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Reproductive biology of NW Mediterranean tonguefish *Symphurus nigrescens* and *Symphurus ligulatus*

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The population structure and reproductive biology of deep-water continental margin Cynoglossidae is poorly known. Here, we focused on two highly abundant species of the upper slope of the NW Mediterranean Sea, Symphurus nigrescens *and* S. ligulatus. *Megafaunal sampling of 37 hauls was conducted between April 2003 and May 2004 in the Blanes canyon and the adjacent open slope between 300–1500 m depth. The differential bathymetric distribution between the two species was surveyed by standardizing catch data per unit of swept surface and by pooling resulting densities by 100 m depth interval. Females of both species were classified into a five-stage maturity scale according to their external appearance and their gonads dissected for histological analysis. The analysis of the species' bathymetric distribution revealed a differential and only partial overlap. In both species, an increase in size with depth was observed coinciding with the dominance of females in deeper depth strata. The shallower species,* S. nigrescens *showed a marked spawning period with a peak in summer. In contrast, the deeper-living species,* S. ligulatus, *presented a longer spawning period ranging from spring to autumn. Moreover, histological examination demonstrated that the ovaries of both species had an asynchronous organization. Present results on spatial-temporal size frequency distribution, spawning temporality and oocyte size are discussed assuming interspecific competition and the different environmental conditions related to their bathymetric distribution.*

Keywords: NW Mediterranean, reproductive strategies, seasonal cycles, size-frequency distribution, *Symphurus*

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INTRODUCTION

The NW Mediterranean continental margin is the historical focus of a strong fishing pressure, which started during the middle of the past century (Sardà *et al.*, 1994). Large proportions of species caught by commercial bottom trawls have no market value and are discarded (Sánchez *et al.*, 2004). Several of these by-catch items are of ecological importance because of their large abundance and overall biomass within the catch itself (Alverson *et al.*, 1994). Therefore, any human impact assessment in the continental margins requires detailed data on the life history of both target and non-target species, in order to foster an ecosystem management approach to fishery, as required by modern marine EU policies (Marine Strategy Framework Directive; 25-0-2008). However, the available biological data on by-catch species are scarce worldwide (Hall & Mainprize, 2005). The Mediterranean tonguefish *Symphurus nigrescens* and *Symphurus ligulatus* are examples of this situation. Both species are of ecological importance in local demersal communities given their abundance (Stefanescu *et al.*, 1994, Fernandez-Arcaya *et al.*, 2013a). Therefore, their populations often appear in large numbers within commercial hauls (Carbonell *et al.*, 1995).

The available information on *S. nigrescens* and *S. ligulatus* reproductive biology is scarce. These two species are the only pleuronectiform of the family Cynoglossidae inhabiting the Mediterranean slope (Lloris *et al.*, 1984), between 100–700 m and 400–1300 m, respectively (Massutí *et al.*, 1995; Fernandez-Arcaya *et al.*, 2013a). *Symphurus nigrescens* is distributed throughout the Mediterranean (Blauchot, 1987), while *S. ligulatus* is restricted to the western (Matallanas, 1984) and eastern basins (Koukouras, 2010). The two species are mesobenthonic (Massutí *et al.*, 1995) and feed on benthic invertebrates (Macpherson, 1978; Cau & Deiana, 1979). *Symphurus nigrescens* is normally associated with the asteroid *Brisingella coronata* and the coral *Funicula quadrangularis* (Maurin, 1962), while *S. ligulatus* is commonly found on bamboo coral (*Isidella elongata*) communities (Maurin, 1962; Torchio, 1971; Tortonese, 1975).

Mediterranean demersal resources are exposed to a continuous seasonal and spatial clinal variation acting on individual species biology (Aguzzi *et al.*, 2013a, b; Fernandez-Arcaya *et al.*, 2013a). In the NW Mediterranean slope, particle fluxes from the photic zone to the deep sea increase in autumn–winter in response to major coastal storms and during spring–summer water stratification prevents such a

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flux, decreasing the overall energy availability in deep-sea realms (Lopez-Fernandez *et al.*, 2013). In this context, *S. nigrescens* apparently spawns between June and October (Macpherson, 1978), whereas *S. ligulatus* spawns between May and November (Cau & Deiana, 1979). However, a more in-depth analysis of their reproductive strategy has not yet been conducted. Accordingly, our aim is to present a comparative analysis along a bathymetric gradient of the size frequency distribution and reproductive patterns of *S. nigrescens* and *S. ligulatus* in the NW Mediterranean over an annual cycle.

MATERIALS AND METHODS

Sample area and data collection

Megafaunal sampling was conducted between April 2003 and May 2004 in the Blanes canyon and the adjacent open slope (Figure 1). A total of 37 hauls were conducted between 300 and 1500 m depth with commercial and scientific vessels, even though the stratum between 701 and 800 m depth was not sampled. The individuals of *Symphurus nigrescens* and *S. ligulatus* were caught in 28 of those hauls each (Table 1). Fish were caught using a commercial Otter Trawl Bottom System (OTBS) and its scientific equivalent, the Otter Trawl Maireta System (OTMS), both with a cod-end net of 12 mm stretch mesh size (Sardà *et al.*, 1998). The catchability of these two different gears does not differ significantly in either the catch composition or the size frequencies of the species sampled (Sardà *et al.*, 2002).

