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Reproductive pattern of the anchovy, *Engraulis encrasicolus* (Linnaeus, 1758), in the Boka Kotorska Bay (Montenegro, southern Adriatic Sea)

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The annual alteration of gonad morphology in anchovy, Engraulis encrasicolus *(Linnaeus,1758), caught in the period from July 2006 to June 2007 using beach seines in the region of Boka Kotorska Bay (Montenegro, southern Adriatic) was studied. Samples were taken each month. Total length of individuals ranged from 6.6 to 12.2 cm, while weight ranged from 1.58 to 11.27 g, Their ovaries were extracted and weighed, and a piece of female gonad tissue was sampled for histological analysis. The length–weight relationship of all anchovy specimens was described by the equation:* $W = 0.0032\ L_T^{3.2537}$; ($r^2 = 0.9528$). *The lowest GSI values were found in November and remained at low levels until April, which corresponded to the gonad rest stage. The GSI increased gradually during April and May, which coincides with the begining of spawning period. Although primary oocytes (stage I) were present during all months, their percentage increased from October and was the highest during the winter months (November-March 100%). Mature cells, stage IV, were present during April – October period, with highest value in May when they comprised 12.3% of total oocyte number. An increase in the percentage of oocytes in the yolk vesicle (II) and yolk (III) stages occurred in April, remaining almost unchanged until October. Smallest average oocyte size was noticed in period November – February with an average of 59.69 µm, while greatest size was recorded in April 2007, 185.84 µm.*

Key words: *Engraulis encrasicolus,* small pelagic, spawning, oogenesis, length structure, Boka Kotorska Bay

INTRODUCTION

Anchovy, *Engraulis encrasicolus* (L. 1758), is the only species of the family Engraulidae distributed along the coasts of Europe. The species is distributed in the north-eastern Atlantic, and stretches from the North Sea to the coasts of Morocco, including the entire Mediterranean, Adriatic, as well as the Black and Azov Seas (WHITEHEAD, 1986). Anchovy is considered one of the most important commercial pelagic fish species not only in the Adriatic Sea (CINGOLANI

et al., 2003), but also in the entire Mediterranean (LLEONART & MAYNOU, 2003). In recent decades, large inter-annual natural fluctuation of anchovy biomass and catches in the entire Adriatic have been reported (SINOVČIĆ, 2001; CINGOLANI *et al.*, 2003). A severe collapse of anchovy population in the Adriatic was evidenced in the period from 1986 to 1998, believed to be caused by the lower quantities of recruitment in that period and shift of climate regime (SINOVČIĆ & ZORICA, 2006; GRBEC *et al*., 2015).

Montenegrin industrial fishing of anchovy and sardine is still undeveloped, and the fishing fleet targeting small pelagic species is almost non-existent in this part of Adriatic. They are mainly caught through small-scale fishery in the Boka Kotorska Bay, mostly using beach seines of small mesh size (12-16 mm) with artificial light. Sardine and anchovy fisheries using beach seine nets have been traditionally present in this region for centuries (ĐUROVIĆ *et al.*, 2012; PEŠIĆ *et al.*, 2017). This type of fisheries targets mostly juvenile part of population (PEŠIĆ *et al*., 2010) as the Boka Kotorska Bay represents the natural spawning and nursery area of anchovy (MERKER & VUJOŠEVIĆ, 1972; MANDIĆ *et al*., 2011; MANDIĆ *et al*., 2013; MANDIĆ *et al*., 2015; MANDIĆ *et al*., 2017).

Anchovy is a fast-growing species with short life-span that matures early (LA MESA *et al.,* 2009). The species is a multiple spawner with high fecundity and long spawning season during which anchovy tends to migrate (from early spring to late summer, with peaks from May to July in Adriatic) to coastal areas rich in primary production (upwellings, river runoffs and convergence areas) in order to reproduce (GAMULIN & HURE, 1983; PALOMERA, 1992; SINOVČIĆ & ZORICA, 2006; ZARRAD *et al.,* 2006; LA MESA *et al.,* 2009; MORELLO & ARNERI, 2009). After spawning, it migrates towards open waters, forming large shoals (WHITEHEAD *et al.,* 1988).

