BIOLOGICAL SAMPLES COLLECTION FOR GROWTH AND MATURITY STUDIES

EU PORTUGAL AND SPAIN: NORTHEASTERN ATLANTIC AND WESTERN MEDITERRANEAN

ICCAT SMALL TUNAS YEAR RESEARCH PROGRAM

DRAFT REPORT

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SAMPLING

Outline

The contract was signed the 4th of May 2017, one month later than expected; in order to meet the agenda submitted in the proposal, sampling of small tuna (SMT) started before the contract was finalised. Biological sampling of *Auxis rochei* (Bullet tuna, BLT), *Sarda sarda* (Atlantic bonito, BON) and *Euthynnus alletteratus* (Little tunny, LTA) for maturity studies, including the photographs of gonads to develop the Living Working Document, has been undertaken in the Atlantic Ocean (south of Portugal and Spain) and in the Western Mediterranean Sea (Mediterranean Spanish coast) between April and October 2017.

The sampling and lab work undertaken in 2017 included: a total of 267 fish sampled in the Atlantic and 500 fish sampled in the Mediterranean; 360 fresh gonads (Atlantic and Mediterranean) photographed, of which 320 gonad tissues were histologically processed for microscopic examination.

As set out in the proposal submitted on 10th March 2017, a workshop was organized by the authors of the present report at IPMA Olhão (Portugal) from 12th to 14th July 2017, to standardise both the sampling methodology and the macroscopic maturity staging criteria. Six people, four from IPMA and two from IEO attended this workshop. The gonad samples collected in Olhão were transported to the IEO laboratory for further processing.

The Large Pelagic Group of the Instituto Español de Oceanografía (IEO - Málaga Centre) has been routinely collecting samples of the three species mentioned above (BON, BLT and LTA) in the Mediterranean Spanish coast since 2003 and these were included in the study of microscopic maturity staging. The authors considered that the fish sampled in 2017 (until November) would improve the estimation of the length at 50% maturity; therefore those data were also included in the analyses.

MATERIAL AND METHODS

Sampling collection

Fresh individuals of *Auxis rochei* (Bullet tuna, BLT), *Sarda sarda* (Atlantic bonito, BON) and *Euthynnus alletteratus* (Little tunny, LTA) were collected from 38 localities (corresponding to 23 grids 1x1) throughout the Atlantic Ocean and Mediterranean Sea between 2003 and 2017 (**Figure 1**).

Individuals BLT, BON and LTA were caught in the Mediterranean Sea (Spanish coasts) and the Atlantic Ocean (south of the Iberian Peninsula) in a variety of fishing gears (**Table 1**). In decreasing order according to the number of samples collected: trap fisheries (TRAP); purse seine (PS) (targeting coastal species, mainly sardine and anchovy); recreational fisheries (TROL); longline fisheries (mainly targeting *Thunnus alalunga* (LLALB)), and trammel net (TN) targeting small coastal species. On the Portuguese coast individuals were caught by TRAP.

Fork length (FL) was measured either to the nearest 0.1 cm or to the nearest 0.5 cm (fish measured on longline vessels). Total body weight (W) was measured to the nearest gram. A total of 4130 individuals (1281 BLT; 1583 BON; 1266 LTA) were both measured and weighted. The length-weight relationships for BLT, BON and LTA were calculated using a two-parameter power function with a multiplicative error term:

$$W_i = \alpha L_i^{\beta} e^{\epsilon_i}$$

The model is linearised by means of a logarithmic transformation, which has the added benefit of making the errors additive and stabilizing the variances about the model. Simple linear regression methods are used to fit the relationship between $\log_e(W)$ and $\log_e(L)$.

$$log_e(W_i) = log_e(\alpha) + \beta \log(L_i) + \epsilon_i$$

From 2003 to 2017 a total of 3929 measured fish (BLT, 1280; BON, 1388; LTA, 1261) were sampled to identify their sex: male, female, undetermined (individuals with small gonads, which sex cannot be identified visually) and intersex (gonads presented both

testicular and ovarian tissue). A total of 767 undetermined sexed individuals were classified as immature. A subset of 39 gonads of undetermined individuals were histologically processed in order to determine their sex. Two intersex fish were sampled (Macías et al., 2014). Given that intersexual fish did not form part of the study aim, these results are not discussed further. Sex ratio was calculated as the proportion of females by length class.

Maturity stages of gonads (visual stating method) were assigned using similar criteria to those developed for *Sarda sarda*, *Euthynnus alletteratus* and *Scomberomorus tritor* Diouf (1981). The five macroscopic stages are: (1) Immature (virgin); (2) Developing; (3) Spawning capable; (4) Spawning; (5) Regressing and Regenerating (see description of stages in T**able 2**). Some doubts came up when staging and so intermediate stages were assigned. These intermediate stages: immature or developing (1−2) and immature or regressing/regenerating (1−5) were not included in the analysis of the length at 50% maturity (L_{50}). See the submitted *living working document* for small tuna maturity staging with a large amount of detailed photos (macro and micrographs).

Weights of the fresh gonads (GW) were recorded to the nearest 0.01 gram. A 2−3 cm cross-section from the central part of the right or left lobe was cut and fixed in Bouin's fluid for four hours, and then preserved in 70% ethanol. For small gonads, the whole gonad was preserved.

Gonadosomatic index (GSI) was calculated for males and females to identify the spawning periods according to the equation:

$$GSI = GW / (W - GW) \times 100$$

That is, the GSI was calculated from the ratio of gonad weight (GW) to fish gonad-free weight (W - GW) times 10^2; GW and W, both in grams.

Histological processing and microscopic maturity staging of gonads

A representative portion of the preserved gonad tissue was dehydrated in ascending concentrations of ethanol, cleared with n-butanol, and embedded in paraffin. Sections were cut at 10 μm and stained with Mallory's trichrome stain (see **ANNEX I** for protocol details).

Microscopic maturity stating of *Auxis rochei*, *Sarda sarda* and *Euthynnus alletteratus* gonads was based on a modification of the criteria of Schaefer (1998) and Farley *et al*. (2013).

Females: the most advanced group of oocytes (MAGO) was determined for each ovary; primary growth, cortical alveolar (lipid-stage), early vitellogenic, mid vitellogenic, advanced vitellogenic, migratory nucleus and hydrated oocyte stages (see description of oocyte stages in **Table 3**). Ovarian stages were assigned based on the developmental stage of the MAGO, the presence/absence of postovulatory follicles (POFs), the level of alpha and beta atresia of vitellogenic oocytes, the presence/absence of late stages of atresia (gamma/delta) and, the observation of the thickness of the *tunica albuginea* (gonad wall). Six ovarian stages were defined: (1) immature (virgin); (2) Developing which included two substages (2a) LP- Developing, if cortical alveolar oocytes as the MAGO are present and (2b) Developing, when early vitellogenic oocytes as the MAGO are present; (3) Spawning capable; (4) Spawning; (5) Regressing and regenerating (postspawning), with two substages, (5a) Regressing and (5b) regenerating and finally, the stage (6) Abnormal (**Table 4**).

Males: testes were classified according to the most advanced stage of testicular development with respect to sperm maturation. The stages which were differentiated and recorded as to their relative abundance within the cysts were: spermatogonia, spermatocytes, spermatids, and spermatozoa. The presence or absence of spermatozoa within seminiferous tubules and the relative amount of sperm within the central longitudinal sperm duct (vas deferens) were also examined (**Table 5**).

See the submitted *living working document* for small tuna maturity staging with a large amount of detailed photos (macro and micrographs).

Length at 50% maturity

Macroscopic maturity data of both female and male and by sex were used to estimate the length at 50% maturity (L_{50}) (i.e. the length at which 50% of individuals are mature) for each species (BLT, BON, LTA). Fish were classified as either immature or mature (see **Table 2**).

Additionally, L_{50} was estimated using the microscopic maturity data of female of the three species. Fish were classified as either immature or mature depending on the criteria considered. In tuna studies immature females are those with previtellogenic (primary growth and cortical alveolar oocytes) or early vitellogenic oocytes (Schaefer, 1998; Farley et al., 2013), that is, stages 1, 2a and 2b. In contrast, in other fish studies, females whose ovaries contained cortical alveolar oocytes as the MAGO, are considered to be mature (Brown-Peterson et al., 2011; ICES, 2014), i.e only ovaries at stage 1 are considered immature. Therefore in order to assess possible implications in the future assessment, in the present report three calculation of L_{50}, based on microscopic examination of ovaries, were estimated on the basis of the criteria given in **Table 6**.

L_{50} was calculated by fitting the proportion of mature females by 2-cm size classes to a logistic equation, assuming a binomial error distribution, to model the probability of maturity (*p*) by length.

Since the relationship is not linear (primarily due to the constraint that the probability is between 0 and 1), the logistic regression approach transforms *p* to obtain a linear equation. The required transformation is the *logit* transformation. From this transformation a linear model is obtained:

$$log_e\left(\frac{p}{1-p}\right) = \alpha + \beta\, FL$$

Confidence intervals for the parameters of the logistic regression were estimated via bootstrapping. A thousand bootstrap samples (with replacement) were used to derive the sampling distribution of the statistic of interest by means of the bootstrap percentile interval (the empirical quantiles are used to form a confidence interval for the parameter of interest). The 95% bootstrap confidence interval for a parameter was based on the values of the parameter estimate that had ±2.5% of bootstrap sample values (i.e., the 2.5% and 97.5% quantiles of the parameter estimates).

Age at 50% maturity

The age at first maturity (A_{50}) was estimated using size-age relationships for the three species (BLT, BON, LTA) from the literature review (Rey et al., 1986 in N'Guessan et al., 2015; Santamaria et al., 1998 in N'Guessan et al., 2015; Bök and Oray, 2001; Kahraman et Oray, 2001; Zaboukas and Megalofonou, 2007; Valeiras et al., 2008; Hattour, 2009; Plandri et al., 2009; Kahraman et al., 2011; Kahraman et al., 2014 The growth parameters obtained in those studies are shown in (**Table 7**).

RESULTS and DISCUSSION

Length-Weight relationship

Length–weight relationships ($W = aL^b$) were developed for *Auxis rochei* (BLT), *Sarda sarda* (BON) and *Euthynnus alletteratus* (LTA). The sample size, the range of fork lengths and weights, on which the L-W relationships are based, the estimated parameters (a and b) and R^2 values are presented in **Table 8** and **Figures 2−4**.The relationships between the two variables were significant ($p < 0.001$). The slope (allometry coefficient) for each species was higher than 3, indicating a positive allometric growth, that is, fish became more rotund as length increases. Furthermore, the parameters of the relationships estimated in the present study are consistent with those estimated in other Mediterranean areas (see N'Guessan et al., 2015 and references therein).

