SHORT TERM CONTRACT FOR THE BIOLOGICAL AND GENETIC SAMPLING AND ANALYSIS (ICCAT-GBYP 01/2012 B) WITHIN THE GBYP (Phase 3)

Final Report

for:

ICCAT

Scientific coordinator:

Dr. Haritz Arrizabalaga (AZTI-Tecnalia)

Pasaia, February 5th, 2013

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EXECUTIVE SUMMARY:

The main objective of this project is to enhance knowledge about Atlantic bluefin tuna population structure and mixing, but also focusses on age and reproductive dynamics. The sampling protocols and structure of the data bank were revised and agreed with ICCAT Secretariat.

During Phase 3, the consortium has sampled a total of 2813 bluefin tuna (71 larvae, 604 YOY, 701 juveniles, 598 medium size fish, and 839 large fish) from different regions (427 from the East Mediterranean, 286 from the Central Mediterranean, 723 from the Western Mediterranean, 928 from the Northeast Atlantic, 399 from the Central North Atlantic and 50 from the Western Atlantic). From these individuals, 6256 biological samples were taken (2733 genetic samples, 1759 otoliths, 1413 spines and 351 gonads).

By the end of the project, the consortium genotyped 1152 individuals, completed 400 michochemical analyses on otoliths, analyzed 315 hard parts for aging purposes and conducted 158 histological analyses on gonads.

The yet preliminary genetic analyses that were carried out on a subset (n=555) of the total number of individuals genotyped, mainly focusing on the Reference Samples, were encouraging. They revealed that high-performing SNP panels can identify and differentiate at least three ABFT spawning populations (GOM, WMED, EMED), that are genetically well clustered, although the differentiation of the Eastern Mediterranean ABFT Reference Samples needs to be improved by looking for more efficient SNP loci. However, due to the complexity and quantity of the RRSG-generated genomic data obtained for the ABFT, the genomic data needs to be analysed more in depth in the future and various additional analyses are further required to fine tune SNP selection/validation for traceability and management purposes.

Regarding otolith microchemistry, the baseline of yearling fish from known origin has been revised. Classification success (based on quadratic discriminant function

analysis) of the revised baseline was 90% east and 75% west (overall 83%). Results of mixed stock analyses using the revised baseline suggest >99% eastern origin fish in all studied areas (including the Bay of Biscay, Gibraltar and the Mediterranean Sea), except in the Central North Atlantic (70%) and the Atlantic coast of Morocco (27%). However, the sample size was small in the latter case, with very uncertain estimates, and additional analyses are needed to verify the origin of fish caught in this area.

Regarding age determination analyses, 157 otoliths and 158 spines were interpreted. Biannual age-lenth-keys were generated for both calcified structures, i.e. spines and otoliths. Comparison of age estimates between different calcified structures coming from the same specimen was carried out, estimating precision and relative accuracy of spine age interpretations in relation to otolith age interpretations. Results indicated a low discrepancy between spines and otolith for ages 0 to 10 years old, showing that both structures may be used indistinctly for age determination of Atlantic Bluefin tuna for this age range.

A histological analysis was conducted on 158 individuals from the Strait of Gibraltar, Balearics and Sardinia. Although samples on some areas (e.g. Strait of Gibraltar) did not provide much insight on reproductive activity of bluefin tuna, samples from the Balearics and Sardinia showed active reproductive condition for some individuals, and could be used to gather further information about the reproductive biology of bluefin tuna.

In general, Phase3 was importantly affected by the delay in the contract signature. However, the objectives of the Project were met, although a bit delayed. These analyses already started to provide some results on population structure, catch composition, age structure and reproductive ecology that can be refined and further explored in subsequent Phases of GBYP to provide important information relevant for Atlantic Bluefin Tuna management.

1. CONTEXT

On April 27th 2012, the Consortium formed by Fundación AZTI-AZTI Fundazioa, Instituto Español de Oceanografía, IFREMER, Universitá di Genova, University of Bologna, IZOR, University of Cagliari, Euskal Herriko Unibertsitatea / Universidad del País Vasco, National Research Institute of Far Seas Fisheries, Federation of Maltese Aquaculture Producers and Texas A&M University, with subcontracted parties Biogenomics, IPMA, and Drs. Isik Oray, Dr. Saadet Karakulak and Dr. Massimiliano Valastro, coordinated by Fundación AZTI-AZTI Fundazioa, presented a proposal to the call for tenders on biological and genetic sampling and analysis (ICCAT-GBYP 01/2012b). This proposal was awarded by the Secretariat on June 7 2012. The final contract between ICCAT and the consortium represented by Fundación AZTI-AZTI Fundazioa was signed on September 21st 2012.

According to the terms of the contract, a short preliminary interim report was submitted to ICCAT by September 26th 2012, an interim report by November 5th 2012 and the Draft Final Report by January 8th 2013. The last deliverable is the Final Report, including a full description of the work carried out during Phase 3. The present report was prepared in response to such requirement, and represents the final report.

2. SAMPLING

The sampling conducted under this project follows a specific design, aimed primarily at contributing to knowledge on population structure and mixing. As such, the sampling conducted within this project is independent from other routine sampling activities for fisheries and fishery resources monitoring (e.g. the Data Collection Framework).

During Phase 2, the GBYP consortium, together with ICCAT, thoroughly revised the sampling protocols and the structure of the data bank. During Phase 3, the protocols and structure of the data bank have been revised again by the consortium, though no substantial modifications arose from that revision. The final adopted set of protocols and structure of the data bank (included in Appendix 1) was distributed to all members of the consortium.

2.1 Sampling acomplished

A total of 2813 bluefin tuna individuals have been sampled so far. Table 2.1 shows the number of bluefin tuna sampled in each strata (area/size class combination), and Table 2.2. and Figure 2.1 provide summaries by main region and size class.

The original plan, according to the contract, was to sample 1750 individuals (including those to be provided by the tagging cruises), thus the current sampling status represents 161% of the target in terms of number of individuals. By size class, the objectives for age 0, juveniles, medium and large fish were accomplished (201%, 175%, 109% and 210% respectively) and the sampling for larvae was below expectations (71%).

Table 2.1. Number of bluefin tuna sampled by area/fishery and size class. Empty cells indicate that no sampling was planned in that stratum. Green cells indicate strata were no sampling was planned but some sampling was finally accomplished.

		Larvae	Age 0	Juveniles	Medium	Large			
			<=3 kg	>3 & <=25 kg	>25 & <=100 kg	>100 kg	Responsible	Target	%
Eastern Mediterranean	Levantine Sea	0	284		73	70	AZTI (Karakulak/Oray)	200	214%
Central Mediterranean	Malta		0		19	90	FMAP	150	73%
	South of Sicily and Ionian Sea	21	50	0	50		UNIBO	200	61%
	Adriatic Sea		0	56			IZOR	100	56%
Western Mediterranean	Balearic		104	53	15	1	IEO	150	115%
	Southern Spain		13		4				
	Tyrrhenian		132*		50		UNIBO	100	182%
	Sardinia			0	110	60	UNICA	150	113%
	Gulf of Lyon			39	25		IFREMER	100	64%
	Ligurian Sea		21	48	47	1	UNIGE	100	117%
Northeast Atlantic	Gibraltar (small, medium-large): Spanish HL, traps, BB			17	39	65	IEO	150	81%
	Gibraltar: Portuguese traps				21	118	AZTI (IPMA)	100	139%
	Bay of Biscay (small): Spanish BB & French TW			488	107	23	AZTI	150	412%
	Western coast of Africa (medium-large): Morrocan Trap					50	INRH	50	100%
Central North Atlantic	Central and North (medium-large): Japanese & Taiwanese LL				38	361	NRIFSF	50	798%
Western Atlantic	Gulf of Mexico	50					TAMU	0	

- *Includes 62 YOY provided by the GBYP tagging survey*

Table 2.2: Number of bluefin tuna sampled *by main region and size class. Empty cells indicate that no sampling was planned in that strata:*

	Larvae	Age 0	Juvenile	Medium	Large	TOTAL	Target	%wrt target
East Med		284		73	70	**427**	200	214%
Central Med	21	50	56	69	90	**286**	450	64%
West Med		270	140	251	62	**723**	600	121%
NE Atl			505	167	256	**928**	450	206%
Central N Atl				38	361	**399**	50	798%
West Atl	50					**50**	0	
TOTAL	**71**	**604**	**701**	**598**	**839**	**2813**	1750	161%
Target	100	300	400	550	400	1750		
% wrt target	71%	201%	175%	109%	210%	161%		