Anchovy reaches sexual maturity at the end of the first year of life. Length at first maturity is reached by 50% of the total anchovy population at the minimum total length (TL) from 8.2 to 9.0 cm, while 95% of individuals become mature at 11.0 cm (SINOVČIĆ & ZORICA, 2006). At 11.5 cm the entire anchovy population is sexually mature (SINOVČIĆ, 1999).

The objective of this study was to gain information on the reproductive characteristics of anchovy, *Engraulis encrasicolus* (L.) in the Boka Kotorska Bay, a nursery and spawning ground of this species.

MATERIAL AND METHODS

Boka Kotorska Bay is a closed marine bay situated in the southern part of the eastern Adriatic, and it is one of the most indented parts of the Adriatic coast, with the innermost point 15 NM removed from the open sea (MAGAŠ, 2002).

The bay is rich in river and underwater runoffs and freshwater springs, and is prone to significant temperature and salinity variations. During the summer, the inflow of fresh water from the surrounding mountains and small tributaries is minor, causing low circulation of the water mass, with reversed conditions during the winter period. Water from the river runoffs combined with the influence of winds provokes surface streaming from the bay towards the open sea. It is one of the most productive areas of the Montenegrin coast and it seems to be a nursery ground for anchovy, sardine and other small pelagic fish species (MANDIĆ *et al*., 2013).

Anchovy samples were collected monthly from commercial catches by beach seine nets in the Boka Kotorska Bay area (Fig. 1) from July 2006 to June 2007, except in March 2007 when there have been no beach seine fishing activities in the area due to bad weather conditions.

Total length (LT) of 1771 individuals was measured to the nearest 0.1 cm using digital caliper, and weight (W) to the nearest 0.01 g by digital balance. After that, 456 individuals were dissected and gonads were extracted from both sexes (203 males and 253 females). The length–weight relationship was determined according to the logarithmic form of the original exponential equation (RICKER, 1975): $\log W = \log a + b \log L_T$, where a is the proportionality constant, b the allometry coefficient, W is fish weight in grams, and L_T is total length in centimetres. The allometric condition index was determined according to the formula: $Ka = W / aLt^b$ (LE CREN, 1951) where a and b are the coefficient and exponent of the length–weight relationship, respectively.