On board, the length of individuals was measured from the mouth tip to the edge of the tail to the nearest 0.5 cm and they were weighed to the nearest 0.1 g. They were immediately dissected and their sex was determined by macroscopic examination of the gonads (see below).

Population distribution

In order to obtain population density estimation, catch data for both species were standardized to the unit of swept area (ind. km^{-2}), as estimated from Scanmar sensors. These sensors provide information on horizontal average opening of gear doors and the distance travelled between the initial and final position of the gear. To assess the occurrence of a differential bathymetric distribution between the two species, standardized density data were pooled by every 100 m depth interval and represented by box-plotting.

Kolmogorov–Smirnov non-parametric tests were used to evaluate the occurrence of significant depth-related differences in species' size-frequency distribution. An Analysis of Variance (ANOVA) was used to test overall differences in size between the two species. Chi-squared tests were used to analyse sex ratios in relation to depth. Additionally, Mann–Whitney non-parametric tests were used to assess the occurrence of any significant sex dimorphism in size.

Reproductive biology

Sex was determined by macroscopic examination of the gonads. Females of both species were classified into a five-stage maturity scale according to their external appearance, based on currently accepted standardized procedures (Brown-Peterson *et al.*, 2011 modified by Fernandez-Arcaya *et al.*, 2012): I: immature (with undifferentiated gonads); II: regenerating-developing; III: spawning capable; IV: actively spawning; and V: regressing.

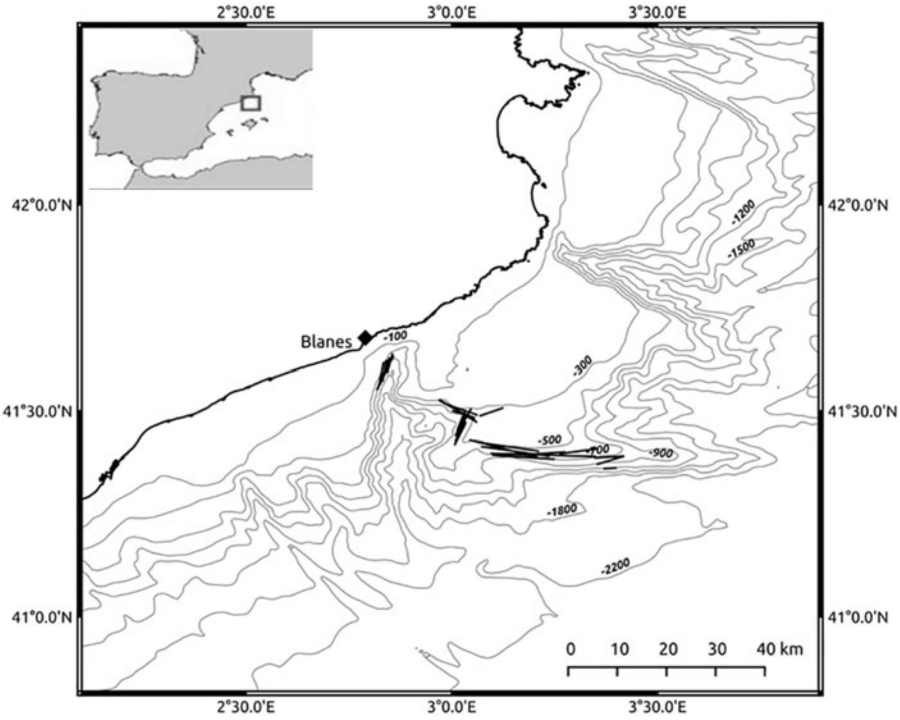

Fig. 1. Study area and bottom trawl fishing stations (lines).

Table 1. Depth range (m) and season for the number of individuals caught, measured, sexed and macro and microscopically analysed of *Symphurus nigrescens* (Sn) and *Symphurus ligulatus* (Sl). SPR, spring; SUM, summer; AUT, autumn; WIN, winter.

