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SOME REPRODUCTIVE PATTERNS OF CULTURED MEDITERRANEAN MUSSEL (*Mytilus galloprovincialis* Lamarck, 1819) IN BOKA KOTORSKA BAY, ADRIATIC SEA

SUMMARY

Results about annual reproductive cycle of Mediterranean mussel (*Mytilus galloprovincialis* Lamarck, 1819) in Boka Kotorska Bay are given in this paper. Sex ratio, gonad developmental stages, oocytes diameter, mean gonad index, condition index as well as correlation to water temperature were analysed. Sex ratio was 1:1. Maturation and spawning occurred over a great part of the year, from October to June, with short resting period during the late summer, when water temperature was above 25°C. Mean oocytes diameter ranged from 56.68 ± 10.52 µm in October 2015 to 66.58 ± 12.09 in December 2015. The highest mean oocytes diameter appeared during period when most of individuals were in ripe stage. Significant negative correlation between mean gonad index and sea water temperature was found, as well as weak negative correlation between condition index and sea water temperature. Positive correlation between condition index and mean gonad index showed that condition index followed the mean gonad index pattern. Obtained qualitative and quantitative data on reproduction cycle of cultured *M. galloprovincialis* are of great significance for farmers.

Keywords: *Mytilus galloprovincialis*, reproduction, oogenesis, marine aquaculture, Adriatic Sea

INTRODUCTION

Despite great natural potential, marine aquaculture is still poor developed in Montenegro. At this moment marine aquaculture is developed only in Boka Kotorska Bay. Since 2020, with the support of the Montenegrin Ministry of Agriculture, Forestry and Water Management, studies regarding potential locations for marine aquaculture along the open part of the Montenegrin coast have begun (Mandić *et al*., 2020).

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Mediterranean mussel (*Mytilus galloprovincialis* Lamarck, 1819) is the main cultured bivalve species in Montenegrin marine aquaculture. Initial exploration on the possibility of mussel farming goes back to the early 1960s, while commercial farming began to develop in the second half of the 1980s (Mandić *et al*., 2016). Today, there are about twenty mussel farms situated in Boka Kotorska Bay (Gvozdenović *et al*., 2020; MONSTAT, 2022). On all farms the traditional farming method is implemented (floating parks), and annual mussel production during 2021 was 186 tons (MONSTAT, 2022). Additionally, on mussel farm in settlement Ljuta, raft culture (a method of growing mussels using a floating platform with rope or socking material hanging from its bottom) is implemented. The consumption size of cultured mussels is 5 cm according to the law of Montenegro (Službeni list Crne Gore 65/15, 2015) and mussel reaches that length in the second year of their life.

Mediterranean mussel has fan-shaped, triangular or elongated shells. Shell valves are equal, pointed at the front and rounded at the back. The shell is black-blue on the outside, and nacreous inside. The mussel secrete a byssus, which plays a role in attaching to hard substrates and even in moving. It lives in dense colonies, and the densest settlements are located in the tidal zone. The mussel primary feeds on phytoplankton. Shell length can be up to 15 cm, and weight up to 200 g. The sexes are separate, and mature in its first year of life. As mussels are good examples of bivalves with a flexible reproductive strategy, adjusting their cycle according to prevailing environmental conditions (Gosling, 2003), annual reproductive cycle of cultured mussel is very important for farmers, mainly because of spawning period and subsequently period of young collection from nature environment.

Although, reproductive biology is described in many bivalve species, especially in economically important species, there is a lack of data related reproductive biology of *M. galloprovincialis* in Montenegro. Stjepčević (1974) gave some results about reproductive biology of cultured *M. galloprovincialis* in Boka Kotorska Bay, and described prolonged spawning period with spring and autumn peak.

Thus, the aim of this study was to investigate the annual reproductive cycle of cultured *M. galloprovincialis* in Boka Kotorska Bay, using quantitative and qualitative analysis (gonad stage analysis and oocyte diameter), as well as sex ratio, mean gonad index and condition index.

MATERIAL AND METHODS

Sampling was done monthly on two mussel farms (Orahovac and Sv. Nedjelja) in Boka Kotorska Bay (Figure 1). During each month, starting from February 2015 till January 2016, 15 individuals of *M. galloprovincialis* were sampled from pergolas at depths 2-3 m. In total, 180 individuals were collected. For each specimens following parameters were measured: shell length (SL), total weight (TW) and wet meat weight (WMW). SL was measured as maximal anterior-posterior axis (Prgić, 2019), using vernier caliper to the nearest 0.1 mm, while weighing was done by balance to the nearest 0.01 g. Condition index (CI) was calculated as ratio between wet meat weight and total weight (Almeida *et al.*, 1999). Sea water temperature was measured each month at depth 2-3 m using the Multiline P4 WTW Probe.

