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Sardine (*Sardina pilchardus*) spawning in the light of fat content analysis

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Summary: Sardine samples from commercial catches obtained by purse seiners operating in Croatian fishing grounds (eastern Adriatic Sea) were collected monthly from March 2017 to February 2018 (excluding January due to a fishing ban). A total of 1085 sardines were analysed. Their total body length and mass ranged from 11.5 to 16.9 cm (mean±SD: 13.9±0.03 cm) and from 11.30 to 54.03 g (mean±SD: 20.31±0.161 g), respectively. Analysis of the length-mass relationship showed positive allometric growth (b=3.3573, r=0.948). Female specimens were predominant (m/f=0.404). According to the monthly gonadosomatic index values, spawning occurred from November to February, which was consistent with previous investigations. The monthly analysis of fat content in the gonads, liver and muscles indicated that the fat content in each studied tissue oscillated seasonally regardless of sex. These alternations were linked to the sardine's reproductive cycle.

Keywords: small pelagics; lipids; reproduction; Adriatic; Mediterranean

El desove de la sardina (*Sardina pilchardus*) a la luz del análisis del contenido de grasa

Resumen: Las muestras de sardina de las capturas comerciales obtenidas por los cerqueros que operan en los caladeros croatas (este del mar Adriático) se recogieron mensualmente desde marzo de 2017 hasta febrero de 2018 (excluyendo enero debido a una prohibición de pesca). Se analizaron un total de 1085 sardinas. Su longitud corporal total y masa variaron de 11.5 a 16.9 cm (media±DE: 13.9±0.03 cm) y de 11.30 a 54.03 g (media±DE: 20.31±0.161 g), respectivamente. El análisis de la relación longitud-masa mostró un crecimiento alométrico positivo (b=3.3573, r=0.948). Predominaron los especímenes femeninos (m/f=0.404). De acuerdo con los valores del índice gonadosomático (GSI) mensuales, el desove se produjo de noviembre a febrero, lo cual fue consistente con las investigaciones anteriores. El análisis mensual del contenido de grasa en las gónadas, el hígado y los músculos indicó que el contenido de grasa en cada tejido estudiado oscilaba estacionalmente independientemente del sexo. Estas alternancias estaban vinculadas al ciclo reproductivo de la sardina.

Palabras clave: pequeños pelágicos; lípidos; reproducción; Adriático; Mediterráneo.

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INTRODUCTION

Sardine (*Sardina pilchardus* Walbaum, 1792) is one of the most important pelagic fish species due to its huge ecological (Cury et al. 2000, Palomera et al. 2007) and economic role. It is distributed along the Atlantic continental shelves of Europe and Africa as well as in the Mediterranean, including the adjacent Black Sea. It is a fast-growing and short-living small migratory pelagic fish species. Sardine is a batch spawner with indeterminate fecundity (Blaxter and Hunter 1982, Ganias et al. 2003), tending to spawn from September to March (Ganias et al. 2007, Sinovčić et al. 2008, Bouhali et al. 2015). This species is known as an omnivore that prefers zooplankton rather than phytoplankton prey items, and its diet changes seasonally in terms of its feeding activity and prey quantity (Costalago and Palomera 2014, Zorica et al. 2017). In the Adriatic, the

sardine is mainly targeted by purse seiners, which use artificial light to attract and aggregate the fish near the surface (Cingolani et al. 1996, Kraljević et al. 2014). Over the last few decades, the official sardine catch and biomass have fluctuated in the Adriatic area (FAO 2017). Such fluctuations in sardine and small pelagic fish populations in general are common and most probably linked to numerous environmental factors to which incoming year-class individuals have to adapt (Fréon et al. 2005).