	Depth (m)	301–400				401–500				501–600				601–700				801–1200				
	Season	SPR	SUM	AUT	WIN	SPR	SUM	AUT	WIN	SPR	SUM	AUT	WIN	SPR	SUM	AUT	WIN	SPR	SUM	AUT	WIN	Total
Sn	No of Ind.	0	5	239	27	0	33	89	0	27	56	103	27	0	0	0	0	0	0	0	0	606
	Ind. measured	0	5	210	27	0	20	89	0	27	56	103	27	0	0	0	0	0	0	0	0	564
	Ind. sexed	0	5	180	10	0	10	69	0	9	54	82	9	0	0	0	0	0	0	0	0	428
	Females macroscop. analysed	0	5	106	4	0	10	31	0	1	40	55	5	0	0	0	0	0	0	0	0	257
	Females microscop. analysed	0	3	26	2	0	10	0	0	1	13	18	3	0	0	0	0	0	0	0	0	76
Sl	No of Ind.	0	0	10	8	0	6	11	0	92	2	86	107	51	3	15	6	0	8	0	0	405
	Ind. measured	0	0	10	8	0	6	11	0	84	1	77	107	50	3	11	6	0	8	0	0	382
	Ind. sexed	0	0	10	3	0	6	11	0	40	1	68	96	42	3	11	6	0	8	0	0	305
	Females macroscop. analysed	0	0	10	1	0	6	3	0	25	1	39	52	21	3	11	3	0	5	0	0	180
	Females microscop. analysed	0	0	10	1	0	6	0	0	14	1	0	3	13	0	11	1	0	4	0	0	64

The reproductive period in both species was established as the variation in the percentage frequency of the females' maturity stages. The Gonadosomatic Index (GSI) formula was then computed over the year (Anderson & Gutreuter, 1983):

$$GSI = (Wg)/(Wt - Wg) \times 100$$

where, Wg is the gonad weight and Wt is the total gonad-free weight.

In order to validate the visually classified maturity stages, a subsample of the females was dissected for gonad extraction (76 for *S. nigrescens* and 64 for *S. ligulatus*) (Table 1). The gonads were preserved in 10% buffered formaldehyde for histological analysis. The males were not considered in this analysis due to the difficulty in extracting the entire gonad. Samples were dehydrated in graded alcoholic baths, embedded in paraffin wax and sectioned by microtome. The sections of 7 μm were stained with Harris' haematoxylin and eosin. The oocyte developmental stages were described according to Brown-Peterson *et al.* (2011) (modified by Fernandez-Arcaya *et al.*, 2012). The average size range of oocytes was calculated measuring the diameters of 100 oocytes of each oocyte developmental stage from 10 different females using Sigma Scan Pro4. Three mature individuals of each species were also selected to analyse the oocyte size frequency distribution. A transverse histological section from one gonad of each individual was used to measure the diameters of all the types of oocytes present with a visible nucleus using image software Sigma Scan Pro4. Finally the histogram of only one of the selected individuals of each species was included in the study (Figure 7) (169 oocytes for *S. nigrescens* and 335 for *S. ligulatus*).

RESULTS

Population structure characteristics

A total of 606 *Symphurus nigrescens* and 405 *S. ligulatus* were caught in 37 hauls (see Table 1). The analysis of species distribution with depth revealed a differential and only partial overlap between their populations (Figure 2). *Symphurus nigrescens* occurred in high number at the shallowest sampling

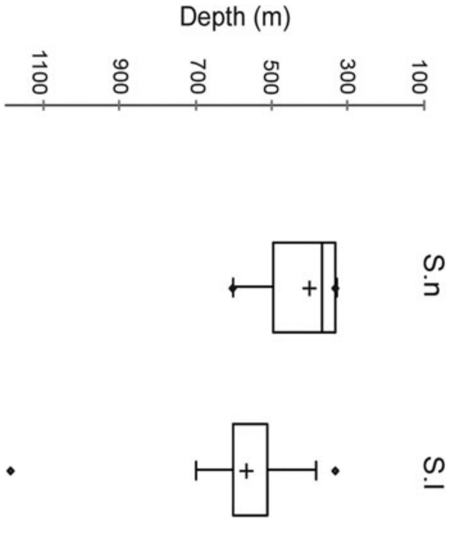

Fig. 2. Box-plot showing the bathymetric distribution of *Symphurus nigrescens* (S.n) and *S. ligulatus* (S.l).

Table 2. Percentage of females and males over total mature individuals by each sampling depth for *Symphurus nigrescens* (Sn) and *S. ligulatus* (Sl)

Depth (m)	301–400		401–500		501–600		601–700		801–1200^a		Total*	
	Sn	Sl*	Sn	Sl	Sn*	Sl*	Sn	Sl*	Sn	Sl*	Sn*	Sl*
Female (%)	58	38	49	47	73	60	0	71	0	60	62	61
Male (%)	42	62	51	53	27	40	0	29	0	40	38	39
No. of individuals	195	13	79	17	154	205	0	62	0	8	428	305

*Significant differences in the sex ratio between females and males (Chi squared, $P < 0.05$).
^{a}The deepest ranges have been grouped because of the low number of individuals.

depth (i.e. between 330 and 420 m), while *S. ligulatus* showed a deeper range, with a maximum density between 500 and 600 m depth. For the latter species few (N = 8) individuals were caught down to 800 m depth.