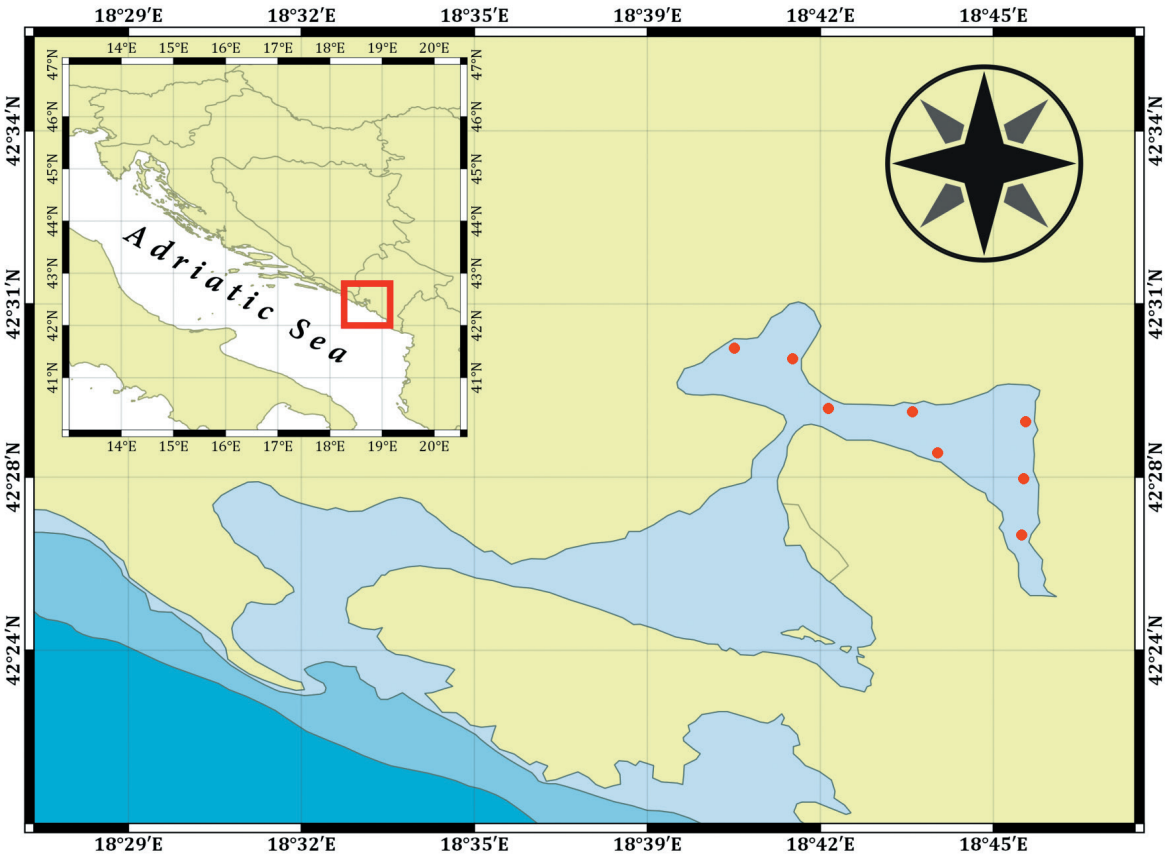


Fig. 1. Map of the study area

To avoid a length/size bias in the condition analysis, only fish less than 10.5 cm in length were used in the analysis. Sex ratio was calculated based on the equation R = M / F, where M is number of males, F is number of females, and R is sex ratio. The gonadosomatic index (GSI) was determined as percentage of their ovary mass in total body mass.

Female gonads were weighted to the nearest 0.01 g and preserved in 4% formaldehyde solution. A piece of tissue from each of the anterior, median and posterior parts of the gonad was dissected and histologically analysis. Tissue samples were dehydrated, clarified in xylol, and embedded in paraffin. Sections (4–6 μm) were cut and stained with Mayer's hematoxylin and eosin Y (CLARK, 1981). The oocytes were counted and measured under a Carl Zeiss Axio Imager Microscope with AxioImage ICC3 camera. Due to a peculiar elliptical shape of anchovy oocytes, the maximal and minimal oocyte diameters were averaged, in order to decrease variance and to avoid artificial increase in size and overlapping of oocytes in different stages when comparing them (WEST, 1990).

Histological slices were examined under microscope and processes of oogenesis were investigated according to morphological characteristics of oocytes. The stages of oogenesis were described according to ZIMMERMANN (1997): Stage I – Peri-nucleolus stage (Primary oocytes), Stage II - Yolk vesicle stage, Stage III - Yolk stage, Stage IV - Mature stage (Migratory nucleus).

RESULTS

Sample structure

The total length of anchovy individuals ranged from 6.6 cm to 12.2 cm, with a mean of 9.58 ± 1.03 cm (SE 0.05), while its weight

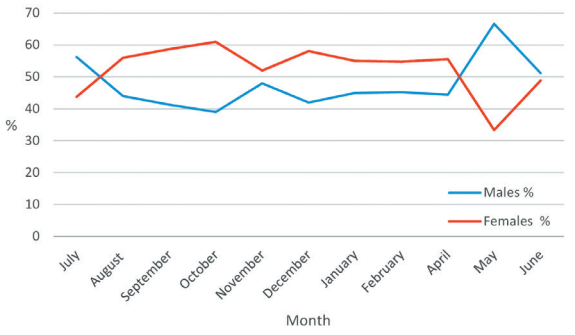
(W) ranged from 1.58 to 11.27 g, with a mean of 5.19 ± 1.85 g (SE 0.09). The total length of females ranged from 6.7 cm to 12.2 cm, with a mean of 9.62 ± 1.05 (SE 0.07), while weight ranged from 1.62 to 11.27 g, with a mean of 5.35 ± 1.96 g (SE 0.12). The length of males ranged from 6.6 cm to 11.7 cm, with a mean of 9.52 ± 0.99 cm (SE 0.07), and weight ranged from 1.58 g to 10.37 g, with a mean of 5.04 ± 1.71 g (SE 0.12). Analysis of average length by month revealed that smallest individuals were present in July (7.44 cm) and August (7.81 cm), while the highest average length was observed in April (9.98 cm) and May (9.79 cm).