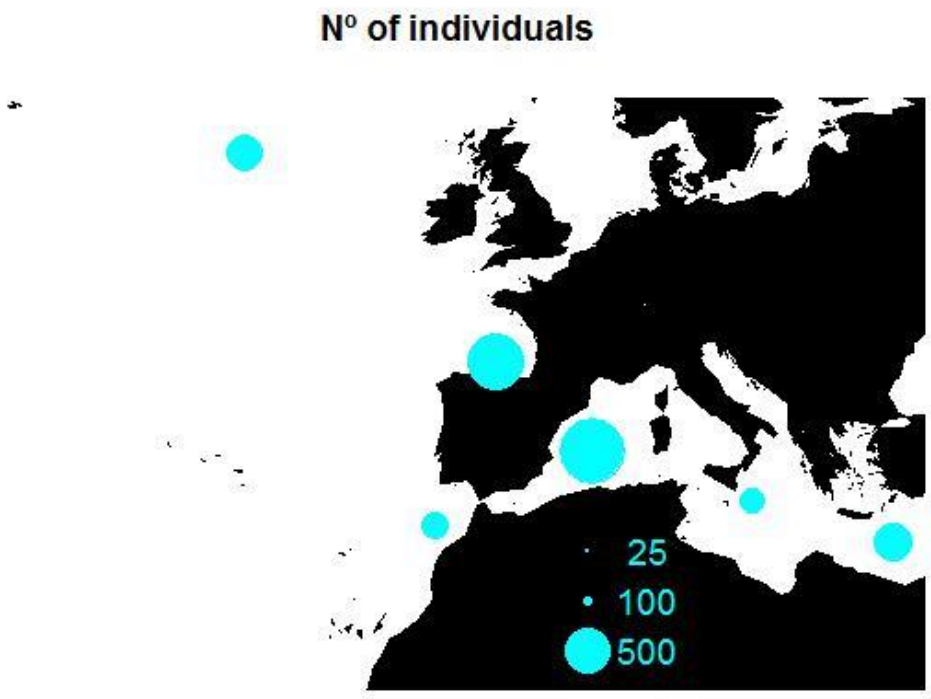

Figure 2.1: Number of individuals sampled, aggregated by main region. Positions of the dots are approximate averages across all samples. In the case of the North East Atlantic region, two dots are presented, one in the Atlantic side of the Strait of Gibraltar and the other in the Bay of Biscay.

In the Eastern Mediterranean, 214% of the target number of individuals has been sampled. The sampling of young of the year fish (YOY) was well above expectations, as was the sampling of medium and large fish. Regarding larvae, a short dedicated survey was conducted and 39 larvae were collected. However, none of them were confirmed as Bluefin tuna using genetic tests for species identification (see section 4).

In the Central Mediterranean, 64 % of the target number of individuals was sampled, including larvae, YOY, juveniles, medium and large fish. One of the main difficulties arouse from the fact that some Italian and Maltese fisheries were closed early (with respect to the date of contract signature). In addition, and in spite of the ICCAT Recommendation that allows for Research Mortality Allowance, the consortium had difficulties to get the local permits required to catch YOY fish from some administrations (e.g. Malta). Finally, the GBYP tagging cruise experienced a lack of Bluefin tuna and thus they were unable to provide a

sample of juvenile fish for the South of Sicily and Ionian Sea. However, they provided a sample of 62 YOY from the Tyrrhenian sea. A total of 92 larvae were collected from the waters off Sicily's southern coast, of which 21 were confirmed as Bluefin tuna using molecular techniques.

In the Western Mediterranean, 121% of the target number of individuals was sampled. The early closure (with respect to the date when the contract was signed) of the Spanish purse seine and Italian trap fisheries affected the sampling in this area importantly. The sampling in Cagliari was affected by the strong change in the fishing practice in Sardinian traps. Namely, the traditional system of capturing tunas by "mattanzas" on-site has shifted to the capture and caging of live bluefin tuna and their subsequent transport to a tuna farm in Malta (Mare Blu Tuna Farm - Ricardo Fuentes and Hijos). These changes have clearly affected the sampling practice of the team at UNICA during their sampling season of GBYP-3, who have established an agreement with the Fuentes Group to carry out samplings in Malta during autumn when those bluefin will be killed for the market. Also, few longliners operated in southern Spain during the spawning season since they mostly sold their quota to purse seiners or traps, but some individuals were finally sampled from the bycatch in other longline fisheries around the Balearics. The tagging cruise in the Gulf of Lyon also experienced a lack of Bluefin tuna in the area and thus was unable to provide a sample of juvenile fish from this area. Overall, the sampling of the different size classes in the western Mediterranean is still significant thanks to the sampling accomplished in cages and other fisheries (traps, artisanal/sport fisheries) where special efforts allowed for sampling beyond initial targets (for instance, the University of Genova was able to get a sample of YOY fish in the Ligurian sea).

In the North East Atlantic, 206% of the target number of individuals was sampled, including juveniles, medium and large fish. Some few strata could not be properly sampled, i.e. juveniles in Gibraltar, medium size fish in Portuguese traps and large fish in the Bay of Biscay, but overall, the three categories are well sampled in the Northeast Atlantic. The lack of sufficient medium size fish in Portuguese traps was due to the large size of the fish caught by traps in this area. Initial sampling in the Gibraltar area conducted by IPMA were affected by difficulties in collecting spines due to fish processing issues. However this was partially solved during October, as fish processing after harvesting the trap catches changed and allowed the collection of additional samples. Sampling in the Bay of Biscay was also affected by the selling of most of the quota to Mediterranean purse seiners, and the predominance of juvenile fish in the remaining landings. However, sampling in this area is well above the target, due to special sampling efforts and the samples provided by the GBYP tagging team. In Moroccan traps, in

spite of the short fishing season, that ended before the signature of the contract, the objective was covered.

In the Central North Atlantic, the fishing season for Japanese longline vessels started in September. Japanese scientists provided otoliths and muscles from 100 and 379 fish, respectively, which were collected in the Central North Atlantic by Japanese scientific observers during the 2011 fishing season. The total number of fish sampled (399) represents 798% of the sampling target in this area during Phase 3, which was 50 large fish. These samples correspond to both the medium (n=38) and the large (n=361) size categories, and are caught mainly in the eastern Atlantic (east of 45ºW, n=253) but some were also caught in the western Atlantic (west of 45ºW, n=146). The samples during 2012 fishing season will be collected and provided in 2013, as it takes more than a year to obtain the samples from the observers when the vessels return to Japan.

Finally, although no sampling was originally planned in this region, Texas A&M provided a sample of 50 larvae collected in the Gulf of Mexico from earlier years (2007-2009).

Tables 2.3. and 2.4 as well as Figures 2.2, 2.3, 2.4 and 2.5 show the number of different tissues sampled in each area. Because not all biological samples have been received at AZTI yet, and thus verified, the list of biological samples available might have some slight changes in the future. According to it, 6256 biological samples have been collected so far. In many cases, not all tissues (otoliths, muscle or fin for genetics, spine, and/or gonad, according to the sampling scheme) were collected from each single fish. However, both the total amount of samples as well as the number of samples by tissue type (1759 otoliths, 1413 spines, 351 gonads and 2733 genetic samples) is high and relatively well distributed over the different main regions (considering the circumstances explained in earlier paragraphs).

Table 2.3: Number of samples collected by area/fishery and tissue type:

		Otolith	Spine	Gonad	Muscle/Fin	Sampler
Eastern Mediterranean	Levantine Sea	340	142		427	AZTI (Karakulak/Oray)
Central Mediterranean	Malta	104			109	FMAP
	South of Sicily and Ionian Sea	100	100		121	UNIBO
	Adriatic Sea	51	56		51	IZOR
Western Mediterranean	Balearic	142	65	74	157	IEO/AZTI
	Southern Spain	14	15	17	17	IEO
	Tyrrhenian	181	182		182	UNIBO/UNICA/AZTI
	Sardinia	34	166	51	168	UNICA
	Gulf of Lyon	63	64	1	64	IFREMER/IEO
	Ligurian Sea	117	110	91	117	UNIGE
Northeast Atlantic	Gibraltar (small, medium-large): Spanish HL, traps, BB	104	105	117	117	IEO/AZTI
	Gibraltar: Portuguese traps	132	63		137	AZTI (IPMA)
	Bay of Biscay (small): Spanish BB & French TW	228	345		587	AZTI
	Western coast of Africa (medium-large): Morrocan Trap	49			50	INRH
Central North Atlantic	Central and North (medium-large): Japanese & Taiwanese LL	100			379	NRIFSF
Western Atlantic	Gulf of Mexico				50	TAMU
TOTAL		**1759**	**1413**	**351**	**2733**	

Table 2.4: Number of samples by main region and tissue type:

	Otolith	Spine	Gonad	Muscle/Fin	TOTAL
East Med	340	142		427	**909**
Central Med	255	156		281	**692**
West Med	551	602	234	705	**2092**
NE Atl	513	513	117	891	**2034**
Central N Atl	100			379	**470**
West Atl				50	**50**
TOTAL	**1759**	**1413**	**351**	**2733**	**6256**
Target	1450	1250	250	1550	4500
% wrt target	121%	113%	140%	176%	139%

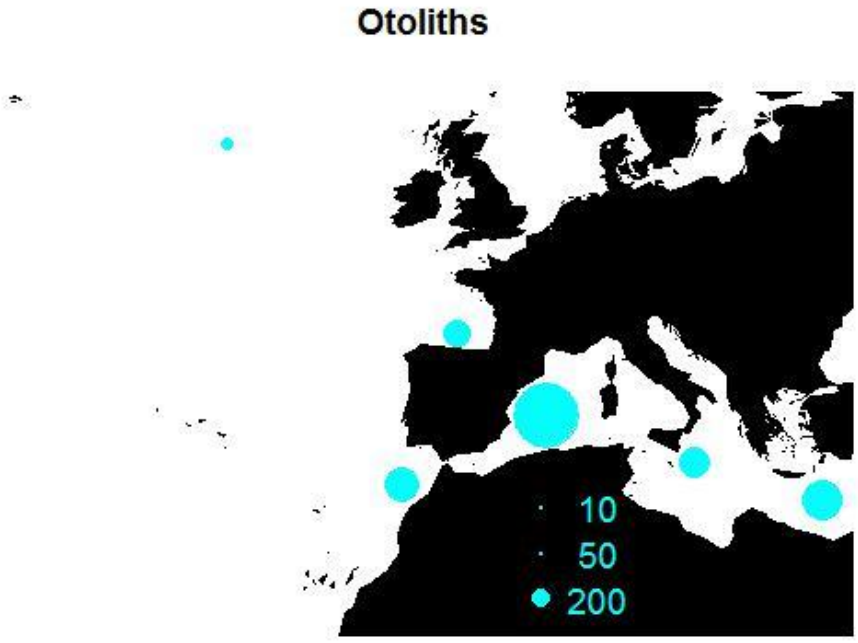

Figure 2.2: Number of individuals with otolith sampling, aggregated by main region. Positions of the dots are approximate averages across all samples. In the case of the North East Atlantic region, two dots are presented, one in the Atlantic side of the Strait of Gibraltar and the other in the Bay of Biscay.

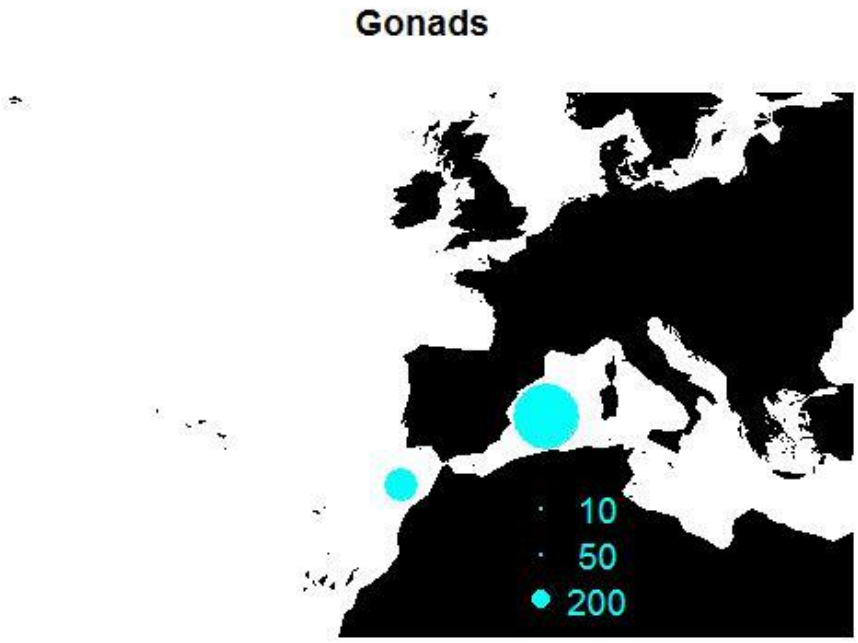

Figure 2.3: Number of gonads collected, aggregated by main region. Positions of the dots are approximate averages across all samples.

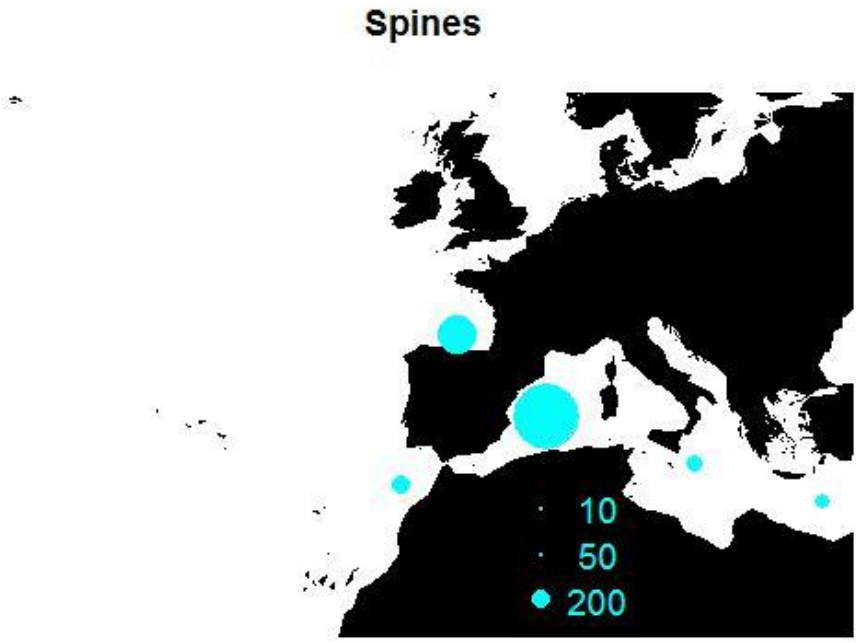

Figure 2.4: Number of spines collected, aggregated by main region. Positions of the dots are approximate averages across all samples. In the case of the North East Atlantic region, two dots are presented, one in the Atlantic side of the Strait of Gibraltar and the other in the Bay of Biscay.

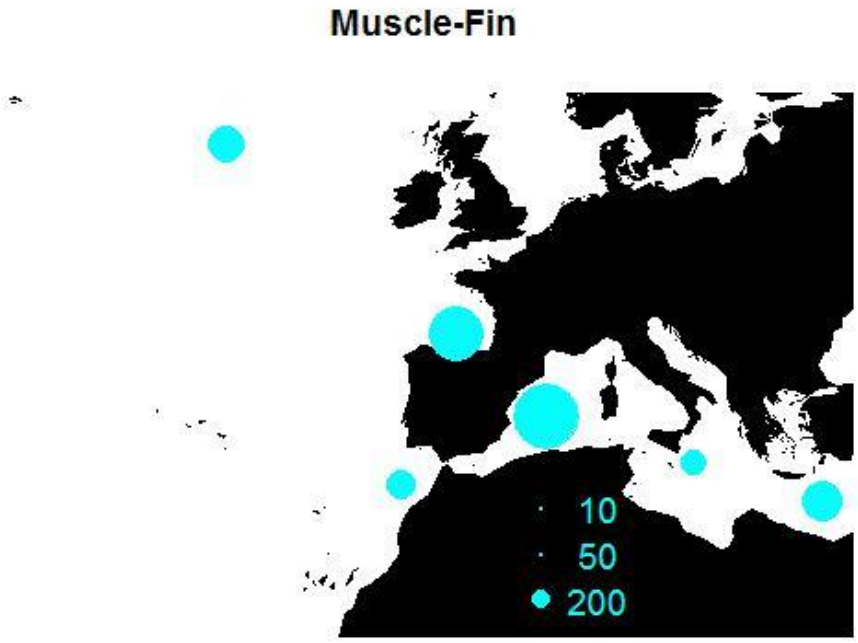

Figure 2.5: Number of muscle or fin tissue samples collected, aggregated by main region. Positions of the dots are approximate averages across all samples. In the case of the North East Atlantic region, two dots are presented, one in the Atlantic side of the Strait of Gibraltar and the other in the Bay of Biscay.

Most of these samples have been sent to AZTI, following the protocols (although some samples were directly sent to the analyst due to time constraints). This step allows for quality control of the samples and the coding, as well as fulfilling the requirement of having a centralized collection of samples for future use. The samples are conserved following the protocols and stored in the central facilities of AZTI-Tecnalia in Pasaia (contact persons: Igaratza Fraile and Nicolas Goñi). The samples already distributed to other labs (for analyses under different tasks) are tagged in the database.