In both species, the overall sex ratio was significantly different (Chi-squared test: *S. nigrescens* $\chi^2 = 5.76$; *S. ligulatus* $\chi^2 = 4.84$; $P < 0.05$) being females:males ratio of 1:0.6 in both species. Also, in both species, the females were significantly larger than males (Mann–Whitney U-test: *S. nigrescens* $U = 36330.5$, $N_{\text{females}} = 265$, $N_{\text{males}} = 163$; *S. ligulatus* $U = 18464$, $N_{\text{females}} = 184$, $N_{\text{males}} = 121$). When the sex ratio was analysed in relation to depth (Table 2), the results showed that *S. nigrescens* presented a neutral sex ratio at its shallower distribution (i.e. 301–400 m, and 401–500 m) (Chi-squared test: 301–400 m, $\chi^2 = 2.56$; 401–500 m, $\chi^2 = 0.04$; $P > 0.05$), while females dominated in deeper ranges (i.e. 501–600 m) (Chi-squared test: 501–600 m, $\chi^2 = 21.16$; $P < 0.05$). *Symphurus ligulatus* also showed a greater proportion of females at deeper depths (i.e. below 500 m) (Chi-squared test: 501–600 m, $\chi^2 = 4$; 601–700 m, $\chi^2 = 17.64$; 801–1200 m, $\chi^2 = 4$; $P < 0.05$), while males were predominant at shallower depths (i.e. 301–400 m) (Chi-squared test: 301–400 m, $\chi^2 = 4$; $P < 0.05$) (see Table 2).

Both species showed a size increase according to depth with a higher proportion of females (i.e. as the largest individuals) on their lower realms (Figure 3). However, the observed pattern was not statistically significant (Kolmogorov–Smirnov test, $P > 0.05$). The size frequency distributions of *S. ligulatus* between 301–400 m, 401–500 m and below 701 m depth were discarded because of the small sample (N < 20).

The analysis of size frequency distribution through seasons for *S. nigrescens* (Figure 4) showed significant differences in autumn (Kolmogorov–Smirnov; $D = 0.15$; $P < 0.05$), where the smallest individuals were also captured (i.e. 4.5 cm). In spring and winter, in contrast, individuals of intermediate size were observed (i.e. 6.5 cm and 10 cm, respectively). *Symphurus ligulatus* also presented differences in its seasonal size distribution. The higher number of small individuals was captured in winter, while in spring the largest proportion of large individuals was found (see Figure 4). No significant differences in the size-frequency distribution between all seasons except in the individuals captured in summer and winter (Kolmogorov–Smirnov; $D = 0.689$; $P < 0.05$) were found. The size comparative analysis showed an overall larger size for *S. nigrescens* than *S. ligulatus* (ANOVA; $F = 63.9$; $P < 0.005$).

Reproductive biology

Germinal cells showed similar morphology but differed in size for both species. The vitellogenic oocytes of *S. nigrescens* were significantly larger than those of *S. ligulatus* (Mann–Whitney U-test: $U = 4432$, $N_1 = 60$, $N_2 = 103$; $P < 0.001$). The characteristics of the different gonad developmental stages and the range of oocyte sizes for each maturity stage of the two species are shown in Table 3 and Figure 5.

Table 3. Description of macroscopic and microscopic features in relation to the ovary developmental stages in *Symphurus nigrescens* and *S. ligulatus*. AVtg, advance vitellogenic oocyte; EVtg, early vitellogenic oocyte; GVM, germinal vesicle migration; PG, primary growth oocyte; POF, postovulatory follicle; Sn, *Symphurus nigrescens*; Sl, *Symphurus ligulatus* (based on Brown-Peterson *et al.*, 2011 modified by Fernandez-Arcaya *et al.*, 2012).

Stage	Macroscopic features	Microscopic features
II. Regenerating-Developing	Small ovaries, whitish in colour or very pale orange	Presence of oogonia, PG (Sn: 20.07–103.22 µm; Sl: 25.15–85.38 µm) and EVtg (Sn: 91.42–157.17 µm; Sl: 73.52–139.76 µm). Small cells with the nucleus occupying most of the cytoplasm (PG). Cytoplasm increase and appearance of yolky granules in its periphery (EVtg) (Figure 5A). Reduced blood vessels may be present
III. Spawning capable	Ovaries increase in size. Pale orange in colour	Presence of AVtg (Sn: 143.46–217.67 µm; Sl: 115.52–209.29 µm) with big size yolky granules. The other cell types described previously also present (Figure 5B)
IV. Actively spawning	Ovaries reach its maximum size. Orange colour	Presence of GVM (Sn: 193.50–400.31 µm; Sl: 194.92–397.59 µm). Fusion of yolky granules and nucleus migration to the periphery (GVM). All the other cell types described previously also present (Figure 5C)
V. Regressing	Ovary flaccid with pale orange colour. Presence of blood vessels and empty spaces	Characterized by the presence of PG oocytes, recent POF and atresia (Figure 5D). Prominent blood vessels