Sex ratio

Auxis rochei

The size of males ranged between 26.0 and 48.7 cm FL (n = 540) and females ranged between 24.0 and 47.5 cm FL (n = 554). Significant differences in the proportion of females were found between length classes ($P = 0.02$). The analysis showed that males

were more abundant in the length classes greater than 42 cm FL. The sex ratio was 1:1 in the length class group between 34 and 36 cm FL. Females predominated in length class less than 26 cm FL; however this result should be considered with caution due to the fact that ovaries are easier identified than testes when gonads are small. Predicted sex-ratio (% females) by length (FL, cm) for BLT is shown in **Figure 5**.

Sarda sarda

The size of males ranged between 25.6 and 79.3 cm FL (n = 520) and females ranged between 23.9 and 71.0 cm FL (n = 671). Significant differences in the proportion of females were found between length classes (P = 0.02). The analysis showed that females predominated in almost all of the length class groups. The sex ratio was 1:1 in the length class group between 40 and 42 cm FL. Predicted sex-ratio (% females) by length (FL, cm) for BON is shown in **Figure 6**.

Euthynnus alletteratus

The size of males ranged between 30.6 and 101.0 cm FL (n = 414) and females ranged between 28.5 and 99.0 cm FL (n = 461). Significant differences in the proportion of females were found between length classes (p <0.001). The analysis showed that males were more abundant in the larger length classes (> 96 cm FL). Predicted sex-ratio (% females) by length (FL, cm) for LTA is shown in **Figure 7**.

Maturity classification of gonads

A total of 3808 individuals measured (FL, cm) were macroscopically classified. By species: BLT, 1200 fish; BON, 1377 fish; LTA, 1231.

A total of 1509 fish (40%) were immature (stage 1); 2227 fish (58%) were mature (stages 2 – 5); and 73 fish (2%) were classified as immature or mature (i.e. immature or developing (1−2) and immature or regressing/regenerating (1−5)), which were not included in the analysis of the length at 50% maturity (L_{50}). A total of 54 gonads out of the 73 fish were further histologically processed in order to determine the maturity status.

Microscopic examination of the gonads

A total of 39 gonads of undetermined individuals were histologically processed in order to determinate their sex, 29 of them were successfully identified as male or female. Histological analysis showed that 20 fish were incorrectly sexed. After microscopic examination of the 54 gonads macroscopically classified as immature or mature (1−2 and 1−5) it was determined that 24 of them were immature, 27 were mature and 3 remained uncertain.

A total of 1008 gonads were microscopically examined, including the 3 gonads which reproductive status remains uncertain and the 4 gonads at abnormal stage (see the description in Tables 4 and 5). The number of females and males by microscopic maturity stages for each species (BLT, BON; LTA) is given in **Table 9**.

Spawning season

The gonadosomatic index (GSI) values were calculated monthly for both mature males and females by stock (Atlantic and Mediterranean) for each species.

Auxis rochei

The mean monthly variation in mean GSI of mature males and females in the Mediterranean Sea indicated that fish spawn between May and August, and peak in June and July, decreasing from September to November (**Figure 8**). The monthly pattern in frequency of gonad stages for mature females was consistent with the GSI data, spawning and spawning capable were sampled predominantly in June and July (**Figure 9**)

Similarly, the mean monthly GSI values in the Atlantic waters showed that spawning occurs from May to August, and declined in September. Unfortunately there was not data available in July (**Figure 8**). Spawning BLT was sampled predominantly in May and August (**Figure 9**).

Sarda sarda

The monthly variation in mean GSI values of mature males and females in the Mediterranean Sea indicates that fish spawn between April and July, and declined from September to October; unfortunately any fish was sampled in August (**Figure 10**). The monthly pattern in frequency of gonad stages for mature females was consistent with the GSI data, spawning capable females were sampled from April to July, and spawning females predominated in June and July (**Figure 11**).

The estimated GSI values of BON from the Atlantic waters were smaller than those calculated for BON in the Mediterranean; GSI values greater than 2.0 GSI were only observed in May (**Figure 10**). The monthly pattern in frequency of gonad stages for mature females showed that spawning capable ovaries were sampled from April to June and spawning in May and June (**Figure 11**). GSI values calculated for females in June, July and August were close (≈0.85); however the histological analysis of ovaries showed that in August regressing ovaries predominated.

Euthynnus alletteratus

The monthly variation in mean GSI values of mature males and females in the Mediterranean Sea suggest that the spawning season for LTA is between June and August, with the highest values in July. Mean GSY values decreased sharply from September to December (**Figure 12**). Histological analysis of ovaries confirmed that spawning activity occurred from June to August (**Figure 13**).

Few samples of LTA in the Atlantic were collected; therefore spawning season for this species cannot be assessed (**Figures 12 and 13**).

In summary:

BLT: histological analysis of the ovaries and GSI values (for males and females) suggested that the spawning season for *Auxis rochei* in the western Mediterranean Sea and in the Northeastern Atlantic (south of the Iberian Peninsula) extends from May to August.

BON: histological analysis of the ovaries and GSI values (for males and females) suggested that the spawning season for *Sarda sarda* in the western Mediterranean Sea takes place from April to July (note that no samples were collected in August) and, from April to June in the Northeastern Atlantic (south of the Iberian Peninsula).

LTA: histological analysis of the ovaries and GSI values (for males and females) suggested that the spawning season for *Euthynnus alletteratus* in the western Mediterranean Sea extends from June to August.

Our results for the three species are in line with other studies conducted in the Mediterranean Sea in the Gibraltar Strait (Rodríguez-Roda, 1966; Hajjej et al., 2010; Kahraman et al., 2008; Kahraman et al., 2010; Kahraman et al., 2014).

Length at 50% maturity

The estimated length at which 50% of *Auxis rochei*, *Sarda sarda* and *Euthynnus alletteratus* is mature is showed in **Table 10**.

Auxis rochei

A total of 1165 BLT was macroscopically classified, 40% was immature and 60% mature. The estimated length at which 50% of BLT (both sexes) is mature (L_{50}) was 34.81 cm FL (*34.36 cm -35.21 cm*, 95% confidence interval). The estimated length at which 90% BLT is mature (L_{90}) was 40.19 cm FL (*39.53 cm -40.91 cm*, 95% confidence interval). Logistic fit for the proportion of mature BLT is shown in **Figure 14**.

Significant differences in the logistic regression models between sexes were found (p <0.001). The estimated L_{50} were 34.12 cm FL (*33.44 cm -34.66 cm*, 95% confidence interval) for females and 35.43 cm FL (*34.57 cm - 36.17 cm*, 95% confidence interval) for males. The estimated L_{90} were 37.94 cm FL (*37.25 cm - 38.65 cm*, 95% confidence interval) and 42.86 cm FL (*41.47 cm - 44.54 cm*, 95% confidence interval) for female and male, respectively. Results showed that BLT males matured at smaller sizes than females. Logistic fit for the proportion of mature BLT by sex is shown in **Figure 15**.

The histological analysis of BLT ovaries showed that 36 (18%) were at stage 1 (immature), 62 (32%) at stage 2a (LP-developing), 23 (12%) at stage 2b (developing)

and, 75 (38%) at stages 3-5b (mature individuals in all of the three criteria considered). Logistic regression models are parallel (p = 0.435) with equal intercepts (p = 0.784), which indicates that there is no significant differences in the logistic regressions among maturity scenarios, the calculations that follow are for the shake of completeness:

L_{50} was estimated to be 29.25 cm FL (*12.75 cm - 32.93 cm*, 95% confidence interval) when females with ovaries at stage 2a (ovaries containing cortical alveolar as MAGO) and onward were considered mature. This estimate increased to 38.17 (*35.45 cm - 40.90 cm*, 95% confidence interval) when the second, and 44.31 cm FL (*5.67 cm - 91.41 cm*, 95% confidence interval) when the third, criteria were applied (i.e., when the maturity threshold was defined as at stages 2b and 3, respectively) **Figure 16**.

Sarda sarda

A total of 1362 BON was macroscopically classified, 34% was immature and 66% mature. The estimated length at which 50% of BON (both sexes) is mature was 39.93 cm FL (*39.64 cm - 40.21cm*, 95% confidence interval). The estimated length at which 90% of BLT is mature was 42.28 cm FL (*41.80 cm - 42.72 cm*, 95% confidence interval). Logistic fit for the proportion of mature BON is shown in **Figure 17**.

No significant differences in the logistic regression models for L_{50} and L_{90} between sexes were found (p = 0.971).

The histological analysis of BON showed that 53 ovaries (14%) were at stage 1 (immature), 79 (20%) at stage 2a (LP-developing), 46 (12%) at stage 2b (developing) and, 208 (54%) at stages 3-5b (mature individuals in all of the three criteria considered). Logistic regression models show different slopes (p <0.001) with different intercepts (p <0.001), which indicates that there is significant difference in the logistic regressions among microscopic maturity scenarios. L_{50} was estimated to be 40.60 cm FL when females with ovaries at stage 2a (ovaries containing cortical alveolar as MAGO) and onward were considered mature. This estimate increased to 43.14 when the second, and 45.30 cm FL when the third, criteria were applied (i.e., when the maturity threshold was defined as at stages 2b and 3, respectively) (**Figure 18**).

Euthynnus alletteratus

A total of 1209 LTA was macroscopically classified, 48% was immature and 52% mature. The estimated length at which 50% of LTA is mature was 51.13 cm FL (*50.07 cm - 52.20 cm*, 95% confidence interval). The estimated length at which 90% of LTA is mature was 65.23 cm FL (*63.11 cm - 67.26 cm*, 95% confidence interval). Logistic fit for the proportion of mature LTA is shown in **Figure 19**.

Significant differences in the logistic regressions between sexes were found ($p = 0.002$). The estimated L_{50} were 50.07 cm FL (*48.31 cm - 51.64* cm, 95% confidence interval) for females and 43.44 cm FL (*36.89 cm - 47.34* cm, 95% confidence interval) for males. The estimated L_{90} were 65.24 cm FL (*62.16 cm - 68.65 cm*, 95% confidence interval) and 69.70 cm FL (*65.30 cm - 75.34* cm, 95% confidence interval) for female and male, respectively. These results show that LTA males reached the size at first maturity at smaller sizes than females; however the length at which 90% of LTA matured is smaller for females.

Logistic fit for the proportion of mature LTA by sex is shown in **Figure 20**.

The histological analysis of LTA ovaries showed that 69 (36%) were at stage 1 (immature), 53 (28%) at stage 2a (LP-developing), 30 (8%) at stage 2b (developing) and, 39 (20%) at stages 3-5b (mature individuals in all of the three criteria considered). Logistic regression models show different slopes ($p = 0.020$) with equal intercepts ($p = 0.395$), which indicates that there is significant difference in the logistic regressions among microscopic maturity scenarios. L_{50} was estimated to be 53.19 cm FL when females with ovaries at stage 2a (ovaries containing cortical alveolar as MAGO) and onward were considered mature. This estimate noticeable increased to 68.74 when the second, and 83.33 cm FL when the third, criteria were applied (i.e., when the maturity threshold was defined as at stages 2b and 3, respectively) (**Figure 21**).