3. ANALYSES

In the proposal, the consortium proposed to analyze a subset of 400 otoliths (for microchemistry), 250 hard part structures (for aging), 1000 muscle/fin samples for genetic analyses and 60 gonads. The number of samples obtained is above those targets. However, the number of samples described in the previous section reflects all the samples that are collected at the time of writing this report. As reflected in the Interim Report, the late start of the contract affected the ability of partners to conduct sampling, send samples to AZTI, proceed with planned subcontracts, etc. implying that availability of checked samples for analyses was generally low. On top of this, the tight deadlines for conducting the analyses and the time needed to accomplish them urged to start analyses as soon as possible. This, in some cases, limited the samples that were analyzed to those that were first available.

The following sections reflect the status of analyses conducted by the consortium. The samples that were not analyzed in 2012 remain stored in AZTI for future analyses, where a more optimized design of the different analyses can be approached.

4. GENETIC ANALYSIS OF ATLANTIC BLUEFIN TUNA USING NOVEL GENOMICS TOOLS

Task Leader: Fausto Tinti (UNIBO)

Participants

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UNICA: Piero Addis, Rita Cannas.

Biogenomics-KULeuven: Gregory Maes, Jeroen van Houdt.

IFREMER: Jean-Marc Fromentin.

AZTI: Haritz Arrizabalaga, Urtzi Laconcha, Igaratza Fraile, Nicolas Goñi, Naiara Rodriguez Ezpeleta.

4.1 State-of-the-art

The novel genetic strategy and task carried out in the GBYP-Phase2, namely the Reduced Representation Sequencing and Genotyping (RRSG) permitted to select large panels of outlier and high-divergent SNP loci (from thousands to hundreds in the NGS-RRSG) useful to discriminate preliminarily the ABFT reference spawning population samples. However, the species misidentification of most larvae of the ABFT reference spawning population sample from the Eastern Mediterranean and the low DNA quality/quantity yields have affected and prevented the completion of a wide and representative population genomic structure analysis of ABFT including all candidate populations (i.e. from Gulf of Mexico and Western and Eastern Mediterranean). Therefore, the NGS-RRSG assignment of individuals from feeding aggregate strata to the originating population/s was incomplete and provided contradictory results, which suggested further deep investigations on the composition of these feeding populations.

According to results achieved in the GBYP-Phase2, the Genetic Work Plan in GBYP-Phase3 have been focused on four main tasks with specific objectives and commitments.

- ✓ Task1: To achieve a more exhaustive ABFT strata sampling and genomic profiling for population discrimination and assignment throughout an extension of the population sample analysis design of newly collected reference spawning samples (larvae, Age 0) and feeding samples besides those already available from GBYPPhase2. Specific commitments aimed a) to add new areas from Eastern and Central Mediterranean, b) to include temporal replicates of reference spawning samples and c) to increase the size of spawning samples analysed from 24 to 40 individuals each for increasing the statistical robustness.

- ✓ Task2: To define and analyse the optimal SNP RRSG-derived loci for population structure and feeding aggregate assignment throughout bioinformatic analyses for selecting SNPs suitable for discriminating the GOM vs MED spawning populations as well as the MED spawning populations among themselves. It was expected that a broader representativeness of ABFT reference spawning populations will technically improve the assignment power of RRSG SNP panels.

- ✓ Task3: To generate extensive dataset of spatio-temporal Mediterranean BFT genetic variation and to select a validated RRSG-generated SNP panel for genetic structure and assignment by lab processing ABFT samples for DNA extraction, careful quality check for assessment of DNA quality and quantity and if needed species identification to avoid misidentified samples that could occur in Larvae strata. Specific commitment has been to genotype 1000 individuals with the most cost efficient technology available and to perform a validation test of the most performing 48-96 SNPs on a minimum number of 100 individuals for traceability purposes.

- ✓ Task4: To provide significant and reliable data on Mediterranean population structure and feeding aggregate composition throughout data analyses for population genomic analyses, including commonly-used descriptive statistics to estimate genetic diversity and differentiation among samples with outlier loci (the most performing, with the highest Fst values).

Below we reported the activities and results we have obtained according to GBYP-Phase3 task objectives and commitments.

4.2 Sampling design for genomic profiling by NGS-RRSG

With the objective of achieving a more exhaustive spatio-temporal genetic profile of spawning and feeding Mediterranean populations, the genetic experimental design of NGS-RRSG for GBYP-Phase3 was conceived in order to include suitable samples to:

- ✓ increase the size of reference spawning samples (Larvae/Age0) already analysed by RRSG in GBYP-Phase2 from 24 to 40 individuals each, for having a statistically more robust representation of genetic variation within sample;

- ✓ extend the genotyping to spawning samples already available from GBYPPhase2 Sampling (EMED: Levantine Sea; WMED: South Tyrrhenian, Balearic 2009 and Balearic 2010);

- ✓ include all newly available spawning samples collected in 2012 within GBYP-Phase3 activities.;

- ✓ obtain a more representative geographic sampling adding new areas with respect to the population sample design analysis of GBYP-Phase2, especially from Eastern and Central Mediterranean and Central and Western Atlantic;

- ✓ include temporal replicates of reference spawning samples and feeding aggregates for better assessing interannual variation.

When possible we aimed at reaching a sample size of 40 individuals for reference spawning samples (Larvae/Age0) and of 24 individuals for feeding aggregates samples (from Juveniles to Large adults).

Final selection and scheduling of samples for the RRSG was also conditioned by results of DNA quality and quantity check (exclusion of poor DNA samples) and species identification to avoid misidentified samples that could occur in Larvae strata.

The sampling design and results achieved for genomic profiling are reported in Table 4.1, with a total of **51 strata included**, covering **Mediterranean and Atlantic ABFT spawning and feeding areas**, with special emphasis for **reference spawning samples** which are represented by **20 strata** from **six different geographical areas** and **temporal replicates**.

To cope with time constraints of each activities (sampling, sample lab processing and data analyses required) it was decided to process all samples with the same genotyping RRSG technique, instead of having two separate genotyping step carried out with two different technologies. Moreover, this unique approach was evaluated as more cost efficient, given the multiplexing potential of RRSG where 192 individuals can be processed in the same sequencing lane. Several steps need to be followed for RRSG: testing of DNA quality and quantity, testing of restriction enzymes for optimal fragment number and finally the full analyses using the 192 barcodes which uniquely identify the individuals that will be processed in the same sequencing lane.

4.3 Lab processing of samples

In order to arrange a proper lab processing of samples a scheduling of **three separate batches of 384 individuals each** was planned: samples already collected in GBYP-Phase2 were included in Batch1, while newly collected samples in 2012 from GBYP-Phase3 sampling activities or from additional collaborations were subsequently added to Batch2 and Batch3 according to availability of samples from sampling partners.

The samples of **Batch 1 (384 individuals)** were delivered **mid August**. The samples of **Batch 2 (384 individuals)** were delivered at the **beginning of November**. The samples for **Batch 3 (384 samples)** were delivered in two phases: at the **beginning of December and at the end of December**.

After an initial Quality Check of sample DNA quality and quantity, the RRSG libraries of Batch 1 were constructed and sent for sequencing by the end of August 2012. In total, 384 individuals were processed (see Table 4.1). Two full hiSeq2500 lanes were generated and read numbers were evaluated. The full dataset was available for first bioinformatic analyses on 26/09/2012.

After an initial Quality Check of sample DNA quality and quantity, the RRSG libraries were constructed for a total of **576 samples** (Batch 2 and 50% of Batch 3) and sent for **sequencing by early January 2013**. The remaining 192 samples have undergone a Quality Check and were sent for sequencing end-of January (results expected mid February). In total, during Phase 3 Biogenomics **sequenced 1152 individuals**. In the future, all poorly sequenced individuals, which are crucial for the analyses (reference samples or most important mixed populations) will be resequenced to improve the SNP genotyping quality.