Sex ratio

Of all analysed anchovy individuals, 55.5% were females, while 44.5% were males. Overall sex ratio was 0.92, and females were predominant in most of length classes (Table 1). Sex ratio deviated significantly from the hypothetical distribution of 1:1 (χ^2 = 0.8772; d.f. = 1; p > 0.001).

Analysis of sex ratio by month revealed that females were more abundant during August – April period, while males were more abundant only in May – July period (Fig. 2). Sex ratio varied from 0.64 in October, to 2.0 in May when males were more abundant in a sample.

Fig. 2. Sex ratio of Engraulis encrasicolus *by month (period July 2006 – June 2007)*



Length-weight relationship

Length-weight relationship for the overall sample was: $W = 0.0032\ L_T^{3.2537}$; $r^2 = 0.9528$. Also, the length-weight relationship was calculated separately for females and males. Regression coefficients for both sexes (males: $b = 3.2181$, $r^2 = 0.9501$; females: $b = 3.273$, $r^2 = 0.955$) indicated that deviations of allometric coefficients from the value of 3 were statistically significant (males, $t = 8.0555$; females, $t = 10.8109$; df = 1, p > 0.05).

Monthly variations of allometric coefficient were studied for the whole anchovy sample. The lowest values of allometric coefficient *b* were observed in August 2007 and June 2006, $b = 2.974$ and $b = 2.668$, respectively. During the other months of the study period the value of allometric coefficient *b* was higher than 3. The lowest value was observed in November (b=3.010) and the highest in April (b=3.308).

Condition

The average value of allometric condition coefficient (*Ka*) for anchovy from Boka Kotorska Bay during the study period was 1.004 ± 0.01.

Table 1. Sex ratio (R) by length classes

Length classes (cm)												
	6.5	7	7.5	8	8.5	9	9.5	10	10.5	11	11.5	12
Females	50.0%	50.0%	33.3%	58.8%	58.3%	59.0%	47.1%	56.1%	46.9%	66.7%	73.3%	100.0%
Males	50.0%	50.0%	66.7%	41.2%	41.7%	41.0%	52.9%	43.9%	53.1%	33.3%	26.7%	0.0%
R = M / F	1.00	1.00	2.00	0.70	0.71	0.69	1.12	0.78	1.13	0.50	0.36	0.00

The highest value of *Ka* was observed in July (*Ka* = 1.016 ± 0.07), with a slow decrease in succesive months, until February when *Ka* reached its lowest value (*Ka* = 0.994 ± 0.08).

GSI

A similar pattern of changes in mean monthly values of GSI was observed for females and males separately and for both sexes together (Fig. 3). Values of GSI observed in July 2006 were 4.46 for females and 4.35 for males. GSI decreased rapidly during successive months until November when it reached the lowest values, 0.83 for females and 0.70 for males, which coresponds to spent gonads. Afterwards, the GSI increased rapidly during April and May when it reached its highest values for females 4.85 and 4.08 for males. This coincides with the begining of spawning period.

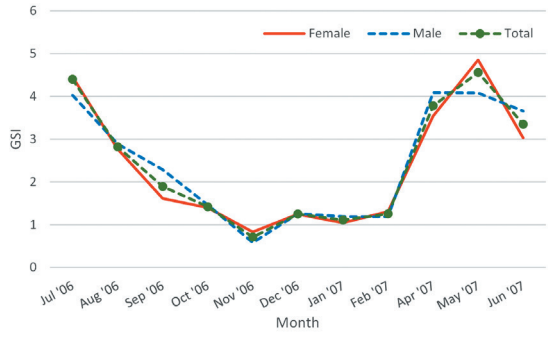


Fig. 3. Annual cycle of the gonadosomatic index (GSI) in females, males and both sexes together of Engraulis encrasicolus*, over the period July 2006 to June 2007.*

Reproductive cycle

Examination of histological slides of anchovy ovaries revealed that all four developmental stages of oocytes were present in the ovaries during most of the study period, except during November – February, when only stage I was present (Fig. 4). Additionally, ovaries were examined for the presence POFs (postovulatory follicles), and they were recorded in all months of the study period, except from November to February. The smallest mature female individual with presence of POF measured 7.5 cm in length and 2.42 g in weight (Fig. 5).