The abovementioned fluctuations are scientifically intriguing and surely linked to the fish's metabolism and its functioning, which is strongly correlated with fish life stages and the environment. Throughout its life span, the sardine (like all other living organisms) needs to accumulate sufficient energy reserves to ensure successful growth, reproduction and survival (Shulman and Love 1999). The required energy reserves are not constant within the year or over the years, since the life cycle adapts along with the abiotic and biotic parameters of an ecosystem. The energy flow through one living organism is difficult to track and even more difficult to measure. In order to gain insight into this complex system, scientists have defined numerous ways of measuring metabolic rates. In the case of teleosts, the principal energy sources that can be used to measure the metabolic rate are lipids, proteins and carbohydrates (Shulman and Love 1999).

Based on the above, the general aim of this study was to link energy reserves with the sardine's reproductive cycle. Lipids (as energy reserves) were evaluated as they seem to play a major role in fish spawning. In fact, their levels have an effect on gonad development, egg quality, fecundity, fertilization, hatching rate and survival of larvae (Sargent et al. 1989, 2002, Rainuzzo et al. 1997, Brosset et al. 2016a). Within this study, the seasonality of lipid storage in the gonads, liver and muscle was studied over one year and analysed in the context of spawning. The findings from this investigation should provide a better understanding of sardine reproductive potential, which will be a step forward to sustainable management of this species.

MATERIALS AND METHODS

Biological sampling and processing

Sardine specimens (N=1085) were collected monthly from March 2017 to February 2018 (with the exception of January when the fishing ban for purse seine was in force) in the middle eastern Adriatic Sea by commercial purse seiner (mesh size of 14 mm) (Fig. 1). Immediately after capture, random samples of sardine specimens weighing approximately 1.5 kg were put on ice, landed and transported to the laboratory. Upon arrival in the laboratory, each sardine was measured (total length, TL) to the nearest 1 mm and weighed (total body weight, W) to the nearest 0.01 g. The sex of each specimen was determined macroscopically. The seasonality of sardine spawning was established by monthly changes in gonad weight (Wg±0.01 g) and the gonadosomatic index (GSI; monthly share of

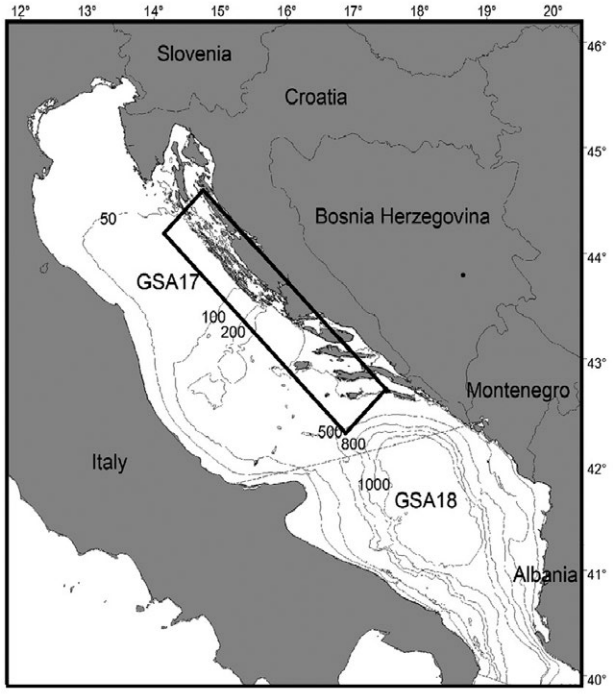

Fig. 1. – Study area, middle eastern Adriatic Sea. The sampled area is marked by a black rectangle.

gonad weight (Wg) in total body weight (W). GSI=100 Wg/W) by sex and overall. The length-weight relationship was determined by sex on a monthly basis and overall, according to the logarithmic form of the standard equation log(W)=log(a)+b log(TL), where W is total fish weight in g; TL is the total length in cm; a is the proportionality constant; and b is the allometric coefficient.