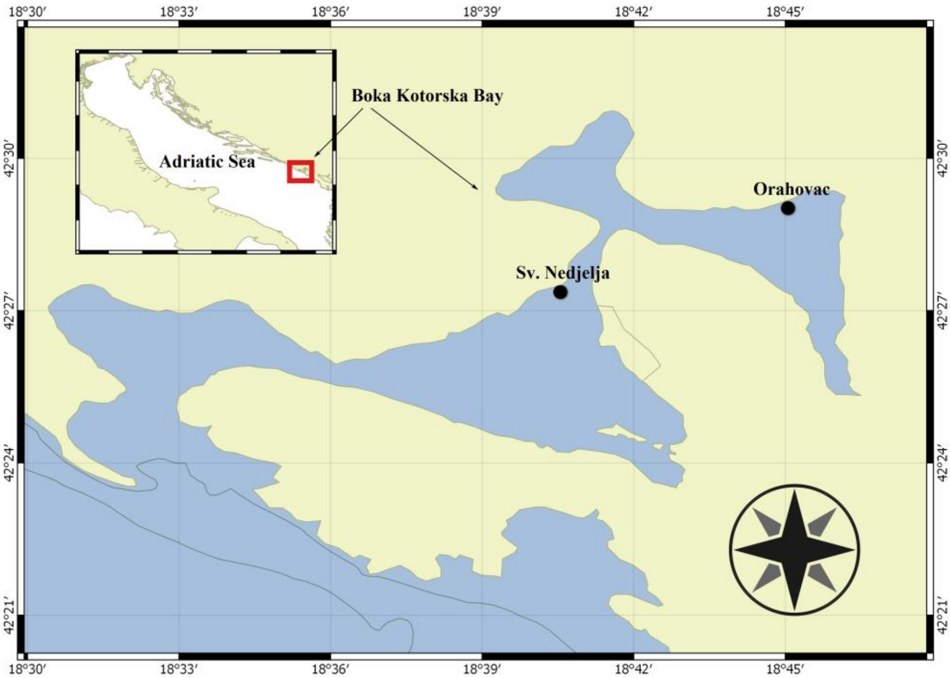

Figure 1. Study area – Sv. Nedelja and Orahovac mussel farms in Boka Kotorska Bay

For quantitative and qualitative analysis, whole gonads were separated from rest of tissue and fixed in 10% formaldehyde in plastic bottles volume of 15 ml. The gonad tissue was then dehydrated, embedded in paraffin and stained by the haematoxylin and eosin (e.g. Crnčević *et al.*, 2013; Popović *et al.*, 2013). Each histology prepared gonad slide was examined at 100×, 200× and 400× magnifications (Zeiss Axio microscope) and development stages were assigned according to Lubet (1959) (Table 1).

A mean gonad index (MGI) was calculated for each month for both sexes together. It was calculated by multiplying the number of individuals from each developmental stage (ni) by the numerical ranking of that stage (Si), and dividing the result by the total number of individuals (N) (e.g. Seed, 1975; Gosling, 2003; Benomar *et al.*, 2006; Bhaby, 2015).

$$MGI=\sum ni*Si/N$$

Quantitative analysis included oocytes counting and diameter measures. Oocytes with visible nuclei within one visual field at 100× magnification were measured as described by Popović *et al.* (2013), using AxioCam ICc3 camera and Axio Vision Rel. 4.6 program.

Table 1. Gonad development stages in mussels (according Lubet, 1959); Si - the numerical ranking of the stages

Stage	**Gonad description**	**Si**
0	Resting	1
I	Beginning of gametogenesis	2
II	Development of gametogenesis and appearance of gamets	2
IIIA	Ripe	3
IIIB	Spawning	2
IIIC	Restoring	2
IIID	Reabsorbing	1

For sex ratio, additional 1080 individuals were used, so for sex ratio in total 1260 individuals were processed (180+1080 individuals). The gender for 180 individuals is determined visual (based on color) and also by histology, while for additional 1080 individuals gender is determined only based on color (the mantle containing the gametes is typically orange in females and creamy-white in males) (Dardignac-Corbel, 1990; Gosling, 2003).