Age at 50% maturity

The age at first maturity (A_{50}) was estimated using size-age relationships for the three species (BLT, BON, LTA) from the literature review (**Table 7**). Resultant ages at 50% maturity are shown in **Tables 11-13**. From our point of view, the growth parameters obtained by Kahraman et al., 2011 for BLT, Kahraman et al., 2014 for BON and, Hattour, 2009 for LTA, are in line with the knowledge about the reproductive biology of the species, that is, BLT would reach maturity at 1 year, BON at 1 year, and LTA at 2 year.

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OUTLOOK

The authors of the present work suggest intensifying the sampling of small tuna, mainly of *Euthynnus alletteratus*, across the Mediterranean Sea and Atlantic Ocean in order to collect enough data for the estimation of maturity and growth parameters and other reproductive traits.

Macroscopic stages of gonadal development are an essential feature in fish stock assessment in order to estimate the maturity ogive and spawning-stock biomass (SSB). The limits of the maturity stages are difficult to identify. Difficulties and doubts not only arise when assigning macroscopic stages, but also when assigning microscopic ones. In September 2017 the first author of the present report participated in the "Workshop on Sexual Maturity staging from histological tools (WKMATHIS)" organised by the International Council for the Exploration of the Sea (ICES). In this Workshop it was observed that maturity staging assigned by different laboratories can often result in significant bias and therefore, a workshop focussed on tuna species is recommended.

The objective of the submitted *living working document* is to create a living bank of macroscopical and histological images in order to facilitate the interpretation of small tunas (SMT) reproductive status, as well as to promote agreement on common criteria. This *living working document* should be a dynamic document, continually updated with new images and information from those laboratories interested in participating. In addition it would be beneficial for a *Workshop* to be undertaken in order to make a general review of the macroscopic maturity criteria and histological studies applied to macroscopic stages, compile international agreed macroscopic and histological descriptions for the different maturity stages, compile an overview of available histological information and to identify the need for further studies of histological tools to validate the macroscopic stages of gonadal development for the different target species of the ICCAT.

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TABLES

Table 1. Number of individuals (BLT, BON and LTA) collected between 2003 and 2017 in the Atlantic Ocean and Mediterranean Sea by gears (sorted in decreasing order). Trap fisheries (TRAP), purse seine (PS), targeting coast species mainly sardine and anchovy, recreational fisheries (TROL), longline targeting *Thunnus alalunga* (LLALB), trammel net (TN) targeting small coast species, longline home-based (LLHB), longline "Stone-ball" (LLPB), bottom or deep longliners (BLL).

	Atlantic Ocean			Mediterranean Sea				
Gear	BLT	BON	LTA	BLT	BON	LTA	Total (%)	
TRAP	229	104	53	760	813	684	2643	(63.49)
PS				195	558	84	837	(20.11)
TROL	19	25	3	78	59	335	519	(12.47)
LLALB					8	93	101	(2.43)
TN					19	11	30	(0.72)
LLHB				2		26	28	(0.67)
BLL					2	2	4	(0.10)
LLPB				1			1	(0.02)
Total	248	129	56	1036	1459	1235	4163	

T**able 2**. Maturity stages of gonads (visual stating method). Based on the criteria of Diouf (1981) (Collect. Vol. Sci. Pap. ICCAT, 15 (2): 327–336).

Sex / Reproductive stages	**Macroscopic criteria**	**Photo**
Undetermined: Immature (virgin)	Gonads are very small (thread-like). Sex not distinguished by naked eye.	
Females		
1. Immature (virgin)	Gonads are small and cylindrical in shape; more or less translucent-pinkish.	
2. Developing (early developing)	Gonads are increasing in size; orange, pink or reddish colour. External blood vessels start to develop around the gonads (vascularisation).	
3. Spawning capable (late developing)	Gonads are well developed; yellow - orange colour. Opaque oocytes are visible.	
4. Spawning	Gonads are greatly enlarged; orange – reddish colour, with conspicuous superficial blood vessels. Large translucent hydrated oocytes visible if those are presented.	
5. Regressing and regenerating (postspawning)	Gonads are bloody and flaccid, show a wrinkled wall; reddish colour.	

Sex / Reproductive stages	Macroscopic criteria	Photo
Males		
1. Immature (virgin)	Gonads are small, thin and flattened; more or less translucent – lightly pink.	
2. Developing (early developing)	Gonads are increasing in size and triangular in cross section; orange, whitish - pinkish colour.	
3. Spawning capable (late developing)	Gonads are well developed; whitish - pinkish colour. Accumulation of sperm in the spermatic ducts, under pressure sperm is expelled.	
4. Spawning	Gonads are greatly enlarged with conspicuous superficial blood vessels; pinkish colour. Large amount of sperm, under very lightly pressure sperm is expelled.	
5. Regressing and regenerating (postspawning)	Gonads are bloody and flaccid, show a wrinkled wall; reddish colour.	

NOTE: in Diouf (1981) stage II refers to Developing or Regenerating stages. Sometimes, at the early beginning of the spawning season, these mentioned stages are difficult to distinguish in individuals that spawned in the previous spawning season.

Table 3. Description of the oocyte stages in *Auxis rochei*, *Sarda sarda* and *Euthynnus alletteratus* ovaries according to their histological characteristics

Stage oocytes	MAGO. Histological characteristics
*Primary growth oocytes (PG)	In an early phase: homogeneous basophilic cytoplasm with no cytoplasmic inclusions.
*Cortical alveolar (CA) or lipid-stage oocyte (LP)	Small lipid droplets in the cytoplasm but still no yolk granules.
Primary or early vitellogenic oocytes (VT1)	Yolk granules in the periphery of the cytoplasm and lipid droplets occupy more of the cytoplasmic area than the yolk granules;
Secondary or mid vitellogenic (VT2)	Yolk granules and lipid droplets are spread throughout the cytoplasm.
Tertiary or advanced vitellogenic (VT3)	Larger yolk granules than that of Vtg2 stage. Lipid droplets fuse and are distributed around the nucleus.
Migratory nucleus (MG)	Germinal vesicle migration (GVM) stage: Lipid droplets fuse into 1−3 large droplets. Migration of the nucleus toward the animal pole. In a later phase, yolk granules fuse progressively.
Hydrated (HY)	Hydration (HYD) stage: The nucleus has disintegrated. All yolk granules fuse into a homogeneous yolk mass and the oocyte increases in size due to hydration. The oocyte is still surrounded by the follicle layer, i.e. ovulation has yet not taken place.

* Commonly PG and CA oocyte stages in tuna species are reported as one oocyte stage called ‘unyolked oocytes’ or ‘previtellogenic oocytes’.

* According to oogenesis studies in bluefin tuna (*Thunnus thynnus*) cortical alveoli appears after the lipid droplets (Abascal and Medina, 2005; Sarasquete et al., 2002). For this reason, the term lipid-stage oocyte is used instead of cortical alveolar stage in studies of tuna species (Corriero et al., 2003; Figueiredo et al., 2008; Aragón et al., 2010).

Table 4. Microscopic classification criteria for *Auxis rochei*, *Sarda sarda* and *Euthynnus alletteratus* females.

Maturity stage. Females	Microscopic characteristics
1. Immature (virgin)	Only oogonia and primary growth oocytes present. No atresia. Absence of POFs. Thin ovarian wall and little space between oocytes.
2. Developing	
2a. LP-Developing (immature or mature)	Cortical alveolus oocytes present as MAGO. Some atresia may be present. Absence of POFs.
2b. Developing (immature or mature)	Early vitellogenic oocytes present as MAGO. Some AT may be present. Absence of POFs.
3. Spawning capable (mature)	Mid or advanced vitellogenic oocytes present as the MAGO. Atresia (<50%) can be present. Absence of POFs.
4. Spawning (mature)	POFs present and /or migratory nucleus or hydrated oocytes present as the MAGO. Atresia, when present at all, only in limited amounts.
5. Regressing and regenerating	
5a. Regressing (mature)	In an early phase: cortical alveolus or early vitellogenic oocytes as the MAGO. Abundant alpha and/or beta atresia. Absence of POFs. In a latter phase: cortical alveolus oocytes as the MAGO. Abundant beta atresia. Absence of POFs. Disorganization of ovary structures, with some spaces. Thick and/or wrinkled gonad wall is observed (in some ovaries).
5b. Regenerating (mature)	Only primary growth oocytes as the MAGO present, with some spaces. Absence of POFs. Late stages of atresia. Thick and/or wrinkled gonad wall is observed (in some ovaries). At the early beginning of the spawning season. Difficulties are found to distinguish between this stage and LP-developing (2a), that is, individuals that spawned in the previous spawning season and immature individuals developing for the first time.
6. *Abnormal	Intersex, both oocytes and spermatogonia are present at the same time; sclerosis, the ovary is dominated by atretic oocytes and large amounts of connective tissue; infections; necrosis (atrophy).

*Abnormal stage was defined according to the International Council for the Exploration of the Sea (ICES) (ICES, 2014).

Table 5. Microscopic classification criteria for *Auxis rochei*, *Sarda sarda* and *Euthynnus alletteratus* males.

Maturity stage. Males	Microscopic characteristics
1. Immature (virgin)	Only spermatogonia present. No sperm in the sperm duct. Small space of lobule lumen.
2. Developing (early developing)	Spermatocytes, spermatids, and spermatozoa. Small space of lobule lumen.
3. spawning capable (late developing)	Abundant spermatids and some spermatozoa within seminiferous tubules. Sperm duct relatively full of sperm.
4. Spawning	Some spermatids and abundant spermatozoa. Greatly enlarged tubules, sperm duct full of sperm.
5. Regressing and regenerating (postspawning)	In regressing, residual spermatozoa. In regenerating stage only spermatogonia present.
6. *Abnormal	Intersex, both oocytes and spermatogonia are present at the same time; sclerosis; infections; necrosis (atrophy).

*Abnormal stage was defined according to the International Council for the Exploration of the Sea (ICES) (ICES, 2014).

Table 6. Immature or mature females according to three criteria considered (microscopic maturity scenerios) to estimate L_{50}.

	Immature. Stages:	Mature. Stages:
Criterion mat_a	(1) Immature	(2a) LP-Developing (2b) Developing (3) Spawning capable (4) Spawning (5a) Regressing (5b) Regenerating
Criterion mat_b	(1) Immature (2a) LP-Developing	(2b) Developing (3) Spawning capable (4) Spawning (5a) Regressing (5b) Regenerating
Criterion mat_c	(1) Immature (2a) LP-Developing (2b) Developing	(3) Spawning capable (4) Spawning (5a) Regressing (5b) Regenerating

Table 7. The growth parameters for *Auxis rochei* (BLT), *Sarda sarda* (BON) and *Euthynnus alletteratus* (LTA) from the Mediterranean Sea.