Total lipid content analysis

A sub-sample was taken each month from the collected sardine sample for total lipid content analysis. Monthly sub-samples were made of ten sardine specimens of each sex, with the total length between 13.0 cm and 14.0 cm. Three pieces of tissue (1, whole liver; 2, piece of gonad; 3, piece of muscle fillet, with skin) were taken from each fish in the area between the anal opening and the caudal fin base for total lipid analysis. Each month, the tissue pieces of each organ were put together, weighed on an analytical balance (to the nearest 0.01 mg) and stored in plastic containers at –18°C.

The total lipid content was determined using a modified Van Handel extraction and analysis (Van Handel 1985). Tissue samples were homogenized using a Polytron PT 1600 E mixer (Kinematica AG, Luzern, Switzerland) and stored at –20°C until analysis. For the analysis, 50 mg of each tissue was mixed with 4 mL of chloroform:methanol mixture (1:1 (v:v)) using a Polytron mixer in a 50-mL polypropylene tube. The homogenate was centrifuged at 3000 rpm for 5 min, and the supernatant volume was recorded. A 250-µl aliquot was carefully transferred directly to the bottom of the 18×100 mm culture tube. The aliquot was placed at the bottom of the tube to minimize variability by

preventing loss of lipids on the sides of the tube where they could not be properly digested.

The six-point standard curve was generated by setting up tubes containing 100 μL of the standards (0, 0.25, 0.50, 0.75 and 1.00 mg cod oil standard per millilitre of acetone). The samples and standards were set up in triplicate and placed in a heating block set at 100°C to allow the solvent to evaporate. After approximately 10 min, the solvent had evaporated and 100 μL of concentrated sulphuric acid was added to each tube by carefully depositing the acid at the bottom of the tube. The samples were vortexed, heated at 100°C for 10 min and after that allowed to cool to room temperature before adding 2.4 mL of vanillin reagent and vortexing. The reagent was prepared according to the standard method (Van Handel 1985): 600 mg of vanillin was dissolved in 100 mL of hot water after the solution had cooled down, and 400 mL of 85% phosphoric acid was added. The reagent was stored in a dark bottle at 4°C and used within two weeks. After incubation at room temperature for 5 min, the absorbance (A, nm; measured at 500 nm) was measured using a Perkin Elmer UV/VIS Lambda Bio 40 spectrophotometer (Perkin Elmer, Waltham, Massachusetts, USA). Solvent blanks were run throughout the procedure and used to "zero" the spectrophotometer.

In order to obtain the percent lipid content, the supernatant volume was multiplied by the concentration of lipid determined from the standard curve, and then divided by the sample weight (in mg) and multiplied by 100.

All solvents were HPLC grade. Sulphuric acid (98%), vanillin, o-phosphoric acid and cod liver oil were obtained from Sigma-Aldrich (St. Louis, MO, USA). The vanillin reagent was used within two weeks and stored in an amber bottle to reduce degradation.

Statistical analysis

In order to conduct a meaningful analysis, the physiology and the reproductive pattern of the investigated species were taken into account and three phases were defined: F, feeding; S, spawning; and R, resting. The dataset consisted of the gonadosomatic index (GSI) and measured fat content values obtained for each tissue (G, gonads; M, muscles; and L, liver). Given the results of the Draftsman Plot, whole data were log (x+1)-transformed. In order to determine the (dis)similarities in the dataset, principal component analysis was applied. A similarity matrix based on the Bray-Curtis index was then constructed and a one-way ANOSIM was applied to assess sex and phase differences in the dataset (Clarke and Warwick, 1994). The PRIMER software routine (Plymouth Marine Laboratories, UK; Clarke 1993, Clarke and Warwick 1994) was used for all analyses and the level of significance was set to $P<0.05$.

RESULTS

Biological parameters

The total body length and mass of the observed sardine specimens (N=1085) varied from 11.5 to 16.9 cm (mean±SD: 13.9±0.03 cm) and from 11.30 to 54.03 g (mean±SD: 20.31±0.161 g), respectively. The monthly variations in mean total sardine length are shown in Figure 2.