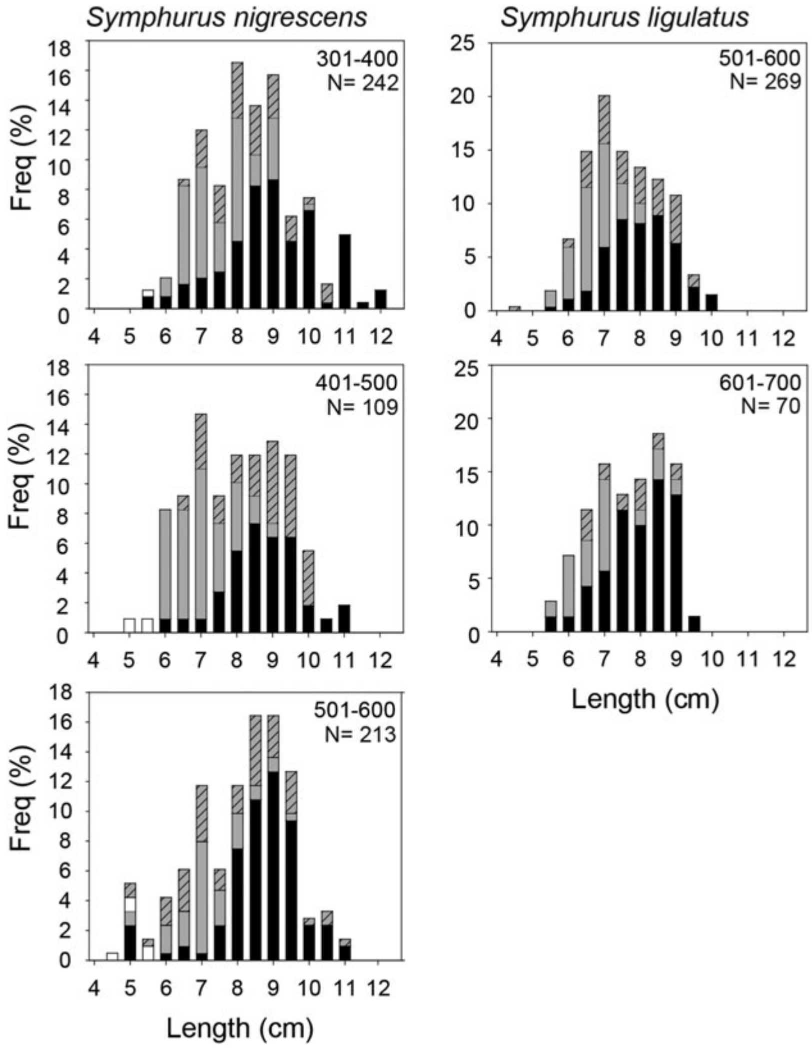

Fig. 3. Total size-frequency distribution histograms at different depth intervals for *Symphurus nigrescens* and *S. ligulatus*. White bars are immature (undifferentiated), grey bars are females, black bars are males and grey bars with lines are sexually undetermined individuals.

Oocytes development in *S. nigrescens* presented a higher seasonality in ovary development than *S. ligulatus* (Figure 6). The former species presented a peak of spawning in summer, when 65.3% of females were found in stage IV (i.e. spawning capable), lasting until the autumn months. In spring and winter, all analysed females were in stage II (i.e. developing gonads). The highest GSI values (4.8%) were found in the summer months, coinciding with the most active spawning period (see Figure 6). *Symphurus ligulatus* showed a semi-continuous reproductive cycle with the presence of females in stages between III and IV (i.e. spawning capable and actively spawning, respectively), starting in spring and lasting until autumn (see Figure 6). However, in winter the large majority of females (94%) were in stage II (developing phase). The GSI data were confirmed by the histological results, which showed a similar trend throughout the year (see Figure 6).

The oocyte size-frequency distribution of *S. nigrescens* and *S. ligulatus* (Figure 7) was continuous in both species according to the criteria of Murua & Saborido-Rey (2003). There was no hiatus between previtellogenic and vitellogenic oocytes, and a random mixture of oocytes at every stage was present in all specimens analysed.

DISCUSSION

This is the first in-depth study of the reproductive biology of *Symphurus nigrescens* and *S. ligulatus*. Both species showed similar reproductive strategies, with asynchronous ovarian organization and similar gonads morphology. However, their depth–size frequency distribution, spawning temporality and oocyte size varied, suggesting that interspecific competition and the different environmental conditions (over their respective depth axes) influence the overall population dynamism.