	Species	Sex	L_∞	k	t_0	Reference
1	LTA	both	117.00	0.19	-1.13	Hattour, 2009
2	LTA	both	123.00	0.13	-3.84	Kahraman et Oray, 2001
3	LTA	both	128.00	0.11	-4.18	Kahraman et Oray, 2001
4	BON	both	51.50	0.74	-1.55	Hattour,2009
5	BON	both	80.70	0.35	-1.70	Rey et al., 1986 in N'Guessan et al., 2015
6	BON	both	80.60	0.36	-1.36	Santamaria et al., 1998 in N'Guessan et al., 2015
7	BON	both	82.99	0.24	-0.77	Zaboukas and Megalofonou, 2007
8	BON	both	67.88	0.46	-1.22	Kahraman et al., 2014
9	BLT	both	46.70	0.37	-0.72	Hattour, 2009
10	BLT	both	57.40	0.18	-4.16	Kahraman et al., 2011
11	BLT	female	49.24	0.31	-3.01	Kahraman et al., 2011
12	BLT	male	60.42	0.16	-4.31	Kahraman et al., 2011
13	BLT	both	44.04	0.70	-0.14	Valeiras et al., 2008
14	BLT	both	47.76	0.39	-2.36	Bök and Oray, 2001
15	BLT	female	45.26	0.40	-1.60	Bök and Oray, 2001
16	BLT	male	45.08	0.34	-1.60	Bök and Oray, 2001
17	BLT	both	45.21	0.71	-0.01	Plandri et al., 2009

Table 8. Number of fish measured and weighted; range of both fork lengths and weights; and estimated parameters of length–weight relationship for *Auxis rochei* (BLT), *Sarda sarda* (BON) and *Euthynnus alletteratus* (LTA). Sampling period: 2003-2017.

Species	N	FL (cm)	W (g)	Parameters L-W relationship ($W = a\ FL^b$)			
		Range	Range	a	b	SE (b)	R^2
BLT	1281	13.8 – 48.7	27.3 – 2055	$3.483\ 10^{-3}$	3.432	0.010	0.990
BON	1583	9.7 – 79.3	8.3 – 7145	$6.321\ 10^{-3}$	3.210	0.005	0.996
LTA	1266	10.5 – 101.0	12 – 15000	$1.242\ 10^{-2}$	3.058	0.006	0.995

Table 9 The number of females and males by microscopic maturity stages for each species (BLT, BON; LTA) is given in **Table 9**.

	Auxis rochei		*Sarda sarda*		*Euthynnus alletteratus*	
Gonad stage	Females	Males	Females	Males	Females	Males
Immature	36	21	53	11	69	9
LP-Developing	62		79		53	
Developing	23	24	46	7	30	45
Spawning capable	16	18	119	33	15	18
Spawning	19	3	70	6	19	1
Regressing	21		11	3		10
Regenerating	19	2	8	6	5	1
Total	196	68	386	66	191	84

Table 10. Estimated length at first maturity (or length at 50% maturity, L_{50}) for *Auxis rochei*, *Sarda sarda* and *Euthynnus alletteratus*.

Species	**Macro**			**Micro**		
	Sex combined	Females	Males	Mat_a	Mat_b	Mat_c
BLT	34.81	34.12	35.43	29.25	38.17	44.31
BON	39.91			40.60	43.14	45.30
LTA	51.13	50.07	43.44	53.19	68.74	83.33

Table 11. Age at first maturity (A_{50}) estimated using size-age relationships for *Auxis rochei* from the literature review (see references in**Table 7**).

	species	parameter	sex	value	hattour_2008	palandri_2009	bok_2001	valeiras_2008	kahraman_2011
1	BLT	l50	both	34.81	2.98	2.06	2.14	2.09	1.02
2	BLT	l50low	both	34.36	2.88	2.00	2.02	2.02	0.91
3	BLT	l50upp	both	35.21	3.07	2.11	2.25	2.16	1.12
4	BLT	l90	both	40.19	4.61	3.09	3.99	3.34	2.53
5	BLT	l90low	both	39.53	4.34	2.91	3.70	3.12	2.32
6	BLT	l90upp	both	40.91	4.92	3.30	4.34	3.64	2.77
19	BLT	l50	female	34.12	2.82	1.97	1.96	1.99	0.85
20	BLT	l50low	female	33.44	2.68	1.89	1.79	1.89	0.69
21	BLT	l50upp	female	34.66	2.94	2.04	2.10	2.07	0.98
22	BLT	l50	male	35.43	3.12	2.15	2.31	2.19	1.18
23	BLT	l50low	male	34.57	2.92	2.03	2.08	2.06	0.96
24	BLT	l50upp	male	36.17	3.31	2.26	2.52	2.32	1.37
25	BLT	l90	female	37.94	3.80	2.56	3.09	2.68	1.85
26	BLT	l90low	female	37.25	3.60	2.44	2.86	2.53	1.66
27	BLT	l90upp	female	38.65	4.03	2.71	3.35	2.86	2.06
28	BLT	l90	male	42.86	6.03	4.15	5.49	5.08	3.47
29	BLT	l90low	male	41.47	5.20	3.50	4.63	3.92	2.96
30	BLT	l90upp	male	44.54	7.59	5.92	6.94		4.15

	species	parameter	sex	value	female_kahraman_2011	male_kahraman_2011	bok_female_2001	bok_male_2001
19	BLT	l50	female	34.12		0.89		2.56
20	BLT	l50low	female	33.44		0.73		2.38
21	BLT	l50upp	female	34.66		1.02		2.71
22	BLT	l50	male	35.43	1.09		2.22	
23	BLT	l50low	male	34.57	0.90		2.01	
24	BLT	l50upp	male	36.17	1.27		2.41	
25	BLT	l90	female	37.94		1.87		3.82
26	BLT	l90low	female	37.25		1.68		3.55
27	BLT	l90upp	female	38.65		2.07		4.13
28	BLT	l90	male	42.86	3.58		5.74	
29	BLT	l90low	male	41.47	2.95		4.60	
30	BLT	l90upp	male	44.54	4.57		8.75	

Table 12. Age at first maturity (A_{50}) estimated using size-age relationships for *Sarda sarda* from the literature review (see references in**Table 7**).

	species	parameter	sex	value	hattour_2008	rey_1986	santamaria_1998	zaboukas_2007	kahraman_2014
7	BON	l50	both	39.93	0.47	0.25	0.54	1.96	0.71
8	BON	l50low	both	39.64	0.43	0.23	0.52	1.94	0.69
9	BON	l50upp	both	40.21	0.50	0.27	0.56	1.99	0.73
10	BON	l90	both	42.28	0.77	0.42	0.71	2.20	0.90
11	BON	l90low	both	41.80	0.71	0.38	0.67	2.15	0.86
12	BON	l90upp	both	42.72	0.84	0.45	0.74	2.24	0.94

Table 13. Age at first maturity (A_{50}) estimated using size-age relationships for *Euthynnus alletteratus* from the literature review (see references in**Table 7**).

	species	parameter	sex	value	hattour_2008	kahraman_med_2001	kahraman_aegean_2001
13	LTA	l50	both	51.13	1.89	0.29	0.46
14	LTA	l50low	both	50.07	1.81	0.18	0.33
15	LTA	l50upp	both	52.20	1.98	0.41	0.58
16	LTA	l90	both	65.23	3.16	1.97	2.30
17	LTA	l90low	both	63.11	2.96	1.70	2.00
18	LTA	l90upp	both	67.26	3.37	2.25	2.60
31	LTA	l50	female	50.07	1.81	0.18	0.33
32	LTA	l50low	female	48.31	1.67	-0.00	0.13
33	LTA	l50upp	female	51.64	1.93	0.35	0.52
34	LTA	l50	male	43.44	1.31	-0.49	-0.41
35	LTA	l50low	male	36.89	0.86	-1.10	-1.09
36	LTA	l50upp	male	47.34	1.60	-0.10	0.02
37	LTA	l90	female	65.23	3.16	1.97	2.30
38	LTA	l90low	female	62.16	2.86	1.57	1.96
39	LTA	l90upp	female	68.64	3.52	2.44	2.81
40	LTA	l90	male	69.70	3.64	2.59	2.97
41	LTA	l90low	male	65.30	3.17	1.98	2.31
42	LTA	l90upp	male	75.34	4.30	3.45	3.89

FIGURES

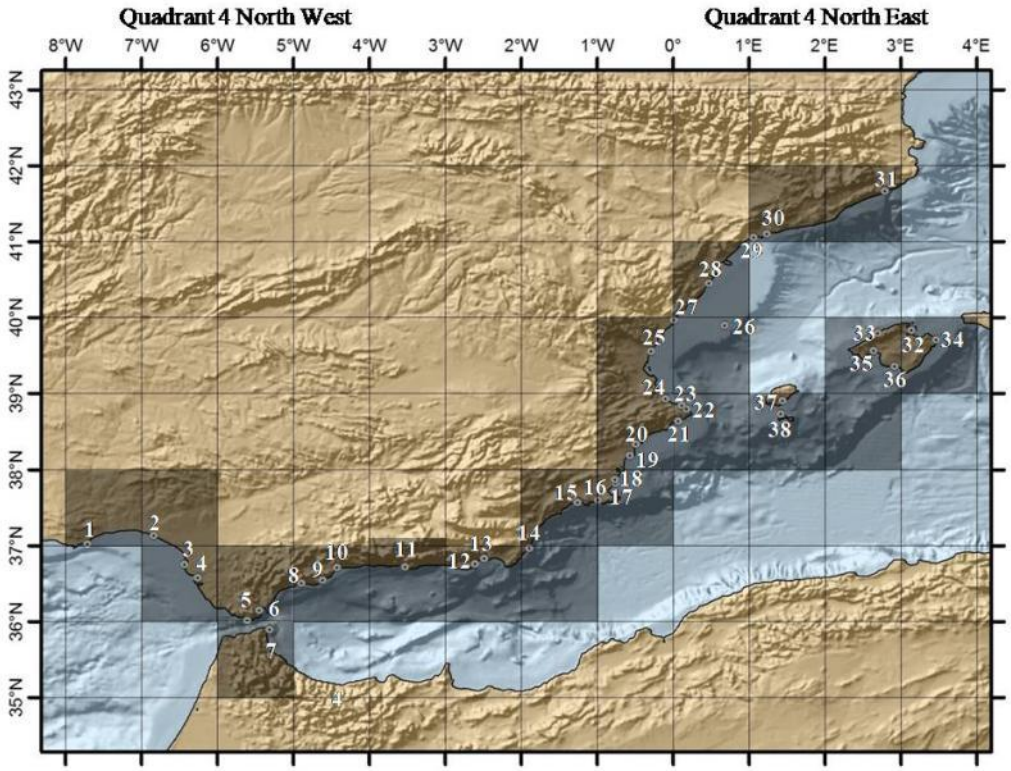

Figure 1. Colored grids 1x1 (squares of 1 by 1 degrees) indicating where BLT, BON and LTA were sample. Sampling period: 2003-2017. Ports: 1, Olhão; 2, Mazagón; 3, Chipiona; 4, Puerto de Santa María; 5, Tarifa; 6, Algeciras; 7, Ceuta; 8, Marbella; 9, Fuengirola; 10, Málaga; 11, Motril; 12, Roquetas; 13, Almería; 14, Carboneras; 15, Mazarrón; 16, Cartagena; 17, San Pedro del Pinatar; 18, Torre de la Horadada; 19, Santa Pola; 20, Alicante; 21, Calpe; 22, Jávea; 23, Dénia; 24, Oliva; 22, Pobla de Farnals; 26, Islas Columbretes; 27, Castellón; 28, Vinaròs; 29, Cambrils; 30, Tarragona; 31, Blanes; 32, Alcúdia; 33, Sóller, 34, Cala Ratjada; 35, Palma de Mallorca; 36, S'Estanyol; 37, Ibiza; 38, Formentera.