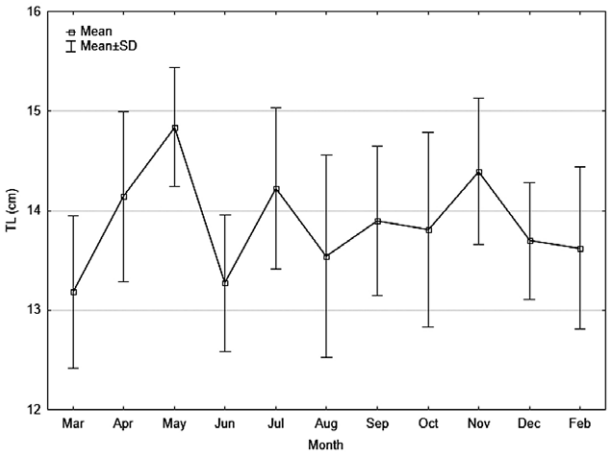

Fig. 2. – Mean monthly total body length of sardines collected by commercial purse seiner operating in the eastern part of the Adriatic Sea; March 2017-February 2018.

The lowest mean monthly values of total body length were recorded in March (mean±SD: 13.2±0.77 cm), whereas the highest values were obtained in May (mean±SD: 14.8±0.60 cm). Sex was determined in 443

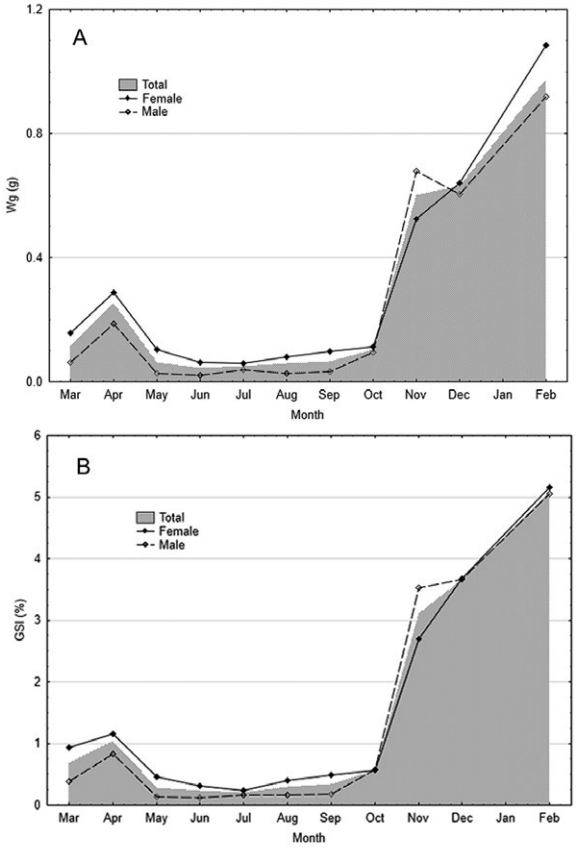

Fig. 3. – Mean monthly values of gonad mass (Wg) (A) and gonadosomatic index (GSI) (B) for female, male and all sardine individuals collected in the eastern Adriatic from March 2017 to February 2018.

individuals (males N=179, mean TL±SD: 13.4±0.66 cm; females N=264, mean TL±SD: 13.9±0.85 cm). The overall sex ratio (m/f=0.404) deviated significantly from the hypothetical distribution of 1:1 (χ^2=15.928, df=1, P<0.05), indicating that females were predominant over males. Seasonal oscillations in mean monthly gonadosomatic indices and gonad mass values, observed separately by sex or overall, showed the same trend (Fig. 3).

There was a slight increase in both values in October (GSI=0.568, Wg=0.11), after which both values increased sharply and stayed at high levels until February, when they reached their peaks (GSI=5.09, Wg=0.976 g) (Fig. 3). Afterwards, there was a sharp decline in the GSI and Wg.