Both species showed different distribution maxima (i.e. *S. nigrescens* on the upper slope above 400 m and *S. ligulatus* on the middle slope between 500–600 m). Variability in environmental factors related to depth, sediment type and diverse feeding habits may account for such a difference (Massutí *et al.*, 1995). *Symphurus nigrescens* and *S. ligulatus* apparently bury themselves partially within the sediment, both moving onto it in search of a common and major item for their diet, the decapod *Calocaris macandreae* (Macpherson, 1978; Cau & Deiana, 1979). The inter-specific competition over space use and associated trophic resources may account for a process of bathymetric segregation in

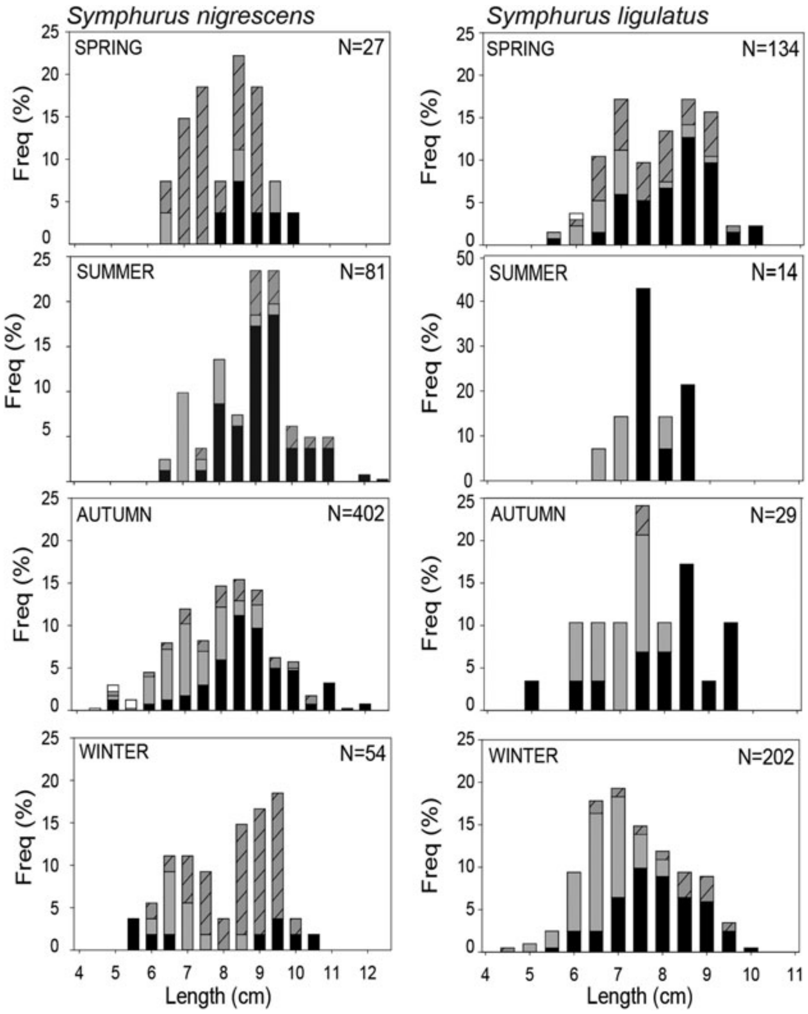

Fig. 4. Total size-frequency distribution by season for *Symphurus nigrescens* and *S. ligulatus*. White bars are immature (undifferentiated), grey bars are females, black bars are males and grey bars with lines are sexually undetermined individuals.

these two ecologically related species, as has been already reported in other local strictly demersal Monkfish *Lophius budegassa* and *L. piscatorius* (Colmenero *et al.*, 2010), other Macrourids (Carrasón & Matallanas, 2002; Fernandez-Arcaya *et al.*, 2013b), as well as in crustacean decapods (Aguzzi *et al.*, 2009).

In this study we observed an increase in size for both species according to the sampling depth, confirming the general 'bigger–deeper' trend phenomenon of continental margin megafauna (Polloni *et al.*, 1979). The observed size–depth related pattern could be attributable to a depth partitioning between sexes. Sex ratio showed a higher proportion of females at greater depths, which have also a larger size than males. Similar results have been found in other species distributed on the NW Mediterranean continental margin (*Trachyrincus scabrus*, Fernandez-Arcaya *et al.*, 2012; *Nezumia aequalis*, Fernandez-Arcaya *et al.*, 2013b). The presence of larger females during their spawning period at deeper depths (i.e. summer-autumn in *S. nigrescens* and from spring to summer in *S. ligulatus*) suggest that both Cynoglossids might undertake migrations toward deeper areas when spawning. Seasonal modifications in fish species distributions seem to commonly occur in this area (Aguzzi *et al.*, 2013a) and have been associated to the seasonal modulation of behaviour over reproductive cycles (Aguzzi & Company, 2010; Aguzzi *et al.*, 2011).

The shallower-dwelling *S. nigrescens* showed a stronger seasonal reproductive pattern than *S. ligulatus*. The spawning females of *S. nigrescens* were concentrated mainly in the summer months, coinciding with other observations of this species in other Mediterranean areas (Sabatés & Maso, 1992; Sabatés & Olivar, 1996; Somarakis *et al.*, 2011) and with the reported overall spawning seasonal pattern of the Cynoglossidae family (Gibson, 2005). The reproductive activity of the shallowest distributed species, *S. nigrescens*, could be strongly affected by the spring-summer phytoplankton blooms (Aguzzi *et al.*, 2013a, b). In contrast, *S. ligulatus* showed a prolonged spawning period, ranging from spring to autumn, and coinciding with the general trend of continuous reproductive cycle found for several species inhabiting between 400–800 m depth in the same study area (Fernandez-Arcaya *et al.*, 2013a). Reproductive characteristics have evolved in response to environmental conditions. However, the factors and processes that ultimately control and shape the reproductive timing of flatfish are still being discussed (Gibson, 2005).