Geographical areas: Atlantic, 1 – 4; Gibraltar Strait, 5 – 7; Alboran Sea, 8 – 13; Mediterranean Sea, 14 – 38.

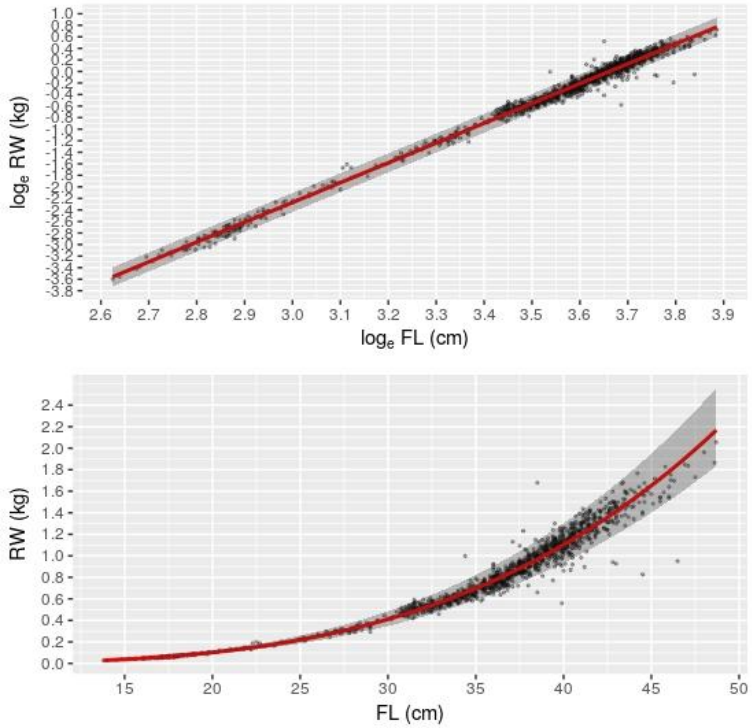

Figure 2: Estimated length-weight relationship for *Auxis rochei* (BLT) (upper panel, logarithmic scale; lower panel, linear scale).

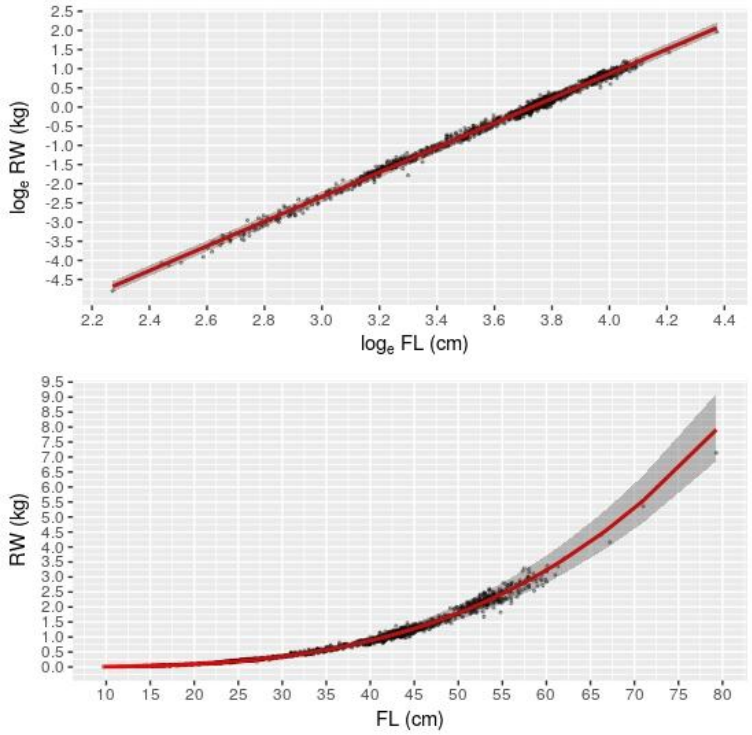

Figure 3: Estimated length-weight relationship for *Sarda sarda* (BON) (upper panel, logarithmic scale; lower panel, linear scale).

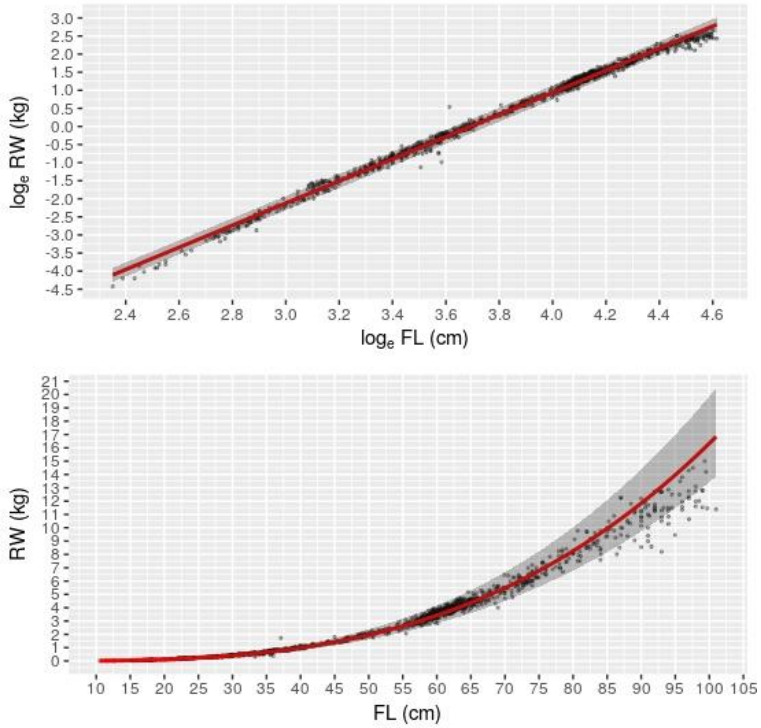

Figure 4: Estimated length-weight relationship for *Euthynnus alletteratus* (LTA) (upper panel, logarithmic scale; lower panel, linear scale).

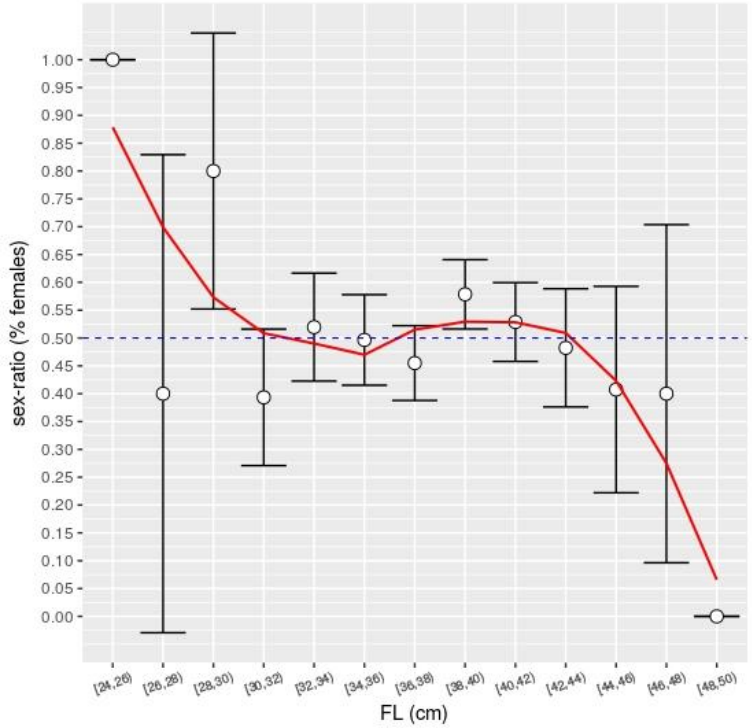

Figure 5: Predicted sex-ratio (% females) by length (FL, cm) for *Auxis rochei* (BLT) with 95% confidence interval (based on normal approximation).

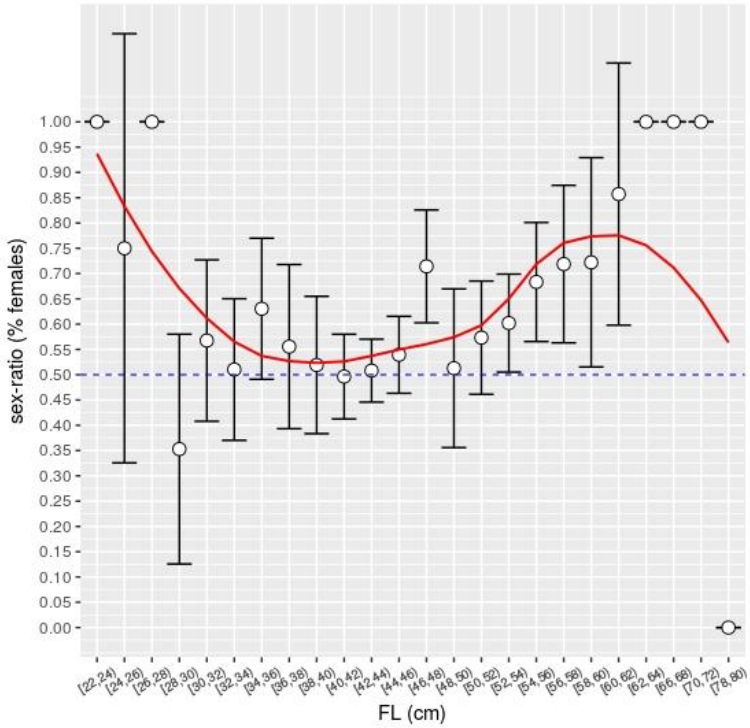

Figure 6: Predicted sex-ratio (% females) by length (FL, cm) for *Sarda sarda* (BON) with 95% confidence interval (based on normal approximation).