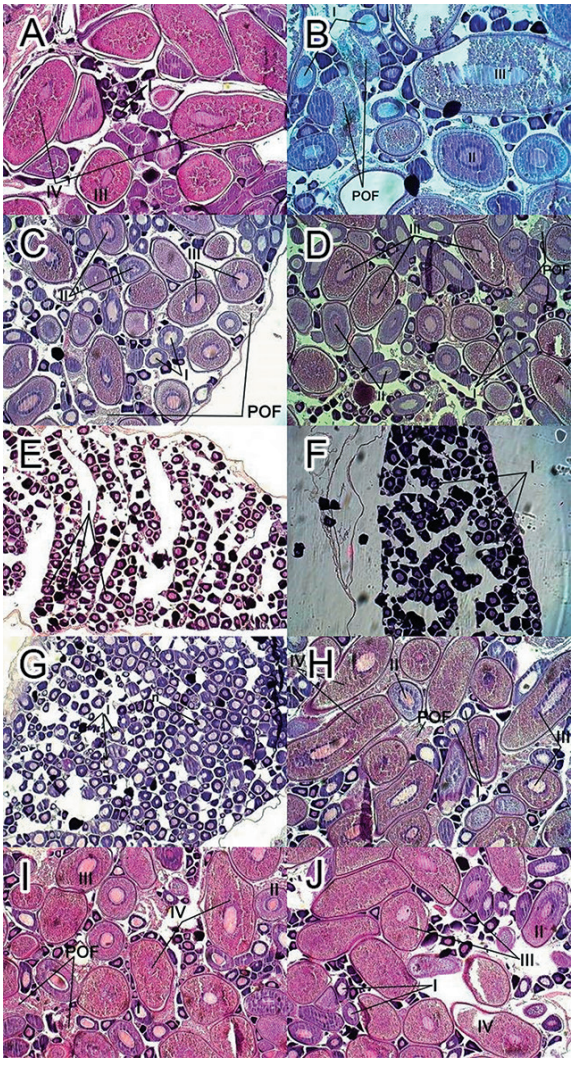


Fig. 4. Consecutive stages of oocyte development in Engraulis encrasicolus *(10 X magnification) – a) July; b) August; c) September; d) October; e) November; f) December; g) February; h) April; i) May; j) June. I – Peri-nucleolus stage; II – Yolk vesicle stage; III – Yolk stage; IV – Mature stage; POF - postovulatory follicles*

During the November – February period, anchovy ovaries were composed of primary oocytes only (stage I), while in the other months all four developmental stages of oocytes were present (Fig. 6). Stage II, the yolk vesicle stage, was present with approximately 25% during the April – September period, and in October its presence decreased to 9%, followed by complete absence in consecutive months. The presence of stage III oocytes, the yolk stage, shows similar pattern, during the April – September period

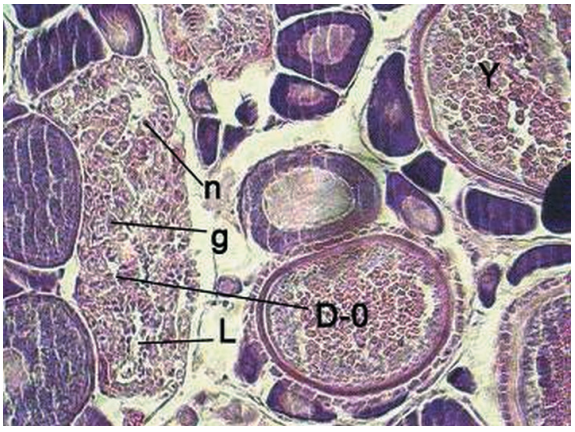


Fig. 5. Postovulatory follicle of Engraulis encrasicolus, length 7.5 cm (20× magnification), Y – yolk globules, n – nucleus of the granulosa cell, g – granulosa cell, L – lumen of follicle, D-0 – postovulatory follicle of age Day 0

stage III comprised 11-25% of total number of oocytes in ovaries, and its presence decreased in October to 9.8%. Stage IV, mature cells, were present during the April – October period, with the highest values in May, when they comprised 12.3% of the total oocyte number.