To test sex ratio, a chi-square test was used. Differences in shell length with respect to sex were analysed using Mann-Witney U test, as Levene's test showed that variance were not homogeneity. Correlation between CI and MGI, CI and temperature, MGI and temperature was determined using Pearson correlation.

RESULTS

There was in total 627 females and 605 males, while for 28 individuals sex could not be determined. There was no statistical differences in sex ratio (chi-square=3.12, p=0.07). Percentage ratio between males and females is presented on Figure 2. Mean shell length of analysed females was 57.7±8.1 mm, while for males was 55.5±5.9 mm.

There was no statistically significant differences in the shell lengths between males and females (U=3178, p= 0.0536).

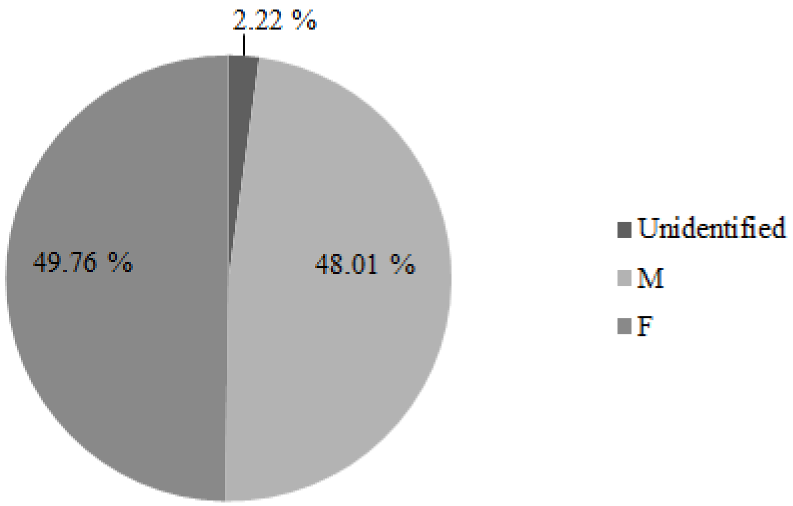

Figure 2. Sex ratio of *Mytilus galloprovincialis*

The reproductive cycle of *M. galloprovincialis* showed prolonged maturation and spawning period, from October to June (Figure 3).

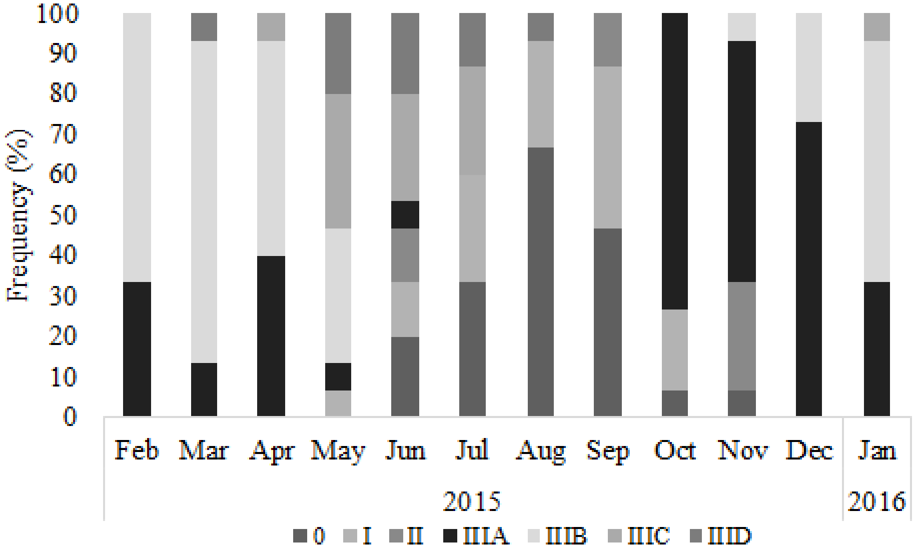

Figure 3. The frequency of developmental stages of *Mytilus galloprovincialis* (both sexes)

During the late summer period resting stage was dominant, especially in August when 70% individuals were in resting stage. Gametogenesis began in

September 2015 when about 50% individuals were in stages I and II (early and late development gametogenesis stages). First ripe individuals appeared during October 2015, while spawning started already in November 2015. During the colder period of the investigated year (February 2015 – April 2015 and October 2015 – January 2016) most individuals were in ripe or spawning stage. During May and June individuals in all development stages were present with dominance of restoring and reabsorbing stages, while in July stages 0, I, IIIC and IIID were equally represented. The most intensive spawning occurred from February to April 2015 and in January 2016.