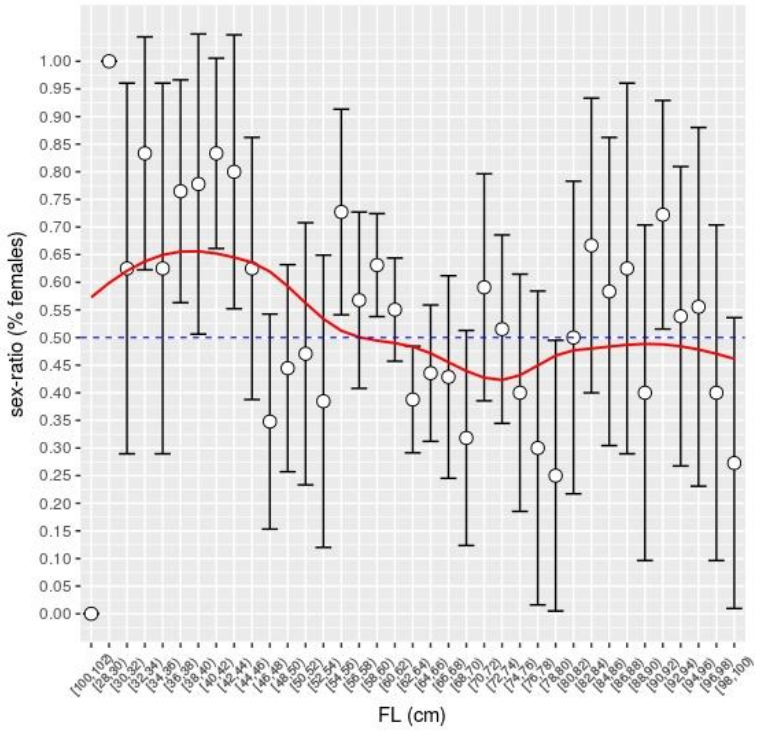

Figure 7. Predicted sex-ratio (% females) by length (FL, cm) for *Euthynnus alletteratus* (LTA) with 95% confidence interval (based on normal approximation).

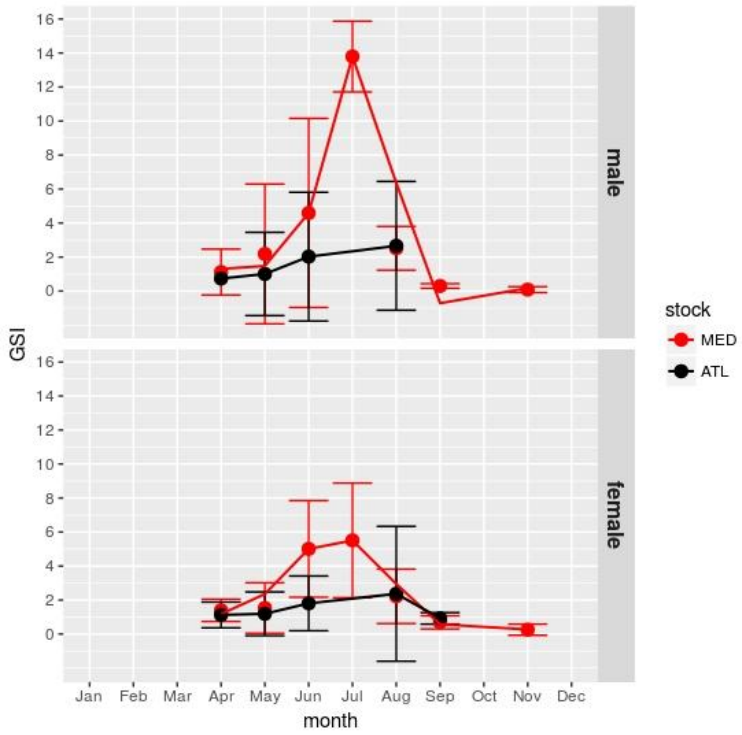

Figure 8. Monthly variation of the gonadosomatic index (GSI) for Mediterranean and Atlantic mature female and male *Auxis rochei* (BLT).

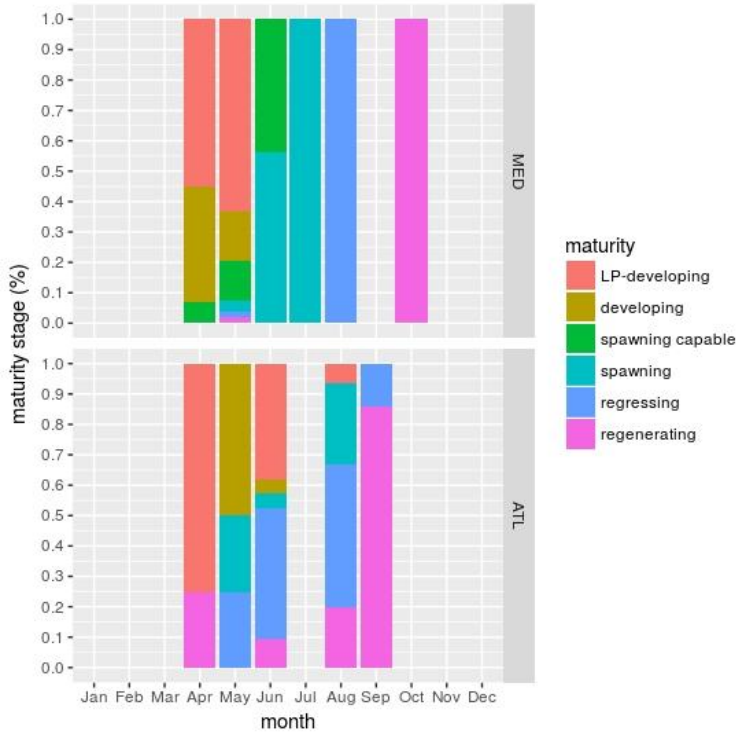

Figure 9. Monthly relative frequency of gonad stages (by microscopic examination) for Mediterranean and Atlantic mature female *Auxis rochei* (BLT).

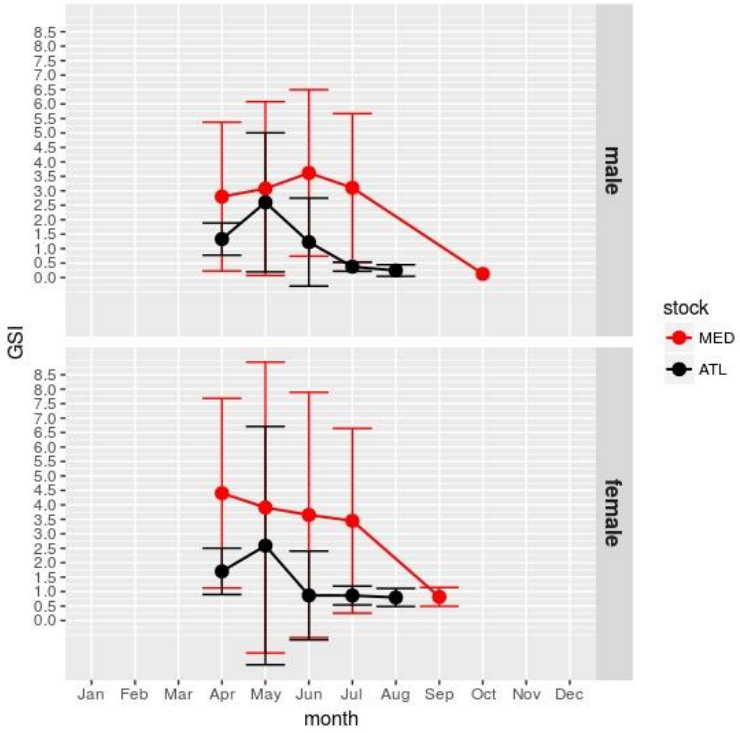

Figure 10. Monthly variation of the gonadosomatic index (GSI) for Mediterranean and Atlantic mature female and male *Sarda sarda* (BON).

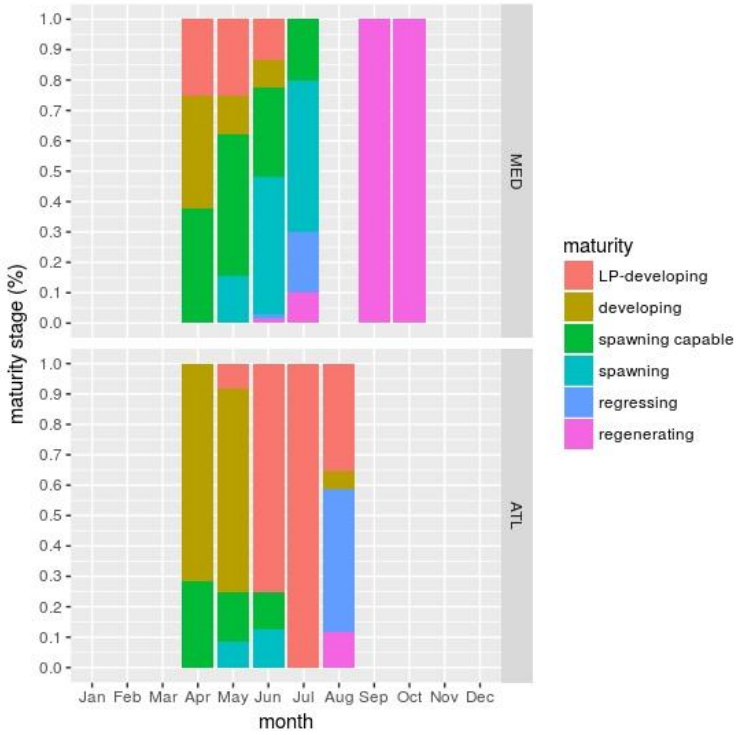

Figure11. Monthly relative frequency of gonad stages (by microscopic examination) for Mediterranean and Atlantic mature female *Sarda sarda* (BON).

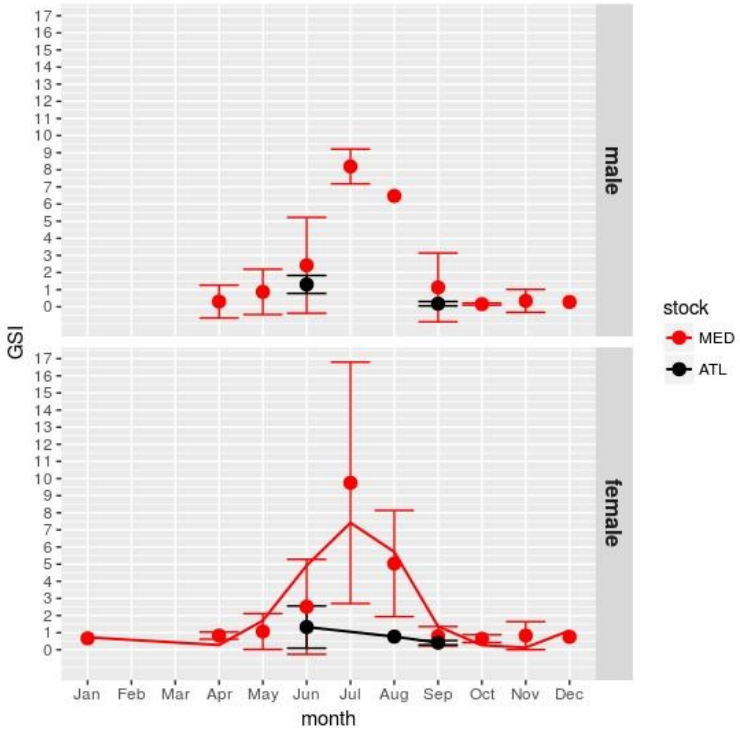

Figure 12. Monthly variation of the gonadosomatic index (GSI) for Mediterranean and Atlantic mature female and male *Euthynnus alletteratus* (LTA).