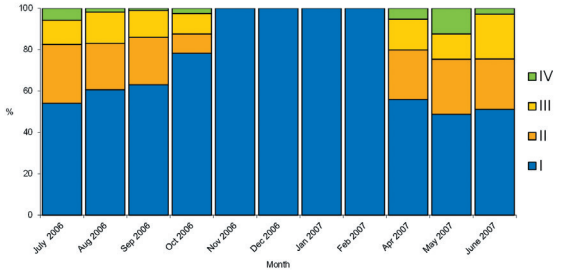


Fig. 6. Annual oocyte composition in the ovaries of Engraulis encrasicolus. *I – Peri-nucleolus stage; II – Yolk vesicle stage; III – Yolk stage; IV – Mature stage.*

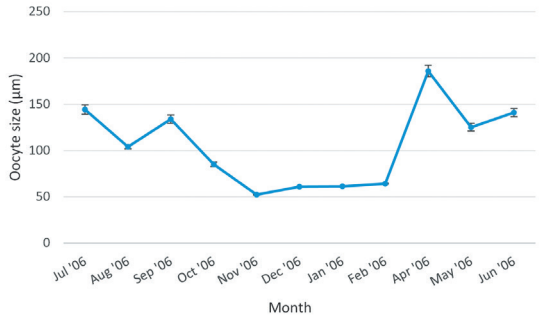


Fig. 7. Annual change of average oocyte size (mean ± SE) in ovaries of Engraulis encrasicolus

In July 2006 the average oocyte size in anchovy ovaries was 144.19 ± 5.07 µm, in August 103.92 ± 2.06 µm, September 133.84 ± 4.55 µm and decreased in October to 83.93 ± 2.42 µm. Smallest average oocyte size was noticed in the November – February period, with an average of 59.69 ± 0.38 µm, which coincided with the presence of only primary oocytes in ovaries (Fig. 7). Average oocyte size was largest in April 2007, 185.84 ± 6.29 µm, and slightly decreased in May (125.12 ± 4.14 µm) and June (140.93 ± 4.36 µm).

DISCUSSION

Boka Kotorska Bay represents an important spawning and nursery area for small pelagic species, especially anchovy and sardine (MERKER, 1971; MERKER & VUJOŠEVIĆ, 1972; MANDIĆ *et al*., 2015). At the same time, this area is the only area in Montenegro where the centuries-old traditional fishery is performed, using beach seine nets with mesh size of 12-14 mm targeting mainly juvenile part of anchovy and sardine population (ĐUROVIĆ *et* al., 2012; PEŠIĆ *et al.*, 2017). In order to protect the juvenile part of population from overfishing, but at the same time to allow (on the principles of sustainable development) this type of fishery with strong social and cultural significance for the population of the Boka Kotorska Bay, it is important to determine the population dynamics and to issue conservation measures.

Adult individuals of anchovy spawn in deeper waters at the open sea (MUŽINIĆ, 1956, 1972; REGNER, 1973; SINOVČIĆ, 1978), while the younger, juvenile individuals spend winter in shallow coastal waters, spawn for the first time at lengths of 8-9 cm and afterwards migrate towards the open sea, forming large shoals (WHITEHEAD *et al*., 1988; SINOVČIĆ, 2000; MARANO, 2001). The results from studies on the Zrmanja River (Central Adriatic) confirm that small anchovy spawns in the bays (SINOVČIĆ & ZORICA, 2006), and our results from Boka Kotorska Bay agree with these findings.

