Grove	-	11-13°C	-	16-19.5°C	-	Ruiz *et al.*, 1992
NE Atlantic	Spain	Ría de Arousa	11.8-19.9°C	~11-13°C	~14°C	>15°C (♂) >18°C (♀)	0.2-3.4 µg l^{-1}	Antonio & Camacho, 2019
SW Pacific	New Zealand	Mahurangi harbour	14-20°C	14°C	16°C	18-20°C	-	Dinamani, 1987
NW Pacific	South Korea	Gamakman Bay	7.5-26.1°C	7.5°C	-	25-27°C	0.1-3.5 µg l^{-1}	Kang *et al.*, 2010
Mediterranean	France	Thau lagoon	11-29°C	-	-	>22°C	up to ~6 µg l^{-1}	Ubertini *et al.*, 2017
Mediterranean	Tunisia	Bizerte lagoon	10.9-28°C	14-15°C	16-20°C	23-27°C	1.2-2.4 µg l^{-1}	Dridi *et al.*, 2006
Mediterranean	Tunisia	Gulf of Tunis	12.2-28.7°C	12.7°C	20.2°C	-	0.5-1.75 µg l^{-1}	Dridi *et al.*, 2014
Mediterranean	Israel	Gulf of Eilat	11-29°C	>12°C	18-22°C	22-26°C	-	Shpigel, 1989
Mediterranean	*Croatia*	*Lim Bay*	*9.7-28.7°C*	*12°C (♂) 10°C (♀)*	*21°C*	*24°C*	*0.18-9.88 µg l^{-1}*	*This study*

Tunisia, an area with similar temperature range as in our study (12.2 - 28.7°C; Dridi *et al.*, 2014). Furthermore, there were differences in chlorophyll *a* concentration between the regions, with higher concentrations in Lim Bay (0.18 - 9.8 μg l^{-1}) compared to the Gulf of Tunisia (0.5 - 1.75 μg l^{-1}; Dridi *et al.* 2014). Consequently, in Lim Bay, both temperature and chlorophyll *a* were favourable for oocyte growth.

This research provides the first knowledge on reproductive biology of *C. gigas* in the Adriatic Sea. Information on reproductive biology of non-indigenous species in new areas is crucial for prediction of their future existence and possible spread in invaded habitats. A recent study conducted by Ezgeta-Balić *et al.* (2019) confirmed the presence of Pacific oyster larva in Lim Bay during sampling in May and September of 2018. Furthermore, Stagličić *et al.* (2020) found that the Pacific oyster in Lim Bay often forms dense clumps with mean density of 107.5 ± 13.6 individuals m^{-2}. In their study, Stagličić *et al.* (2020) found that around 8% of analysed specimens were smaller than 30 mm and around 28% were smaller than 40 mm. Furthermore, in their study the smallest measured individuals in Lim Bay were 21 mm long. Complete gonad maturation and spawning reported in this study, together with reported presence of Pacific oyster larva (Ezgeta-Balić *et al.*, 2019) and presence of small individuals (Stagličić *et al.*, 2020) suggest that Lim Bay has optimal environmental conditions for successful and complete reproductive cycle and settlement of *C. gigas*. In addition, habitats along the entire Croatian coast of the Adriatic Sea might be suitable for the Pacific oyster. However, the species has currently only established populations along the western Istrian coast in the northern part of the Croatian Adriatic Sea (Ezgeta-Balić *et al.*, 2019). While introduction vectors of species to this region are unclear, it looks like the main water current circulation of the Adriatic Sea, which goes northward along the eastern coast (i.e., Croatia) and returns southward along the western coast (i.e., Italy; Orlić *et al.*, 1992), counteracts natural dispersal of larvae to southern parts of the Croatian Adriatic Sea. For future investigations, it is important to keep track of the status of Pacific oyster populations in the Adriatic Sea, as well as to investigate other possible vectors of its introduction, such as aquaculture, commercial shipping and natural larval dispersal from southern Adriatic countries (e.g. Montenegro, Albania) where the species has also been recorded (RAC/SPA-UNEP/MAP, 2014; ME, 2015).

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