Analyses were obviously delayed by the late signature of the contract, which affected the ability of partners to sample, send samples, prepare subcontracts, etc. Given the delays throughout the sampling period and subsequent late arrival of samples at Biogenomics, the data for preliminary **population genomic analysis** could only be composed of a **total of 555 individuals (384 individuals of Batch 1** pooled with **171 individuals already genotyped from Phase 2).**

4.4 Screening of larvae using barcoding techniques

In order to avoid unnecessary RRSG analysis of non-ABFT larvae, all 38 candidate larvae collected by AZTI-Tecnalia from the Levantine Sea as well as all 92 candidates collected by UNIBO off the southern coast of Sicily were identified to species level using molecular techniques. Due to the inherent difficulty of identifying larvae based upon morphological features alone, as well as the physical damage suffered by many dispatched samples, it was necessary to utilize a more demanding yet precise methodology. DNA was extracted from all larvae following the same protocol used throughout the study. Subsequent extracts were amplified via PCR targeting a ~650bp fragment of the *cytochrome oxidase 1* gene. All resulting amplicons were then sequenced and aligned using Mega 5 software. The species

identified by the resulting phylogenetic tree (Appendix GEN1) were then verified by submitting each sequence to the NCBI BLAST engine. In total 21 ABFT larvae were identified amongst the 92 samples from Sicily, while ALL larvae collected from the Levantine Sea proved to be NON-ABFT larvae.

REGION	AREA	YEAR	SIZE CLASS	STRATA	STRATA TYPE	RRSG Sample size	GBYP PHASE/ RRSG BATCH	PROCESSING STATUS		
								SEQUENCED AND ANALYZED	SEQUENCED	ONGOING SEQUENCING
EMED	LS	2011	V	EMED-LS-V-2011	**REFERENCE SAMPLE**	10	Phase2+Phase3-Batch1	10		
EMED	LS	2011	0	EMED-LS-0-2011	**REFERENCE SAMPLE**	29	Phase3-Batch1	29		
EMED	LS	2012	0	EMED-LS-0-2012	**REFERENCE SAMPLE**	40	Phase3-Batch2		40	
EMED	LS	2012	0	EMED-LS-0-2012b	**REFERENCE SAMPLE**	21	Phase3-Batch3			21
EMED	LS	2007	M	EMED-LS-M-2007	*FEEDING AGGREGATE*	26	Phase3-Batch3			26
EMED	LS	2011	M	EMED-LS-M-2011	*FEEDING AGGREGATE*	25	Phase3-Batch2		25	
EMED	LS	2011	L	EMED-LS-L-2011	*FEEDING AGGREGATE*	25	Phase3-Batch1	25		
CMED	AS	2011	J	CMED-AS-J-2011	*FEEDING AGGREGATE*	24	Phase2	24		
CMED	AS	2012	J	CMED-AS-J-2012	*FEEDING AGGREGATE*	25	Phase3-Batch1	25		
CMED	MA	2011	M	CMED-MA-M-2011	*FEEDING AGGREGATE*	22	Phase3-Batch2		22	
CMED	MA	2011	L	CMED-MA-L-2011	*FEEDING AGGREGATE*	25	Phase3-Batch2		25	
CMED	SI	2012	V	CMED-SI-V-2012	**REFERENCE SAMPLE**	21	Phase3-Batch3		8	13
CMED	SI	2011	0	CMED-SI-0-2011	**REFERENCE SAMPLE**	21	Phase3-Batch3			21
CMED	SI	2012	0	CMED-SI-0-2012	**REFERENCE SAMPLE**	40	Phase3-Batch3		40	
CMED	SI	2011	J	CMED-SI-J-2011	*FEEDING AGGREGATE*	25	Phase3-Batch3			25
CMED	SI	2011	M	CMED-SI-M-2011	*FEEDING AGGREGATE*	25	Phase3-Batch2		25	
WMED	BA	2009	0	WMED-BA-0-2009	**REFERENCE SAMPLE**	41	Phase2+Phase3-Batch1	41		
WMED	BA	2010	0	WMED-BA-0-2010	**REFERENCE SAMPLE**	45	Phase2+Phase3-Batch1	45		
WMED	BA	2011	0	WMED-BA-0-2011	**REFERENCE SAMPLE**	40	Phase3-Batch1	40		
WMED	BA	2012	0	WMED-BA-0-2012	**REFERENCE SAMPLE**	40	Phase3-Batch2		40	
WMED	BA	2011	J	WMED-BA-J-2011	*FEEDING AGGREGATE*	25	Phase3-Batch2+3		25	
WMED	BA	2011	M	WMED-BA-M-2011	*FEEDING AGGREGATE*	25	Phase3-Batch3		25	

WMED	GL	2011	J	WMED-GL-J-2011	*FEEDING AGGREGATE*	25	Phase3-Batch2			25	
WMED	GL	2011	M	WMED-GL-M-2011	*FEEDING AGGREGATE*	25	Phase3-Batch2			25	
WMED	LI	2012	0	WMED-LI-0-2012	**REFERENCE SAMPLE**	21	Phase3-Batch3				21
WMED	LI	2011	J	WMED-LI-J-2011	*FEEDING AGGREGATE*	24	Phase2	24			
WMED	LI	2012	M	WMED-LI-M-2012	*FEEDING AGGREGATE*	23	Phase3-Batch3				23
WMED	SA	2011	M	WMED-SA-M-2011	*FEEDING AGGREGATE*	24	Phase2	24			
WMED	SA	2011	L	WMED-SA-L-2011	*FEEDING AGGREGATE*	25	Phase3-Batch2			25	
WMED	SA	2012	L	WMED-SA-L-2012	*FEEDING AGGREGATE*	26	Phase3-Batch3				26
WMED	TY	2011	0	WMED-TY-0-2011	**REFERENCE SAMPLE**	37	Phase3-Batch1	37			
WMED	TY	2012	0	WMED-TY-0-2012	**REFERENCE SAMPLE**	40	Phase3-Batch2			40	
WMED	TY	2012	0	WMED-TY-0-2012b	**REFERENCE SAMPLE**	40	Phase3-Batch3			40	
WMED	TY	2011	M	WMED-TY-M-2011	*FEEDING AGGREGATE*	25	Phase3-Batch3			25	
NEATL	BB	2011	J	NEATL-BB-J-2011	*FEEDING AGGREGATE*	24	Phase2	24			
NEATL	BB	2011	M	NEATL-BB-M-2011	*FEEDING AGGREGATE*	21	Phase3-Batch2+3			21	
NEATL	BB	2011	L	NEATL-BB-L-2011	*FEEDING AGGREGATE*	25	Phase3-Batch2+3			25	
NEATL	GI	2011	L	NEATL-GI-L-2011	*FEEDING AGGREGATE*	25	Phase3-Batch3			25	
NEATL	MO	2011	L	NEATL-MO-L-2011	*FEEDING AGGREGATE*	25	Phase3-Batch1	25			
NEATL	PO	2011	L	NEATL-PO-L-2011	*FEEDING AGGREGATE*	24	Phase2	24			
NEATL	PO	2012	L	NEATL-PO-L-2012	*FEEDING AGGREGATE*	25	Phase3-Batch3				25
CNATL	CA	2011	M	CNATL-CA-M-2011	*FEEDING AGGREGATE*	22	Phase3-Batch1	22			
CNATL	CA	2011	L	CNATL-CA-L-2011a	*FEEDING AGGREGATE*	24	Phase3-Batch1	24			
CNATL	CA	2011	L	CNATL-CA-L-2011b	*FEEDING AGGREGATE*	24	Phase3-Batch1	24			
CNATL	CA	2011	L	CNATL-CA-L-2011c	*FEEDING AGGREGATE*	24	Phase3-Batch1	24			
CNATL	CA	2011	L	CNATL-CA-L-2011d	*FEEDING AGGREGATE*	24	Phase3-Batch1	24			
WATL	GM	2009	V	WATL-GM-V-2009	**REFERENCE SAMPLE**	24	Phase2+Phase3-Batch1	24			

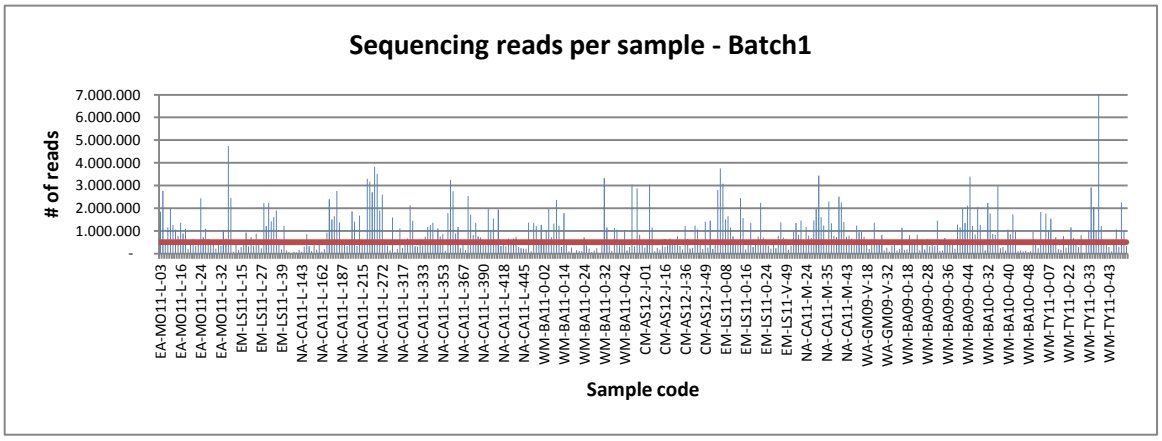
WATL	GM	2007	V	WATL-GM-V-2007	**REFERENCE SAMPLE**	11	Phase3-Batch2		11	
WATL	GM	2008	V	WATL-GM-V-2008	**REFERENCE SAMPLE**	14	Phase3-Batch2		14	
WATL	GM	2009	V	WATL-GM-V-2009b	**REFERENCE SAMPLE**	25	Phase3-Batch2		25	
WATL	GM	2008	0	WATL-GM-0-2008	**REFERENCE SAMPLE**	16	Phase2	16		
			TOTAL	51		1332		555	576	201

Table 4.1: Sampling design and results for genomic profiling of GBYP-Phase3. Samples collected under GBYP 06/2011 and available from other research programs are included.