The mean changes of allometric factor of length-weight relationship (*b*) showed that the

lowest values, less than 3, were observed in June and August, which coincided with the appearance of young, juvenile individuals hatched in that spawning season. Decrease of allometric coefficient is recorded again in November (b = 3.01), after spawning, where individuals are in poor condition (as a consequence of spawning), as their reserves were transferred for gonad maturation and spawning instead of maintenance and physical growth (SINOVČIĆ & ZORICA, 2006). Similar results were observed in other coastal areas of Central Adriatic (SINOVČIĆ, 2000; SINOVČIĆ & ZORICA, 2006), most likely as a consequence of anthropogenic and natural eutrophication which causes high primary production and food availability (REGNER, D. *et al*., 2005, 2008).

The sex ratio of anchovy in this study was slightly skewed towards females (R = 0.92), with males more numerous only in the length class of 7.5 cm (R = 2.0). Greater number of females is adventageous for anchovy population, since sex ratio biased towards females in a population provides a greater rate of reproduction (WOOTTON, 1982). Males were more abundant during May – July period, which coincides with spawning period of anchovy in Adriatic Sea. Namely, the spawning period lasts from March until November, peaking from April to July (REGNER, 1972; REGNER, 1985; SINOVČIĆ, 2000; ZORICA *et al.*, 2013). Our results confirm findings of other authors from the Adriatic Sea. SINOVČIĆ (2000) reports that males were more abundant during spawning season in the Central Adriatic, while females were more abundant during the resting period (winter months), which was also confirmed for the North Adriatic (PADOAN, 1963).

This study of anchovy reproductive cycle revealed that stage I oocites (perinucleoulus stage) were present in ovaries during the entire year (Fig. 6). During the resting period (winter months), all oocites in ovaries were in stage I (100%), while in April, with the begining of the spawning season, all stages of oocite development were present in the ovaries, and continued to be present during the spawning season. Anchovy is a serial batch spawner and produces multiple batches of eggs over a spawning period. The number of batches varies from year to year (REGNER, 1985; SINOVČIĆ & ZORICA, 2006), with a maximum of up to 20 batches (MARANO *et al*., 1998; MARANO, 2001). Presence of stage I oocites started to decrease in April and continued until July, while after August their proportion started to increase again until November, when they represented 100% of all oocytes. After the winter period, with the start of the spawning period in April, stages II, III and IV are present in the ovaries, and their proportion increses until July, and decreases afterwards until October. They were completely absent in November.The proportion of stage IV oocites was greatest in May (12.34%), coinciding with the highest GSI values (4.56%). Compared to other stages, stage IV was the least represented (4.51% on average), which is understandable considering that this stage is the last stage in oocite development and lasts less than 24 hours (MOTOS, 1996). The reason for small proportion of stage IV oocite at histological slides could be that all samples for this study were collected between midnight and 4:00, when the peak of spawning (between 23:00 and midnight (MOTOS, 1996)) has already taken place.

CONCLUSIONS

Our study revealed that the spawning period of anchovy in Boka Kotorska Bay lasts from March to November, which coresponds to earlier studies (VARAGNOLO, 1964; ZAVODNIK, 1970; VUČETIĆ, 1971; REGNER, 1972, 1985; KOLITARI, 2006; SINOVČIĆ & ZORICA 2006; ZORICA *et al*., 2013), although eggs have been reported as early as February (ZAVODNIK, 1970) and as late as November (REGNER, 1972) and December (MANDIĆ *et al.,* 2012; ZORICA *et al*., 2014). Study of GSI parameters also showed that spawning period lasts from April to October. The lowest GSI value during this study was found in November, when specimens' gonad mass accounted for less than 1% of the body mass and remained low until April (Fig. 7). In this period ovaries were very small, composed entirely (100%) of oocites in stage I of oogenesis. The start of oocyte development started in April, and coincided well

with the increase of ovary mass to 3.54%, marking the beginning of spawning period. It then continued to increase through May. Oscillation of GSI during the year corresponded well with changes in number of mature oocites in anchovy ovaries. Study of anchovy egg density in the Kotor Bay during the same study period corresponded well to annual changes in the ovaries and GSI. The reported anchovy egg density was 590 eggs/m^2 of sea surface area (MANDIĆ, 2011).