The length–mass analysis demonstrated positive allometric growth ($W=0.0029TL^{3.3573}$, r^2=0.8987), as the value of parameter b differed significantly from 3 [one-sample t-test (P<0.05)].

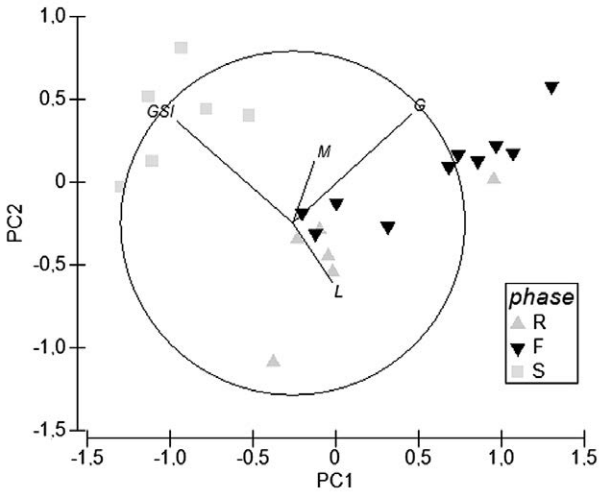

Fig. 5. – Principal component analysis of amount of fat in each tissue (G, gonads; L, liver; M, muscle; and GSI) of sardine specimens collected in the eastern Adriatic, March 2017-February 2018. F, feeding; S, spawning; R, resting.

Fat content

The monthly fat content values in sardine gonads, muscles and liver measured separately for each sex are shown by Figure 4. Overall, the highest levels of fat content were recorded in the gonads, and specifically within male gonads from July (Fig. 4B; 17.6%). The oscillations in gonad fat content in males and females showed a similar trend, as they both reached high values during the warmer part of the year (Fig. 4; females, 4.11% in June and 3.89% in September; males: 9.72% in May and 12.61% in September), although gonad fat content was much higher in males than in females.

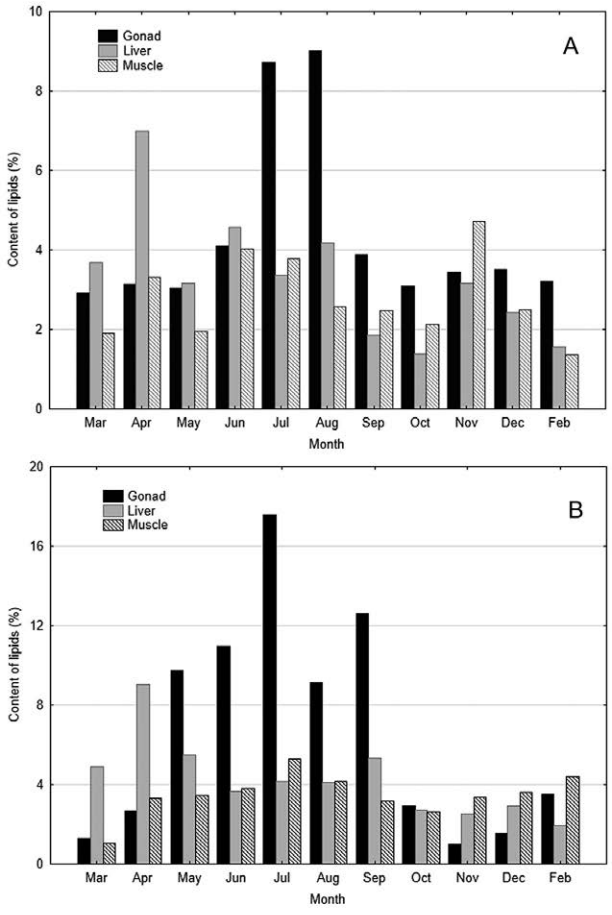

Fig. 4. – Monthly values of fat content accumulated in gonads, liver and muscles of female (A) and male (B) sardine specimens collected in the eastern Adriatic, March 2017-February 2018.