A total of 5776 oocytes diameters were measured. Oocyte diameter ranged from 13.8 to 132.63 µm. Mean oocytes diameter ranged from 56.68±10.52 µm in October 2015 to 66.58±12.09 in December 2015 (Figure 4). During July, August and September most of the individuals were in 0 stage or were males, while several females which were present were in I or IIID stages so there is lack of oocytes diameter for this months.

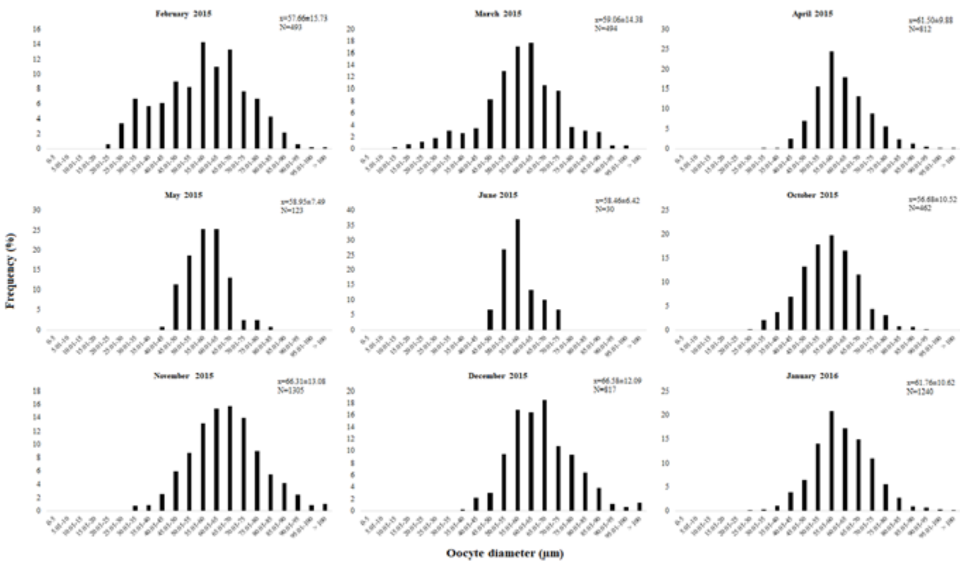

Figure 4. Monthly frequency of oocyte diameters of female *Mytilus galloprovincialis*; X – mean diameter; N – number of measured oocytes

Mean monthly MGI values increased during colder months, when most individuals were in late development, ripe or in spawning stage. The highest MGI value was recorded in December 2015 (2.73) and the lowest in August 2015 (1.26) (Figure 5).

Mean monthly CI values were above 20 during almost all investigated period, except August and September 2015. The highest CI value was recorded in December 2015 (26.63 ± 4.26) and the lowest in September 2015 (19.29 ± 2.63) (Figure 6). There was moderate positive correlation between MGI and CI (r=0.5517, p=0.06), significant negative correlation between MGI and sea water temperature (r=-0.7695, p=0.003), and weak negative correlation between CI and sea water temperature (r=-0.0277, p=0.933).

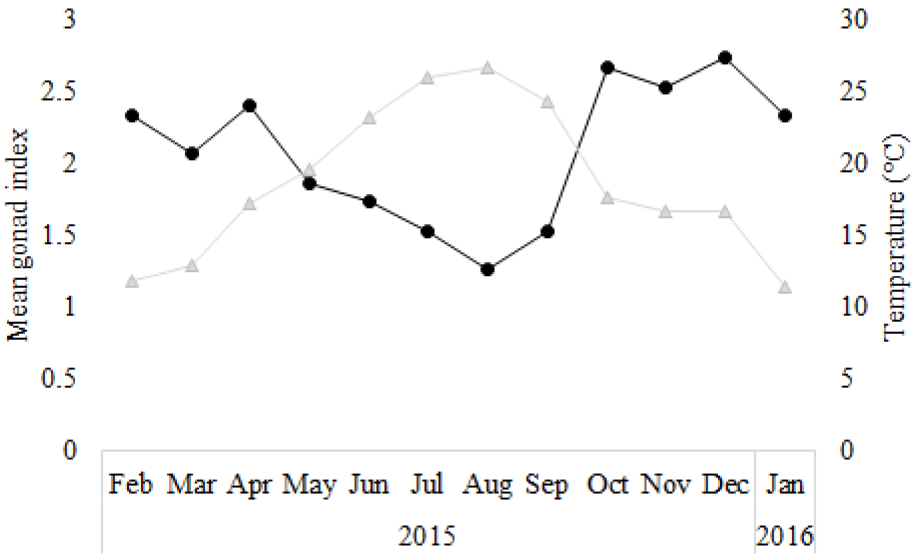

Figure 5. Mean monthly gonad index of *Mytilus galloprovincialis* (both sexes) (black circles) and mean sea water temperature (grey triangles)