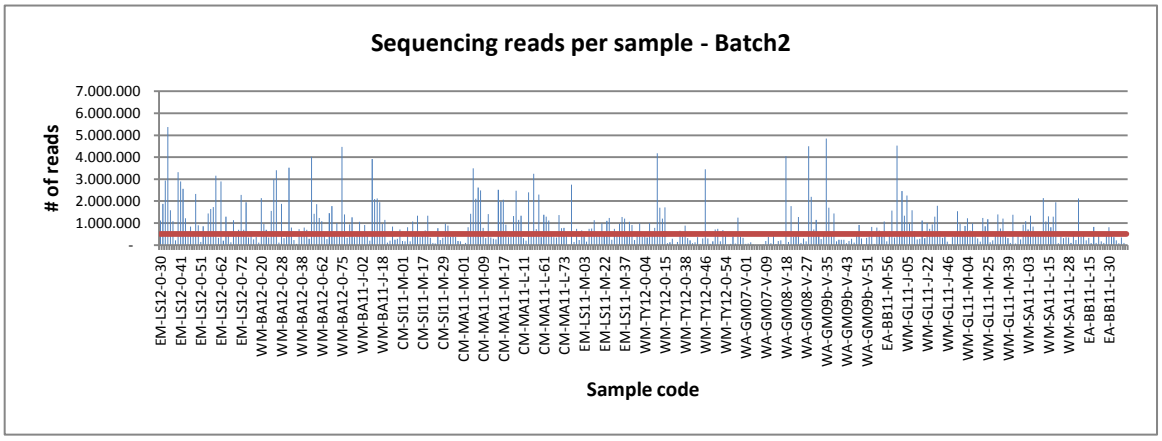
4.5 RRSG results

Given the source, quality, storage history and multiple uses of DNA samples used in this study, many samples did not reach the quality threshold for RRSG (100ng good quality, high molecular weight DNA). Overall, the sequencing results were good, as the RRSG libraries produced a total of **353 million reads** for 384 individuals of Batch 1 (**Figure 4.1a**). For **Batch 2**, we generated **302 million reads** (**Figure 4.1b**), while half of **Batch 3** produced already **202 million reads** (**Figure 4.1c**). To define reliable genotypes for several thousand SNP loci a **minimum coverage of 500,000 reads** (see red line in Figure 4.1a, b and c) has been set and only **70 % of samples meet that threshold**, with different percentage of suitable individuals for downstream analysis in each of the analyzed samples (Figure 4.2a and b). This means that **2-3 repeat sequencing runs will be needed to increase the number of individual samples** used for analysis to 80-90 % of the dataset.

a)



b)



c)

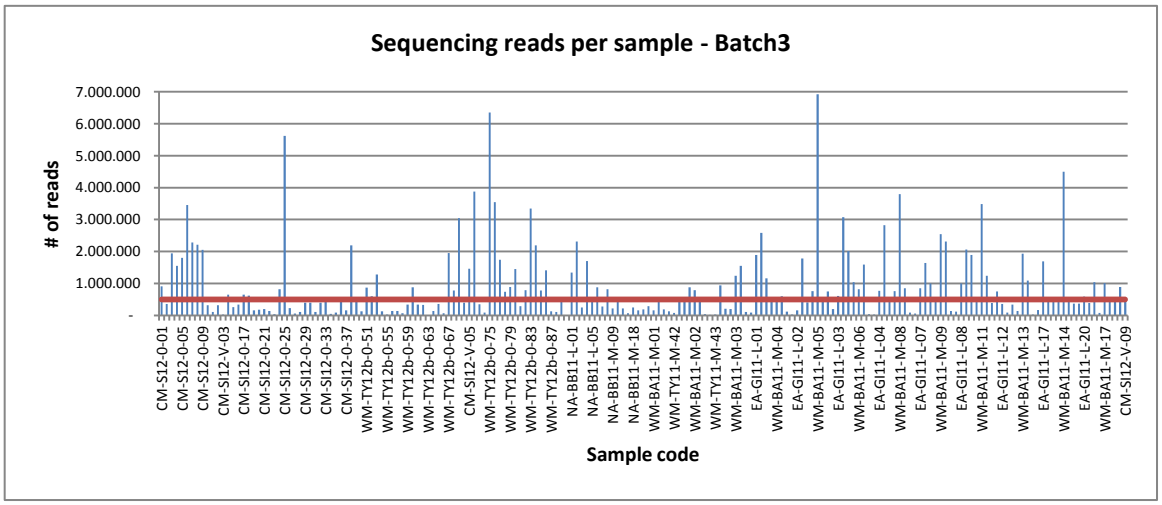
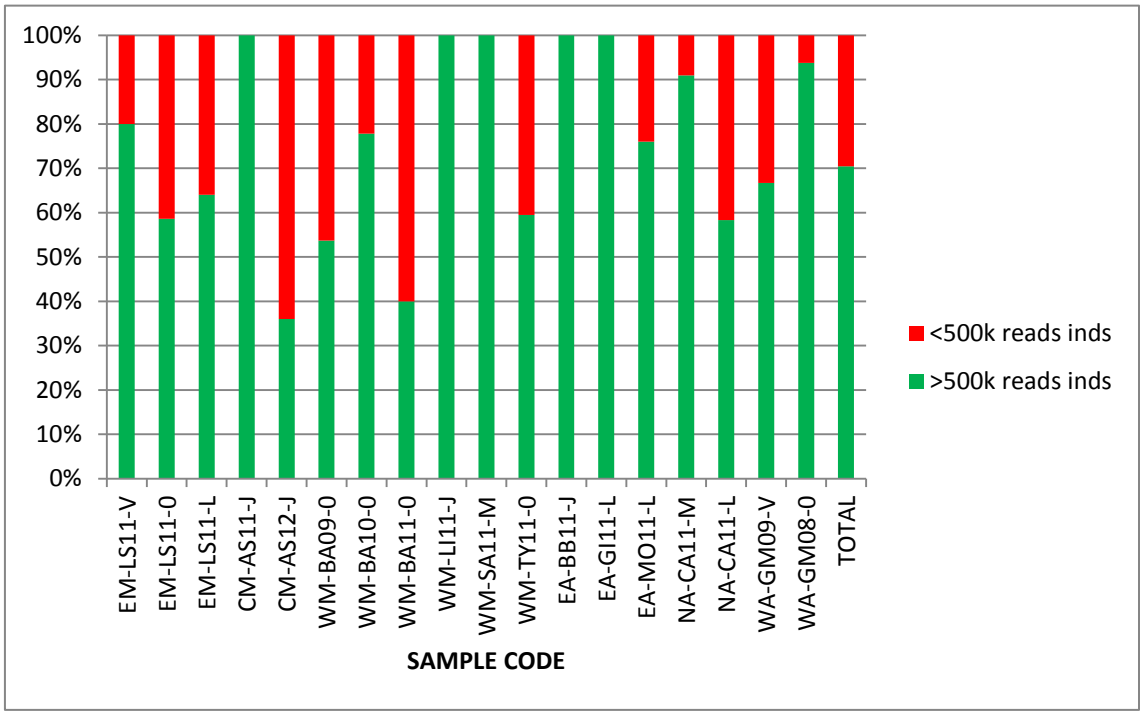


Figure 4.1: Sequencing reads per individual of a) Batch 1 (384 individuals), b) Batch 2 (384 individuals) and c) half of Batch 3 (192 individuals). The red line (500,000 reads) shows the minimum threshold for reliable genotypes per individual.

a)



b)