Slight oscillations in fat content values were observed in the muscles of both males and females. Furthermore, in both sexes the lowest level of fat in muscles was obtained at the end of the colder part of the year (Fig. 4; females, 1.35% in February; males, 1.02% in March), whereas the maximum values occurred at different times for each sex. In fact, the highest accumulation of fat in the muscles of females was recorded in November, and in males the highest accumulation was recorded in July (Fig. 4).

The fat content in the liver in the two sexes varied in a similar manner, and once again was higher in males than females. Both males and females showed higher storage of fat in the liver from March to August (females) or September (males), and maximum values for both sexes were recorded in April (Fig. 4).

Data were collected according to different life phases [spawning (S), feeding (F) and resting (R)]. Based on the high values of GSI and gonad mass (Fig. 2), spawning occurred from November to February, which was considered as the spawning phase. The resting phase occurred from March to June, coinciding with the lowest GSI and gonad mass values. Finally, the feeding phase occurred from July to October, as primary production is high during this part of the year (Kovač et al. 2018) and sardines have to recover energy in order to continue reproducing.

By means of principal component analysis, 84.8% of the variability of four variables (fat content in gonads, liver and muscle; GSI) was explained by the first two components (Fig. 5). The most important components, PC1 and PC2, accounted for 65.0% and 19.8% of the total variation, respectively (Table 1). For PC1, the fat content within the gonads and gonadosomatic index

Table 1. – Results obtained by principal component analysis on gonadosomatic index (GSI) and fat content in gonads (G), liver (L) and muscles (M) of sardine specimens collected in the eastern Adriatic, March 2017-February 2018.

PC	Eigenvalues	Explained variation (cumulative, %)	Explained fitted variation (cumulative, %)	
1	0.608	65.0	65.0	
2	0.185	19.8	84.8	
3	0.102	10.9	95.7	
4	0.040	4.3	100.0	
Variable	Eigenvectors			
	PC1	PC2	PC3	PC4
GSI	–0.677	0.595	0.281	0.330
G	0.689	0.632	–0.086	0.346
L	0.228	–0.342	0.830	0.378
M	0.127	0.360	0.475	–0.793

Table 2. – Results of the one-way ANOSIM (R, Global R; P, significance level) of differences between the spawning, resting and feeding phase and between males and females of sardine specimens collected in the eastern Adriatic, March 2017-February 2018.

Phase	R	P
Resting versus feeding	0.273	0.002
Resting versus spawning	0.763	0.0002
Feeding versus spawning	0.810	0.0001

gave the highest percentages, whereas for PC2, the highest percentages were obtained for the fat content within the gonads and liver (Table 1). In fact, the observed data were clustered into three groups that were more or less in line with the determined phases. The specimens in the spawning phase were thus grouped together with a high loading for PC1 and a low loading values for PC2 (highest GSI and lowest fat content in gonads). Although the resting and feeding phases were not as clearly separated as the spawning phase, they were differentiated on the basis of the fat content in the gonads and liver and the gonadosomatic index: lower values of all three factors defined the feeding phase, whereas a higher fat content in the liver and low fat content in the gonads defined the resting phase (Fig. 5).

The results of one-way ANOSIM showed that the four measured parameters (fat content in gonads, liver, muscle; GSI) did not significantly differ in relation to sex, whereas significant differences were observed according to the defined phases (spawning, resting and feeding) (Table 2).