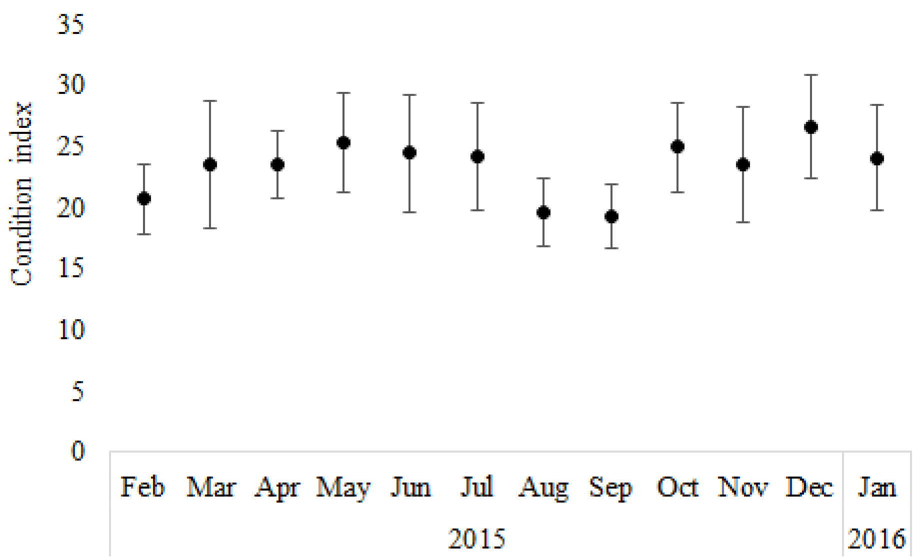

Figure 6. Mean monthly condition index and standard deviation of *Mytilus galloprovincialis* (both sexes)

DISSCUSION

This study relates to the annual reproductive cycle of cultured *M. galloprovincialis* in the main aquaculture area in Montenegro – Boka Kotorska Bay. Reproductive pattern of cultured bivalves are of great significance for farmers, therefore, it is not surprising that research on the reproductive cycle is mostly related to economically important bivalve species (e.g. Da Ros *et al*., 1985; Peharda *et al*., 2006; Crnčević *et al*., 2013; Popović *et al*., 2013; Matias *et al*., 2013; Bhaby *et al*., 2014; Bhaby, 2015; Ćurković, 2017; Egzeta-Balić *et al*., 2020; Puljas and Lukić, 2021).

Obtained data showed that *M. galloprovincialis* is a dioecious species with a sex ratio 1:1. Similar sex ratio was reported by other authors for *M. galloprovincialis* (Da Ros *et al*., 1985; Suárez *et al*., 2005; Bhaby *et al*., 2014; Bhaby, 2015; Azpeitia *et al*., 2017), as well as for *M. edulis* (Sunila 1981; Toro *et al*., 2002). Contrary to those authors, Chelyadina *et al*. (2018) in *M. galloprovincialis* populations from Black Sea found more males during all investigated period (M:F=2.8:1). Sex determination mechanisms in bivalves seems neither purely genetic nor purely environmental (Dalpé, 2020), and sea water temperature is considered as one of the most important environmental factor affecting sex determination in animals (Wedekind, 2017).

In 180 individuals which sex was examined both, by mantle color and by histology, we found a high overlap between these two methods, even in 76% specimen sex was exactly determined. Although in ripe mussels the mantle containing the gametes is typically orange in females and creamy-white in males (Gosling, 2003), sex determination based only on color is considered as not reliable without histology, as it is known that carotenoid pigments, responsible for the orange coloration, are involved in defense against oxidative stress, thus males could also display high concentrations of carotenoids which could lead to gender misidentification (Puljas and Brkić, 2020).