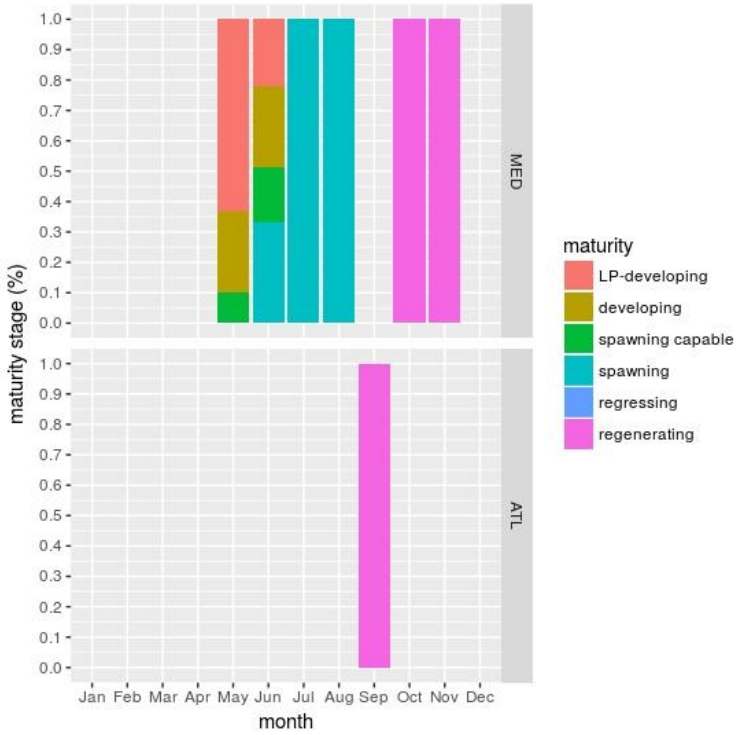

Figure13. Monthly relative frequency of gonad stages (by microscopic examination) for Mediterranean and Atlantic mature female *Euthynnus alletteratus* (LTA).

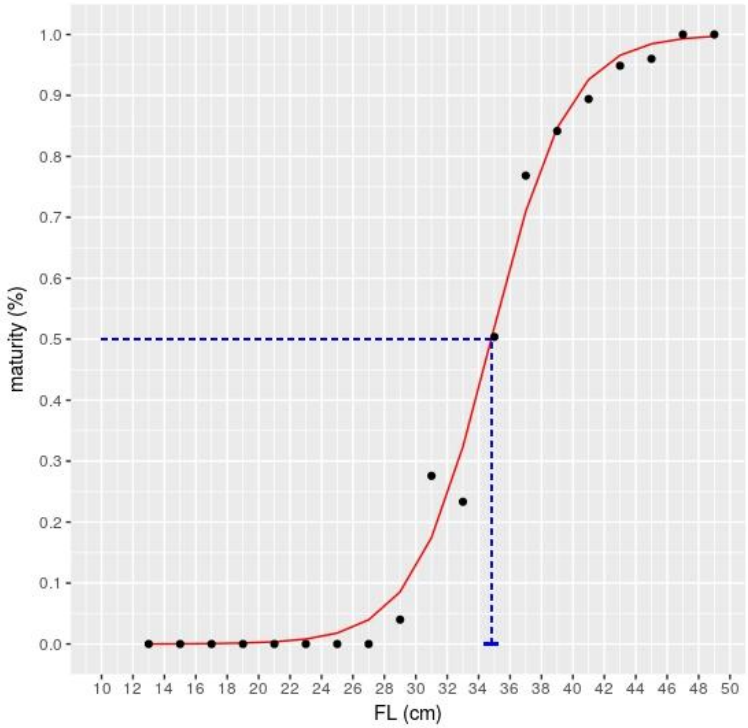

Figure 14. Logistic fit for the proportion of mature *Auxis rochei* (BLT). Macroscopic maturity staging (n = 1165).

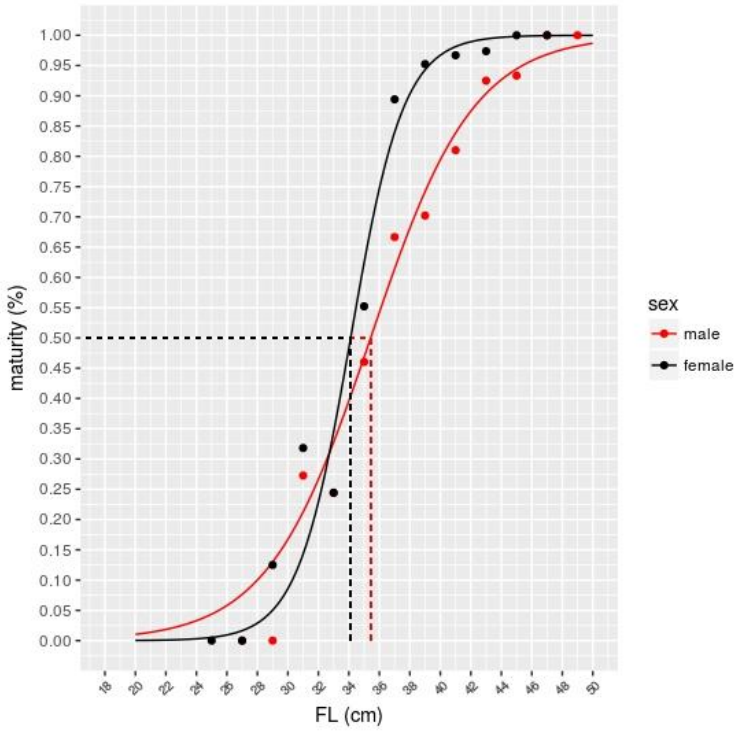

Figure 15. Logistic fit for the proportion of mature *Auxis rochei* (BLT) by sex. Macroscopic maturity staging

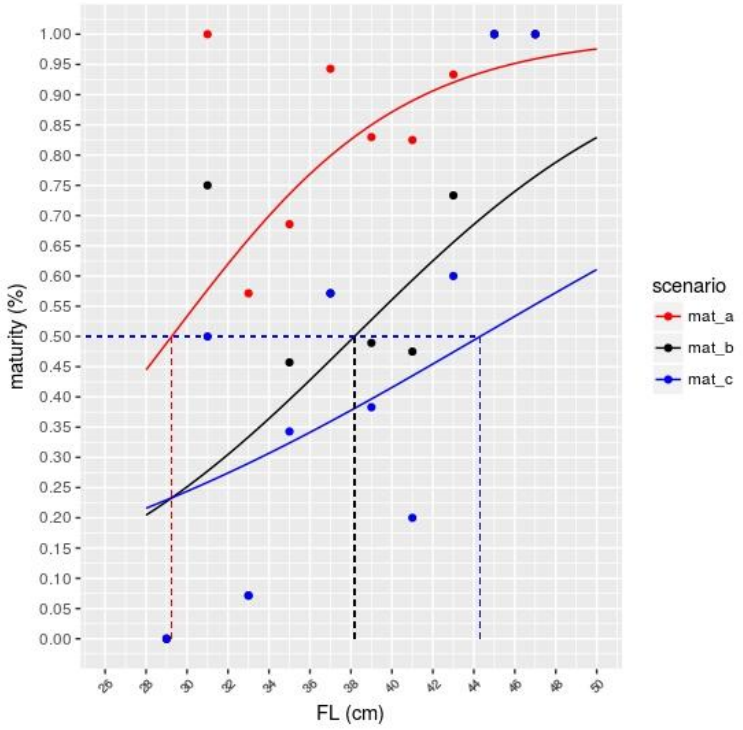

Figure 16. Logistic fit for the proportion of mature female *Auxis rochei* (BLT) by criterion (see Table 5). Microscopic maturity staging.

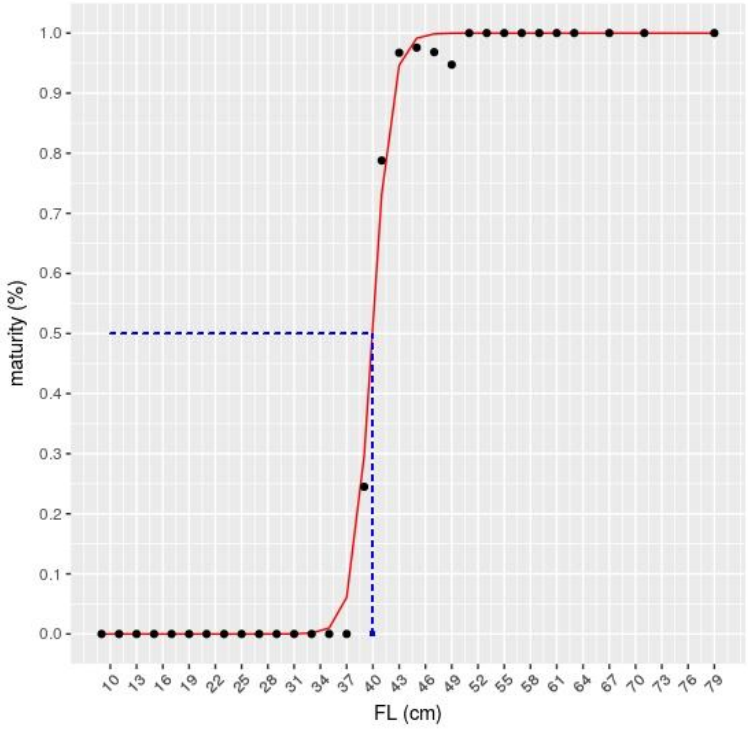

Figure 17. Logistic fit for the proportion of mature *Sarda sarda* (BON). Macroscopic maturity staging (n = 1362).