DISCUSSION

Biological parameters

The overall biological data obtained in this study for the sardines collected by commercial purse seiners in the eastern Adriatic Sea throughout a one-year period (March 2017-February 2018) were consistent with the review by Morello and Arneri (2009) for this Adriatic sardine stock. The length distribution of the sardines investigated was within the ranges obtained for this type of fishing gear operating along the eastern side of the Adriatic, specifically the Croatian fishing grounds (Sinovčić et al. 2008, Kraljević et al. 2014), and their reproductive traits were consistent with those of previous studies (Brosset et al. 2015, Zorica et al. 2016, 2017). Females dominated the sardine population investigated in this study, in agreement with previous studies, with the exception of Mustać and Sinovčić (2010), who reported that the sex ratio of the Adriatic sardine favoured males. This difference might be a matter of sampling strategies that covered limited areas of the Adriatic, as only two small areas of the middle Adriatic were sampled in the study of Mustać and Sinovčić (2010). The allometric coefficient (b=3.3573) obtained fits within the wide range (2.757<b<3.657) given for this population (Morello and Arneri 2009, Brosset et al. 2015).

Fat content

The fluctuations in fat content in all the sardine tissues analysed in the present study (gonads, liver and muscles) were already reported for the same species by Garrido et al. (2007), Nunes et al. (2015) and Brosset et al. (2016b). Due to the measured values of fat content, it seems that the sardine deposits its fat reserves mainly in the gonads, which presumably makes them the organs that are most affected by all physiological processes during the lifetime of an indeterminate batch spawner such as the sardine (Fig. 5).

It was clear that the majority of the fat/energy stores were accumulated in the feeding period (July–October), corroborating the food habits of sardines originating from the same area (Zorica et al. 2017) and elsewhere (Costalago et al. 2012, Le Bourg et al. 2015, Brosset et al. 2016b). In fact, Zorica et al. (2017) found that in the warmer period of the year (June–September), here defined as the feeding phase, sardines consumed higher energy prey such as fish eggs, cladocerans and decapod larvae, resulting in the highest stomach fullness index values. The pattern of acquiring energy reserves before the onset of spawning has previously been reported not only for the sardine (Mustać and Sinovčić 2009, Marin et al. 2010, Bandarra et al. 2018) and other small pelagics (Hunter and Leong 1981, Cubillos et al. 2001, Pacetti et al. 2013), but also for many other fish (El Kebir et al. 2003, Lloret et al. 2008, Grande et al. 2016).

The outcomes of the present study also showed that the fat content in gonads, liver and muscle decreased with the increase in the GSI. The decline in fat content was consistent with that reported by previous studies on small pelagic fish species (Pecquerie et al. 2009, Nunes et al. 2011, 2015, Pacetti et al. 2013). However, this decline did not continue for long, which was somewhat surprising considering the fact that the sardine has a relatively long spawning season during which it has to produce many batches of eggs (Ganias et al. 2004, Somarakis et al. 2006). This is most likely due to the fact that the sardines continued to feed throughout the entire spawning season (Zorica et al. 2016, 2017) in order to obtain and maintain the required, although low, level of energy reserves in all tissues.

As soon as spawning ended, the sardines started their recovery in a resting phase that coincided with the start of spring and lasted until the start of summer. In the Adriatic, this period coincides with the peak of primary production (Ninčević Gladan et al. 2010,

Kovač et al. 2018), which leads to the increased feeding intensity of sardines (Zorica et al. 2016, 2017). The revitalization of fat stocks in all tissues was evident, although the most pronounced accumulation was noted in the liver, whose fat level was the lowest by the end of the spawning phase. It is known that the temporary energy storage within the liver is crucial for fish growth, reproduction and migration (Tocher 2003, Nunes et al. 2011). Furthermore, the quality of energy stored in the liver most probably affects the development of future generations (health and proper development of eggs and larvae) (McBride et al. 2013). Hence, the collection, storage and utilization of fat/energy stored in the liver might be one of the triggers that leads to the oscillation in spawning stock biomass of this and other small pelagic fish species.

In summary, the present results demonstrated that the fat reserves stored in gonads and liver were strongly affected by the spawning season. In general, gonads are widely used to determine the reproductive pattern, but, as shown within this study, the fat content in the liver might be of additional use. The fat content in the liver, other than being used as an indicator of spawning, may be an indicator of the recruitment success through the tracking of its temporal variations.

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