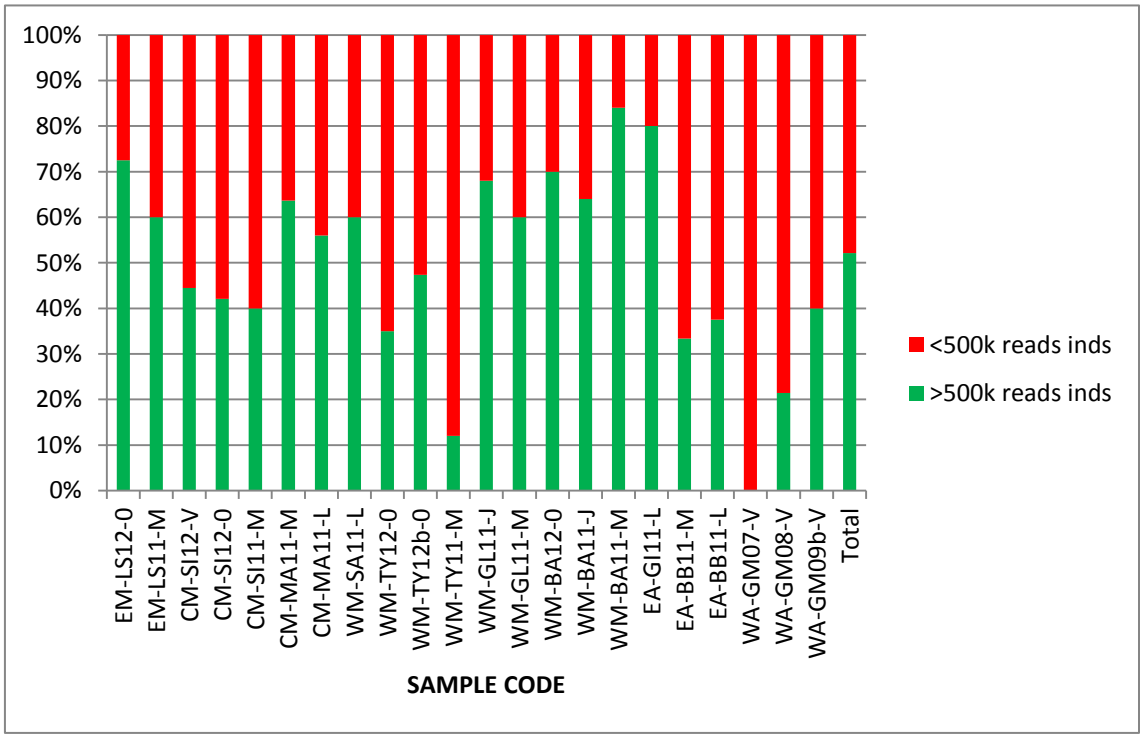


Figure 4.2: Percentage of optimal (>500,000 reads, green bar) and suboptimal (<500,000 reads, red bar) individuals per samples of a) Batch 1 b) Batch 2 (384 individuals) and half of Batch 3 (192 individuals).

4.6 Data-analysis

We first carried out the bioinformatic analysis of the RRSG data, allowing the simultaneous discovering and genotyping of a large number of genetic markers for connectivity/traceability purposes. To do so, we analysed raw sequencing data, summarized this into tags, where after a set of SNPs per individual could be genotyped. We used two methodologies to do this, namely a ***de novo*** (only using RRSG data) and a **reference map** (using also the reference assembly from GBYP) approach. These SNP genotypes could then be analysed in specific downstream population genomic software. For population genomic analyses, commonly-used descriptive statistics to estimate genetic diversity and differentiation among samples were performed. Using population genetic statistical tests implemented in the up-to-date versions of population genetic software (improved in order to deal with large datasets) resolution of analyses was greatly increased (Genepop, Genetix, FSTAT v 2.9.4, Arlequin v 3.5). Outlier analyses were performed using various software (Lositan, Bayescan) to define the most discriminative markers (those with the highest Fst values). Descriptive, multivariate (PCA and MDS) and clustering methods in order to discriminate population units, were performed using the specific R package *Adegenet* for the analysis of large datasets. Multiple approaches were applied for population structure analysis, as this analysis represents a crucial step for genetic stock identification and for further analyses concerning individual assignment to population of origin. ABFT spatial population structure will further be explored using Bayesian MCMC clustering approaches implemented in the software Structure v2.3.3 and BAPS.

4.7 Population genomic results

To analyse the 555 individuals and samples from Phase 2 and Batch 1 of Phase 3 together, we first applied a threshold of 500 thousand reads per individual to define the best contributing samples for analysis. As scheduled in the Genetic WorkPlan, the **initial focus was given to "Reference (Spawning) Samples"** (Larvae and Age0 strata) in order to define genetic markers. Once Reference Samples can be clustered and assigned with confidence, only then could a mixed stock analysis be performed

with certainty to assign the individuals of Feeding Aggregate strata (Juveniles, Medium and Large strata) back to the originating spawning population.

Hence, the results presented here are preliminary and limited to the population genomic structure of the Reference Samples. They will be further expanded once the final set of Reference Samples will be analysed (see the paragraph Further Scheduled Activities). We only show results here of the ***de novo*** **approach**.

4.8 SNP numbers and datasets

In total **we defined 1 million SNPs for ABFT** over the complete datasets, from which subsets will be analysed in depth for all initial 555 individuals (phase 2 and phase 3-Part 1). Inherent to the RRSG, the number of SNPs (or proportion) that can be genotyped and analysed per individual is dependent on the number of reads per individual. Poor quality individuals will be sequenced less, thereby yielding fewer suitable loci for downstream analyses. Further re-sequencing of these individuals is the only solution to increase their information content. The Figure 4.3 shows the proportion of genotyped SNPs of the 1 million maximum related to sequencing reads per each individual. We clearly see that for our threshold of 500,000 reads per individual (red bar), we will be able to **genotype and analyse from 100,000 to 200,000 SNPs**.

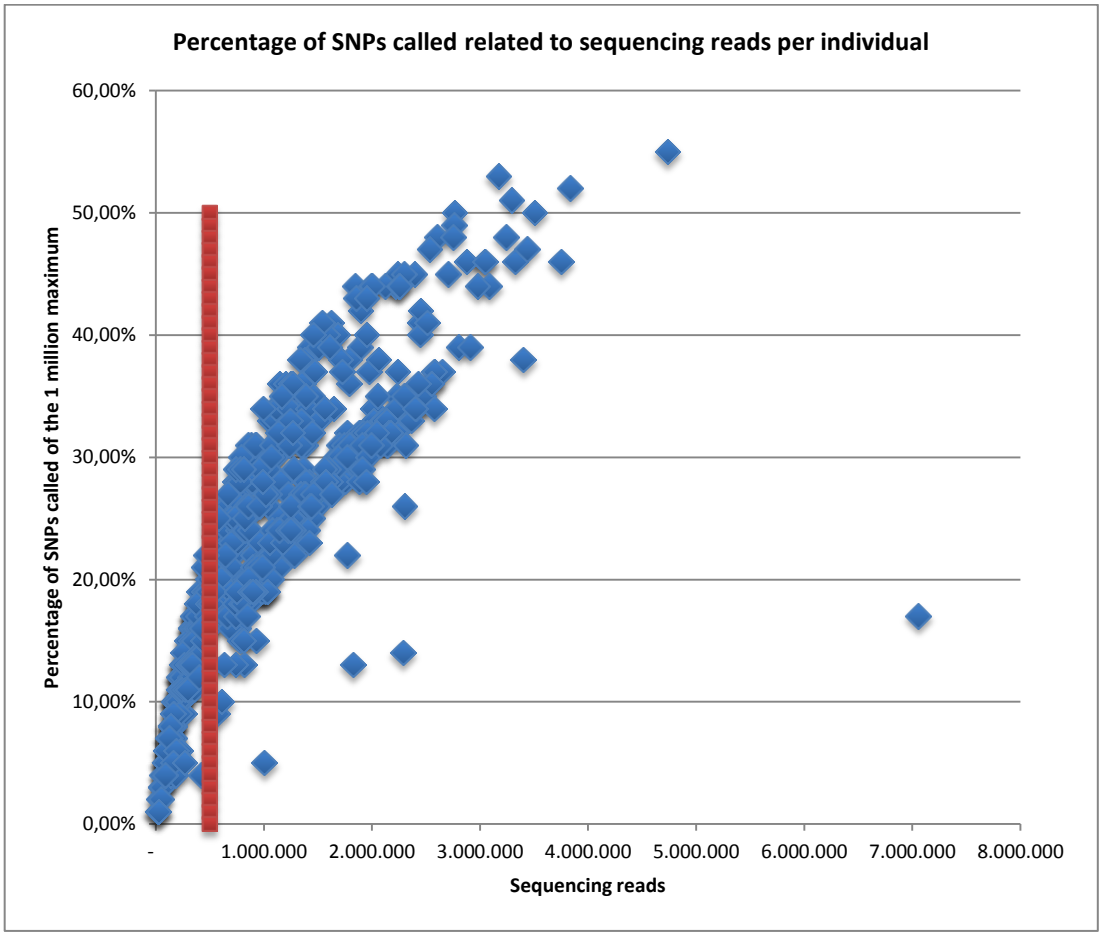


Figure 4.3: Proportion of genotyped SNPs (Y-axis) of the 1 million maximum related to sequencing reads (X-axis) per individual. The vertical red bar indicates the threshold of optimally genotyped individuals (>500,000 reads).