Microscopic analysis of anchovy ovaries during this study revealed that smallest mature female measured 7.5 cm in length. POFs were present in the ovaries of this individual, proving that spawning had already taken place. This individual was caught in August, and according to daily otolith readings, and its age was estimated to be 3 months, which corresponds to April – May hatching of this species. This implies that a certain part of anchovy population from the Boka Kotorska Bay becomes sexually mature in the same season when it was hatched. Length at first maturity of anchovy in Adriatic varies greatly, and is mostly determined based on the macroscopic examination of gonads. Reported length at first maturity of anchovy females varies from 7.1 cm (SINOVČIĆ & ZORICA, 2006), 9.7 cm (SINOVČIĆ, 1978), to 10.9 cm (MUŽINIĆ, 1956) for Central Adriatic, 9.28 cm for the Boka Kotorska Bay area (MANDIĆ *et al*., 2015), and 10 cm for Albanian waters in the South Adriatic (KOLITARI, 2006). Based on hystological analysis of anchovy gonads RAMPA *et al.*, (2005) found that 50% of anchovy population in Northern Adriatic becomes mature at length of 8.1 cm. Early maturing is most likely the result of population response to certain environmental factors, high natural mortality and fishing presure. It has been established that the reproductive characteristics of anchovies, sardines and sprats are characterized by a high degree of plasticity (ALHEIT, 1989), in terms of changes in particular phases in spawning (batch fecundity, spawning frequency, age/length at first maturity), whenever the environmental conditions suddenly change either positively or negatively for the population (MILLAN, 1999).

Long spawning period of anchovy in the Boka Kotorska Bay and histological analysis of gonads confirms that this species is a multiple spawner. In our study the description of oogenesis confirms previous reports that spawning of the anchovy population in the study region of the southern Adriatic Sea begins in April and lasts until October.

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Razmnožavanje brgljuna, *Engraulis encrasicolus* (Linnaeus, 1758) u Bokokotorskom zaljevu (Crna Gora, južni Jadran)

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SAŽETAK

U radu su predstavljeni rezultati proučavanja godišnje promjene morfologije gonada kod brgljuna, *Engraulis encrasicolus* (Linnaeus, 1758) uhvaćenog obalnim mrežama potegačama u razdoblju od srpnja 2006 do lipnja 2007na području Bokokotorskog zaljeva (Crna Gora, južni Jadran). Uzorci inćuna uzimani su mjesečno, sredinom mjeseca. Ukupna dužina jedinki kretala se od 6,6 do 12,2 cm, dok je masa jedinki kolebala od 1,58 do 11,27 g. Kod ženki, jajnicima je nakon vađenja izmjerena masa, a dio tkiva jajnika odvojen za histološku analizu. Dužinsko-maseni odnos svih jedinki brgljuna opisan je jednadžbom $W = 0.0032\ L_T^{3.2537}$; ($r^2 = 0.9528$). Najniža vrijednost gonadosomatskog indeksa (GSI), manja od 1, zabilježena je u studenom, a ostala je niska sve do travnja, što odgovara razdoblje odmora gonada. GSI se postupno povećava tijekom travnja i svibnja, što se poklapa sa početkom perioda mrijesta. Iako su primarni oociti (stadij I) bili prisutni u svim mjesecima, njihova postotna zastupljenost u porastu je od listopada, a najviša tijekom zimskih mjeseci (u razdoblju od studenog do ožujka zastupljenost je iznosila 100%). Zrele stanice (stadij IV) bile su prisutne u razdoblju od travnja to listopada, sa najvećom zastupljenošću u svibnju, kada su činile 12,3% ukupnog broja oocita. U travnju se javlja povećanje postotne zastupljenosti oocita u stadijima žumanjčane kesice (II) i žumanjca (III) i ostaje gotovo nepromijenjeno do listopada. Najmanja prosječna veličina oocita zabilježena je tijekom razdoblja od studenog do veljače, kad je iznosila 59.69 μm, dok je najveća zabilježena u travnju 2007., 185.84 μm.

Ključne riječi: brljun, *Engraulis encrasicolus*, oogeneza, dužinska struktura, dužinsko-maseni odnos, kondicijski faktor, Bokokotorski zaljev