Bivalves are known to have annual reproductive cycle with one or more spawning picks depends on species, geographical location and environmental conditions and mussels are therefore good examples of bivalves with a flexible reproductive strategy, adjusting their cycle according to prevailing environmental conditions (Gosling, 2003). Qualitative results which we obtained in this paper showed that maturation and spawning occurred over a great part of the year, through even nine months while spawning peak was from January to April. Dominant resting period (August 2015) overlap with period when sea water temperature was the highest (> 25°C). Almost the same reproductive pattern in *M. galloprovincialis* population from Venice Lagoon (north Adriatic Sea) was reported by Da Ros *et al*. (1985). Da Ros *et al.* (1985) have also found prolonged spawning period with peak in January and February, as well as reproductive quiescent in July and August. Besides, Hrs-Brenko (1971; 1973) and Dardignac-Corbel (1990) reported prolonged spawning in populations across Mediterranean Sea including Adriatic Sea, as well as Stjepčević (1974) in populations from Boka Kotorska Bay and Azpeitia *et al.* (2017) in populations from Bay of Biscay.

Kunduz and Erkan (2008) in populations from Sea of Marmara as well as Bhaby (2015) in populations from Marocco reported spawning during almost all year including summer. Prolonged spawning period of cultured bivalves is of great advantageous for farmers as brood stock may be available over a longer period of the year (Popović *et al*., 2013).

Food availability and temperature are two main environmental factors affecting reproductive cycle in mussels (Suárez *et al*., 2005). Boka Kotorska Bay is characterised mainly as mesotrophic to eutrophic area where chlorophyll a reaches maximum values mainly in winter (sometimes > 10 mg m^{-3}) and phytoplankton abundance quite often reaches values up to 10^7 cells L^{-1} (Drakulović *et al*., 2016), what is very favorable for reproductive activity in cultured *M. galloprovincialis*. Our results showed significant correlation between MGI and sea water temperature indicating that temperature has strong effect on reproductive cycle as it is reported by other autors (Rand, 1973; Lubet, 1987; Brenko, 1980; Bilecik, 1989; Da Ros *et al*., 1985; Egzeta-Balić *et al*., 2020). We also found that most intensive spawning in *M. galloprovincialis* appear at temperatures between 10 and 15°C, what is in coincides with Lubet (1987) and Hrs-Brenko (1980) who pointed that temperatures below 8°C and above 16°C affect gametogenesis inhibition in mussels.

Quantitative analysis included oocytes counting and diameter measures. Quantitative results were in coincides with qualitative results. The largest oocyte was measured in December 2015 and has diameter 132.63 µm. Additionally, the highest mean values of oocytes were during November and December 2015 (66.31 i 66.58 µm, respectively), indicating that the largest oocytes appear in ripe and partly spawned individuals, as observed in other bivalve species (Gribben *et al*., 2004; Meneghetti *et al*., 2004; Peharda *et al*., 2006; Mladineo *et al*., 2007; Moura *et al*., 2009; Crnčević *et al*., 2013; Popović *et al*., 2013). Toro *et al*. (2002) reported mean oocytes diameter from 70 to 75 µm in M. edulis, and 60 to 65 µm in *M. trossolus,* while Smart *et al*. (2020) reported values from 28.7 to 35.2 µm in *M. galloprovincialis* populations from New Zealand.

The CI is highly dependent on reproductive cycle, and was investigated as such in different bivalve species (e.g., Hrs-Brenko, 1973; Peharda *et al*., 2003; Marušić *et al*., 2009). In our study, moderate positive correlation between CI and MGI was found, means that CI followed the MGI pattern. Bhaby (2015) also reported that CI follows the changes in the reproductive cycle in *M. galloprovincialis* as well as other authors for other bivalve species e.g., *Venus verrucosa* (Popović *et al*., 2013) and *Glycymeris nummaria* (Crnčević *et al*., 2013).

Although obtained results give great insight in reproductive biology of cultured *M. galloprovincialis* in area of Boka Kotorska Bay, limitations of the study can be related to sample size, so data analysis (e.g., developmental stages, mean gonad index) were done on total sample – both sexes were analyzed together.

CONCLUSIONS

Obtained data on the reproductive cycle showed that cultured *M. galloprovincialis* in Boka Kotorska Bay has long spawning period with a short resting period what can be defined as "bradyctic" as described in Da Ros *et al*. (1985). Sea water temperature has strong influence on the reproduction pattern, thus temperatures below 20°C has positive effect on gametes activity. Obtained qualitative and quantitative data on reproduction cycle of cultured *M. galloprovincialis* are of great significance for farmers in Boka Kotorska Bay.

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