Related to the ovarian organization, it appears to be asynchronous for both species as shown by the oocyte-size

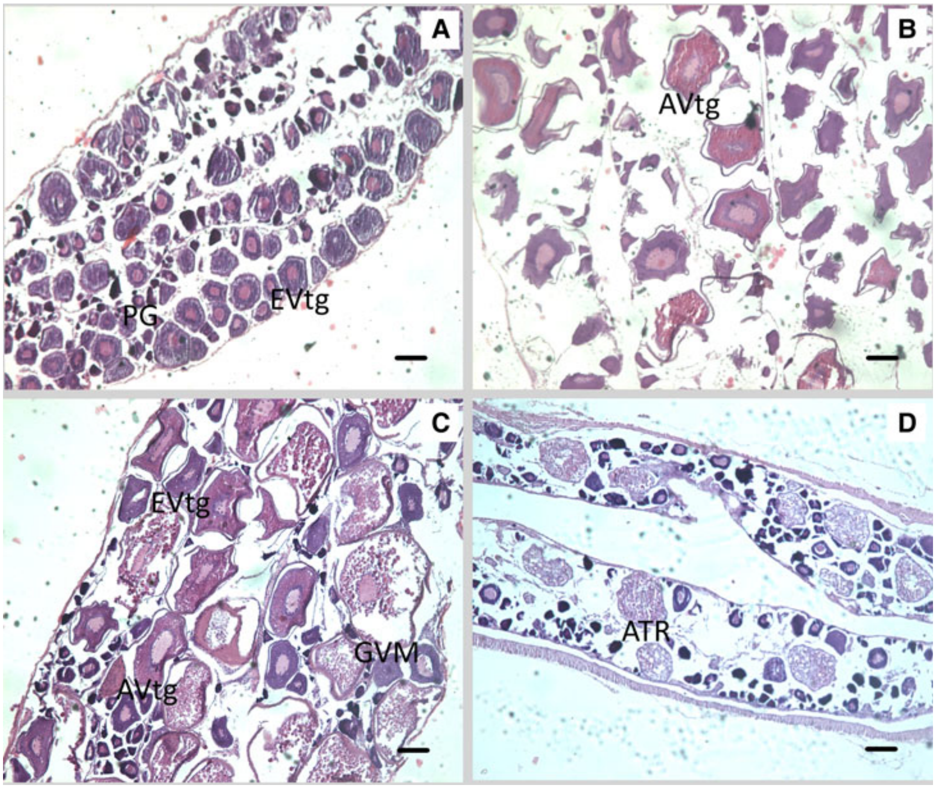

Fig. 5. Histological sections of ovaries of *Symphurus nigrescens* (A–B) and *S. ligulatus* (C–D) at different stages. (A): stage II, developing-regenerating; (B): stage III, spawning capable; (C): stage IV, actively spawning; (D): stage V, regressing. ATR, atretic follicle; AVtg, advanced vitellogenic oocyte; EVtg, early vitellogenic oocyte; GVM, germinal vesicle migration; PG, primary growth oocyte (based on Brown-Peterson *et al*., 2011 modified by Fernandez-Arcaya *et al*., 2012). Scale bars = 100 μm.

distribution, which is characterized by a random mixture of oocytes at every stage of development. A clear separated stock of oocytes between advanced yolked oocytes and hydrated oocytes occurs only at hydration (Murua & Saborido-Rey, 2003). In addition, even though they are not very apparent, we observed two slightly discrete modes of oocyte sizes in both species between early vitellogenic and advanced vitellogenic oocytes. However a deeper analysis of the oocyte size frequency distribution should be conducted in order to confirm these observations. If the presence of these two modes was confirmed, together with the observed regressing phase from autumn to spring in *S. nigrescens* and in spring in *S. ligulatus*, it would suggest that both species have some kind of seasonal modulation in the production of their oocytes. Such a result suggests that during the females' maturation process a continuous recruitment of previtellogenic oocytes into a 'vitellogenic oocyte clutch' might occur. However, that recruitment process seems to cease after the spawning season. Asynchronous ovarian organization has been related to income-breeding species, the energy for reproduction of which is financed by the available energy (Stearns, 1992). The peak of reproductive activity of *S. nigrescens* and *S. ligulatus* is linked to the greater availability of *C. macandreae* in summer (Cartes & Maynou, 1994). Our results suggest that both species might use the demographic increase of *Calocaris* as a timing cue to set their reproduction synchronously, in a way to successfully satisfy the energy requirements for their reproduction.