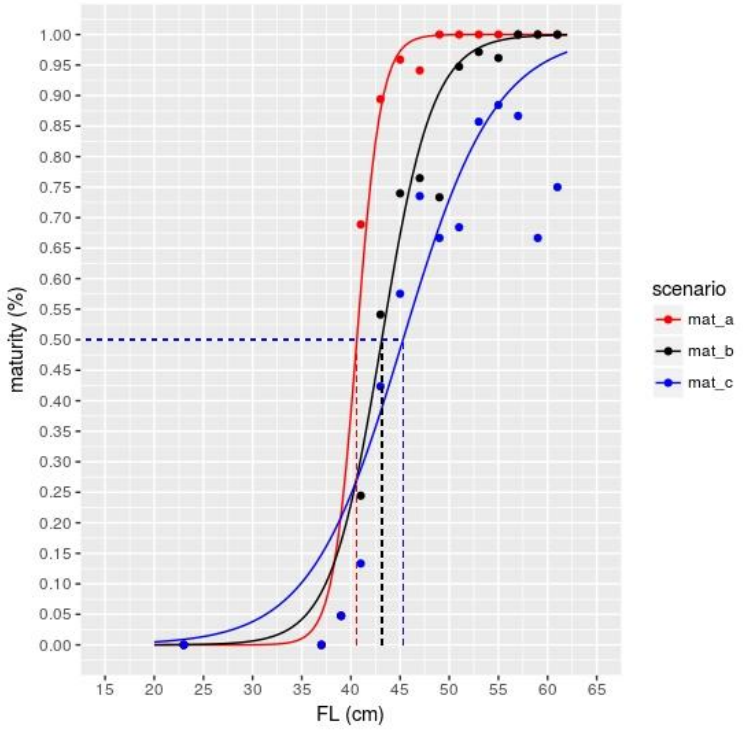

Figure 18. Logistic fit for the proportion of mature female *Sarda sarda* (BON) by criterion (see Table 5). Microscopic maturity staging.

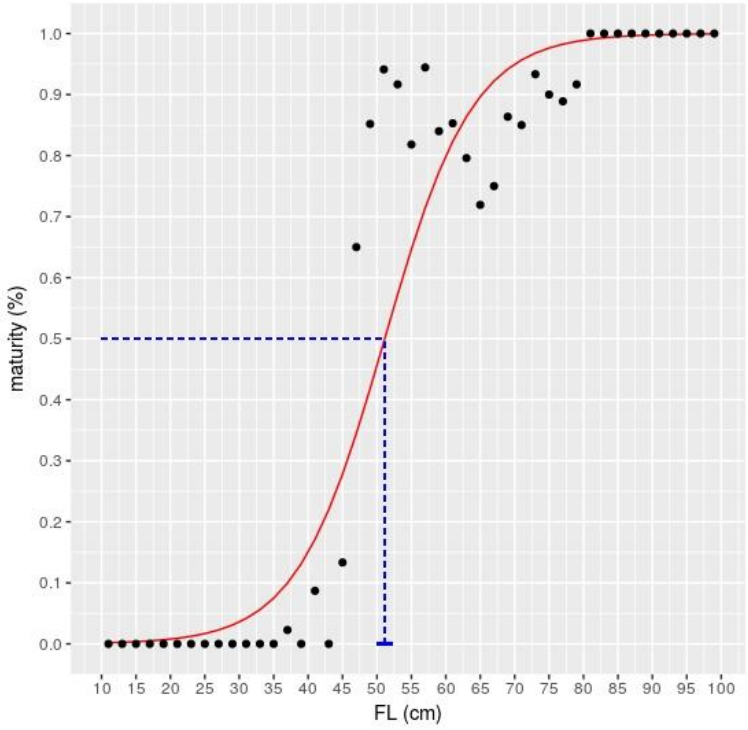

Figure 19. Logistic fit for the proportion of mature *Euthynnus alletteratus* (LTA). Macroscopic maturity staging (n = 1209).

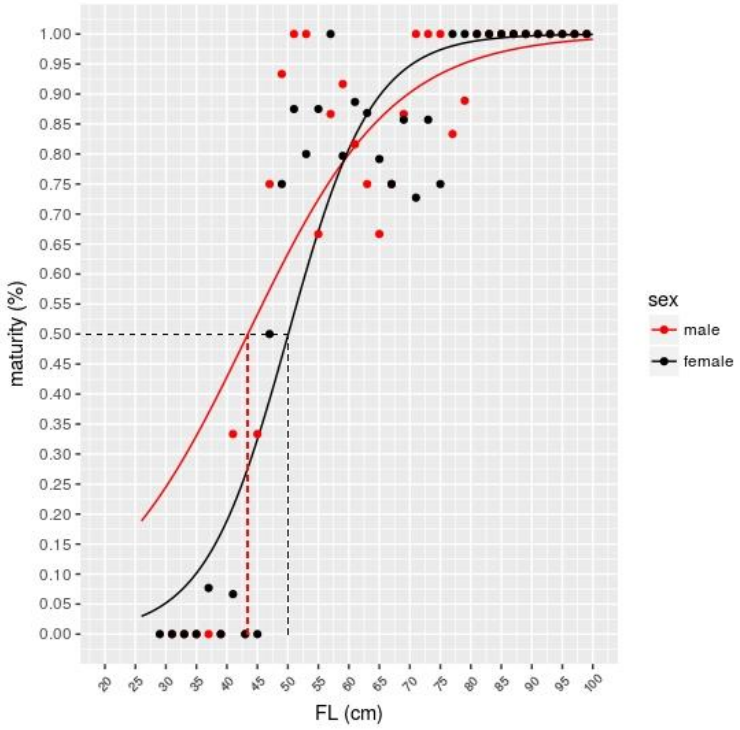

Figure 20. Logistic fit for the proportion of mature *Euthynnus alletteratus* (LTA) by sex. Macroscopic maturity staging.

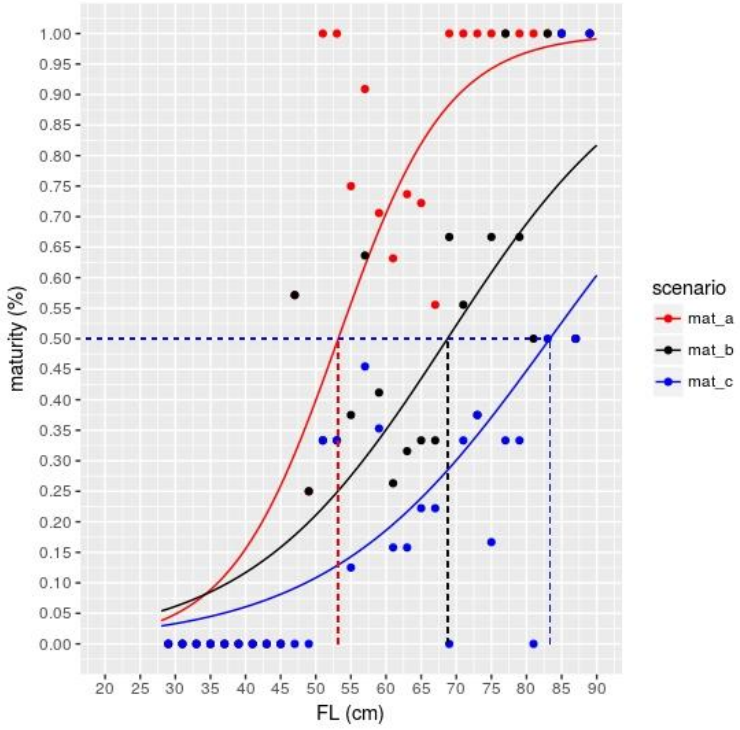

Figure 21. Logistic fit for the proportion of mature female *Euthynnus alletteratus* (LTA) by criterion (see Table 5). Microscopic maturity staging.

GONAD EMBEDDING, SECTIONING AND STAINING PROCEDURE

Large Pelagic Group of the Instituto Español de Oceanografía (IEO), Centro Oceanográfico de Málaga-Fuengirola

A 2−3 cm cross-section from the central part of the right or left lobe was fixed in Bouin's fluid for four hours, and preserved in 70% ethanol. A representative subsample (from the *tunica albuginea* to the ovarian lumen, see Figure 1) was taken from the preserved ovarian tissue and processed, using gentle agitation (from step 1 to step 7), as follows:

If samples are fixed and preserved in phosphate buffered formaldehyde

Distilled water (2 x 30 min)

50% ethanol (1 hour)

70% ethanol (1 hour)

If samples are fixed in Bouin's fluid and preserved in 70% ethanol

1. 80% ethanol (1 hour)
2. 90% ethanol (1 hour)
3. 96% ethanol (1 hour)
4. 99.6% ethanol (1 hour)
5. 99.6% ethanol (1 hour)

Subsamples can keep in the second bath of 99.6% ethanol (step 5) over night

6. Clearing agent → Butanol* (1 hour)
7. Clearing agent → Butanol (1 hour)

Immersion time in butanol (steps 6 and 7) should be not exceeded

8. Paraffin* at ≈ 62°C (2 hours)
9. Paraffin at ≈ 62°C (2 hours)
10. Paraffin at ≈ 62°C (2 hours)

Subsamples are kept in the third wax (step 10) in the drying oven over night

* butanol used one time in step 7

* paraffin used one time in steps 9 and 10

The infiltrated ovarian tissues are then embedded into wax blocks. To create paraffin blocks:

- Put small amount of molten paraffin in mould/tin, dispensing from paraffin reservoir.
- Using warm forceps, transfer tissue into mould/tin.
- Molten paraffin is added to the mould/tin. Fill mould/tin with enough paraffin to cover the tissue. If necessary, use a warm needle to eliminate air bubbles.

Once the tissue is embedded, it is stable for many years.

Sectioning

Cut at 10 µm. Pick the sections up with forceps or a fine paint brush and float them on the surface of the 37°C distilled water bath. Pick up sections from water by placing the slides under the sections. Dry the slides in the drying oven (35°C) during 24 hours.

Staining procedure

- Deparaffinize and rehydrate sections

  Xylene (3 x 10 min) (blot excess xylene before going into ethanol)
  Ethanol 99.6% (2 x 5 min)
  Ethanol 96% (2 x 5 min)
  Ethanol 70% (5 min)
  Ethanol 50% (5 min)
  Distilled water (5 min)

- Staining (times should be not exceeded)
  Corrosive sublimate (20 min)
  Rinse distilled water (3−4 drips)
  Acid fucsin 1 % (1 min)
  Rinse in running tap water (≈ 10 min)
  Rinse distilled water (3−4 drips)
  ***** Phosphomolybdic acid 1 % (1 min) (keep in darkness, renewed after two days)
  Rinse distilled water (3−4 drips)
  Mallory's trichrome stain (1 min 15 seconds)
  Rinse in running tap water (≈ 10 min)
  Rinse distilled water (3−4 drips)

***** Phosphomolybdic acid 1% is kept in darkness and should be renewed after two days

- Dehydrate and clear sections

  Ethanol 96% (1 min)
  Ethanol 99.6% (1 min)
  Eucalyptol (15 min)
  Xylene (2 x 10 min) (subsamples can kept more time in the final step)

Cover the sections using mountex/cover glasses. Dry the slides in the drying oven (35−37°C) during 24 - 72 hours.