The spawning of small hydrated oocytes and the presence of an oil drop in hydrated oocytes (not quantified here) suggests that *S. ligulatus* and *S. nigrescens* produce pelagic eggs, which are the most common type of eggs in flatfishes. Benthic eggs would otherwise have been larger in these deep-water species due to the usually low temperature and the poor food availability of their environment (Gage & Tyler, 1992). Flatfish are not an exception and in a comparative analyses conducted among species of this group, an increase in egg size with depth has been observed (Gibson, 2005). However, the species of the present study did not follow a similar depth-driven pattern in their size. Although we could not measure hydrated eggs, the deeper dwelling species, *S. ligulatus*, showed smaller oocyte size than the shallower species, *S. nigrescens*. The larger size of the shallower species *S. nigrescens* and the higher food availability at shallower depths might encourage the development of larger oocytes.

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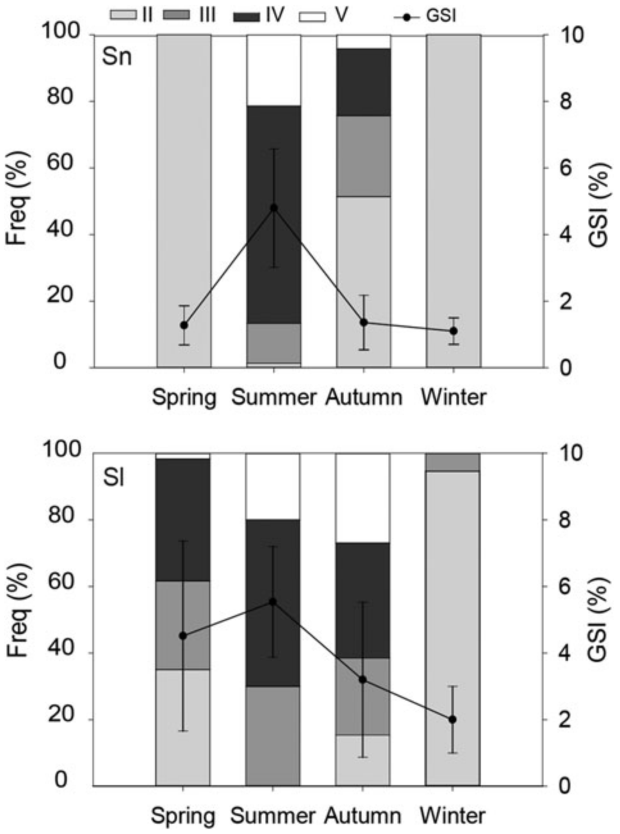

Fig. 6. Ovary maturity stages and gonadosomatic index (GSI) by season for *Symphurus nigrescens* (Sn) and *S. ligulatus* (Sl). II–VI: developing-regenerating stage; III: spawning capable stage, IV: actively spawning stage, V: regressing stage.

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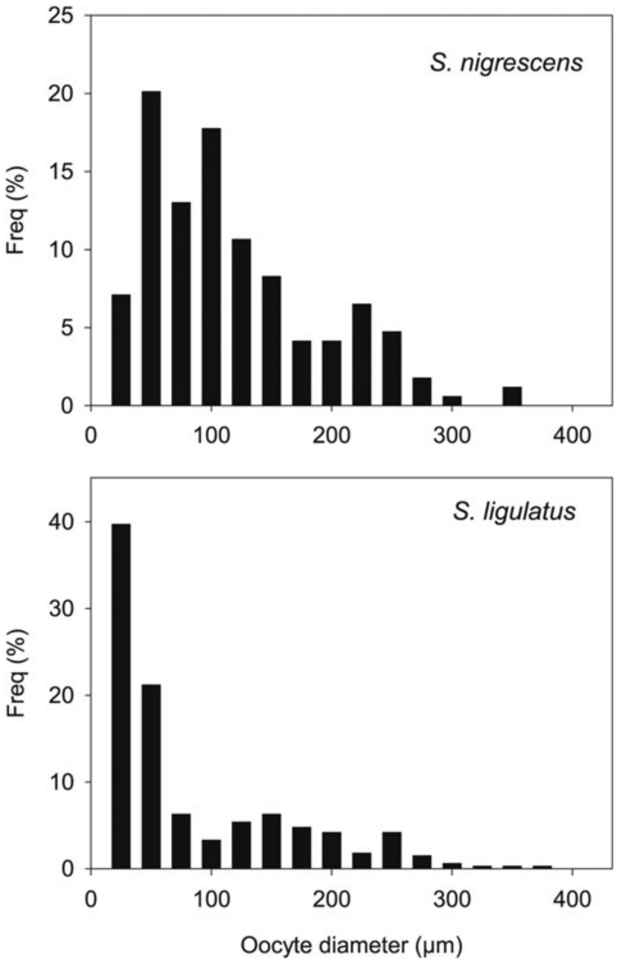

Fig. 7. Oocyte-size frequency histograms for *Symphurus nigrescens* and *S. ligulatus* on one spawning capable individual (stage IV).

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