Based on these results, we defined a strategy to test various genotype thresholds per population sample, to increase the reliability and sample number for population genetic analyses. The table 4.2 shows our strategy, namely the number of SNPs and of individuals selected by 1) choosing a **50 %** (dataset 1 and 2) and **70 %** (dataset 3 and 4) **well genotyped individuals per population** (>500,000 reads), 2) choosing to **input well genotyped individuals only from the reference (REF, a)** or all (**ALL, b) populations** and finally 3) choosing to **output well genotyped (1 and 3)** or **all (2 and 4) individuals** for analysis.

Table 4.2: Datasets produced and analysed for the de novo approach. The datasets in bold were analysed with priority.

	% well genotyped individuals per pop	**Input** pops	**Output** individuals	Number of **SNPs**	Number of **individuals**
Dataset 1a	**50%**	REF	Only well genotyped inds	119,877	151
Dataset 1b	50%	ALL	Only well genotyped inds	74,532	391
Dataset 2a	50%	REF	All inds	119,877	242
Dataset 2b	50%	ALL	All inds	74,532	555
Dataset 3a	**70%**	REF	Only well genotyped inds	44,626	151
Dataset 3b	**70%**	ALL	Only well genotyped inds	16,688	391
Dataset 4a	70%	REF	All inds	44,626	242
Dataset 4b	70%	ALL	All inds	16,688	555

4.9 Outlier analysis

In a first analysis we performed an outlier SNP-based analysis with dataset 3a, to select a list of highly differentiating loci (based on FST) between the reference populations, applying four different *a priori* scenarios of differentiation 1) among All populations, 2) between Gulf of Mexico and Eastern Mediterranean, 3) between Gulf of Mexico and Western Mediterranean and 4) between Eastern Mediterranean and Western Mediterranean (Figure 4.4). This allowed us to construct a dataset with all common outliers/high grading loci for all scenarios.

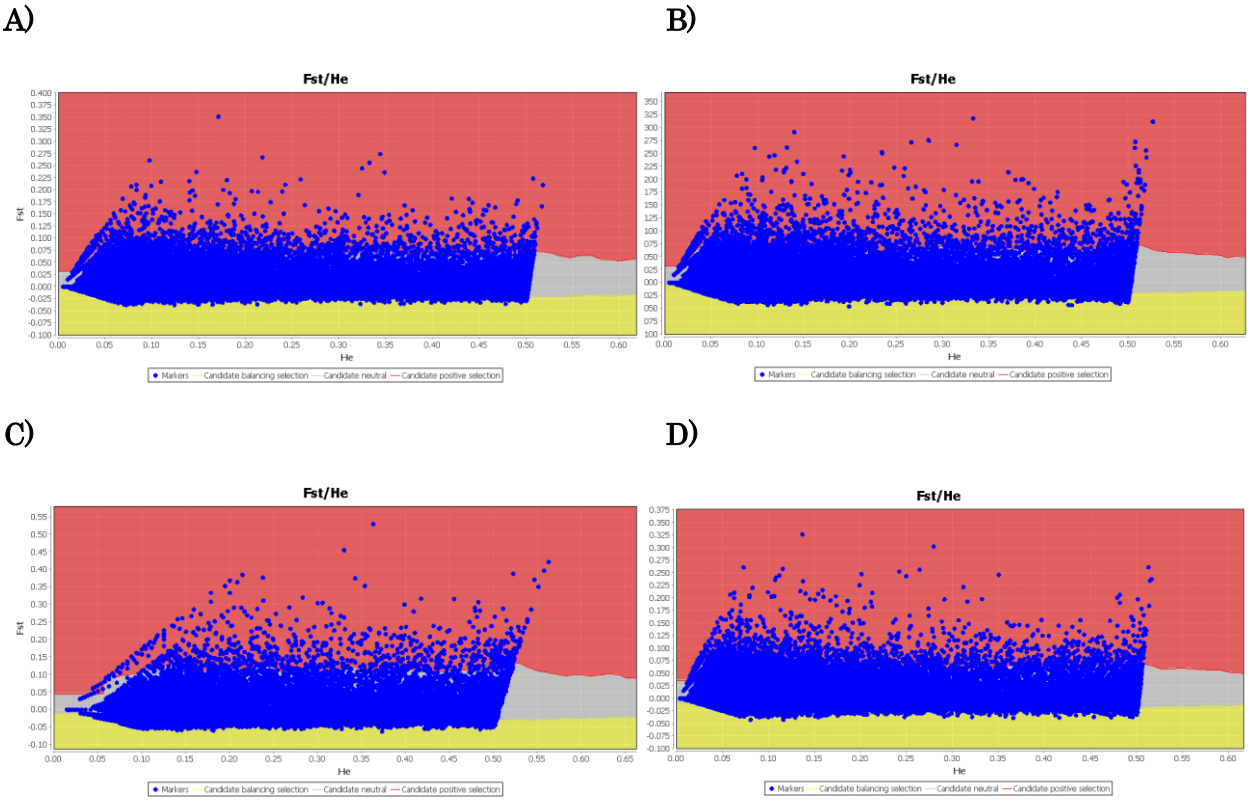


Figure 4.4: Outlier analysis for dataset 3a (source data 44,626 SNPs, 151 individuals) for all 4 scenarios: A) Gulf of Mexico vs. Western Mediterranean; B) Eastern Mediterranean vs. Western Mediterranean; C) Gulf of Mexico vs. Eastern Mediterranean; D) All Reference Populations.

Based on these results, the common outliers for dataset 3a were defined to allow a more focused population genomic analysis. Additionally, an outlier analysis was performed with the larger dataset 1a (data not shown) to define the common high grading loci between both datasets. The figure 4.5 shows a Venn-diagram pooling all outliers from all scenarios in both datasets 3a and 1a and showing the 1676 common loci selected for a first analysis.

A) B)

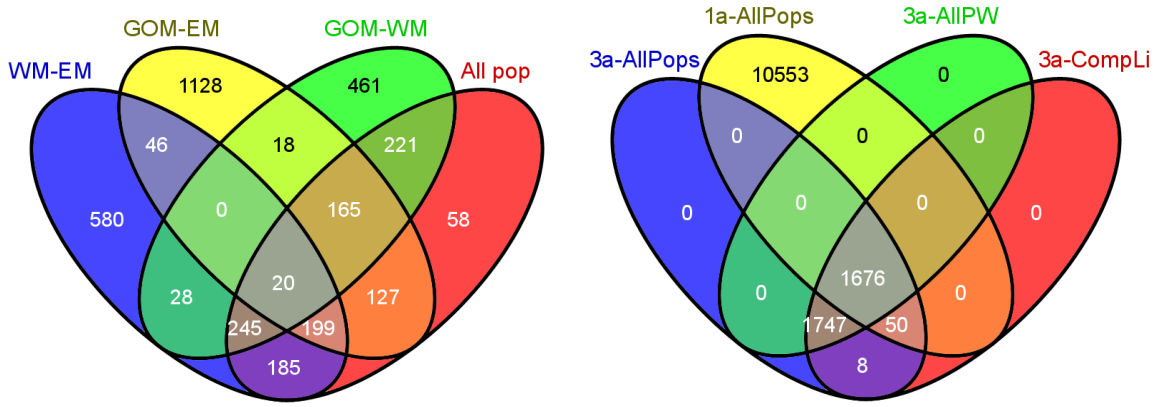


Figure 4.5: Venn-diagram showing the common outlier for each scenario for dataset A) 3a and B) 3a and 1a. Only 20 loci seem common for all 3a scenarios, while 1676 outlier loci are common to all scenarios in dataset 3a and 1a.

4.10 Descriptive analysis

A rapid descriptive analysis of the 1676 outlier loci shows that they are all variable and their genetic diversity is consistent with that expected for SNPs (maximum of 0.5 heterozygosity), despite some evidence of disequilibrium (FIS) for various loci. This will be checked further once a final panel of promising loci is defined, to discard loci with too few observations or potential technical artifacts (inherent to genomic analyses).

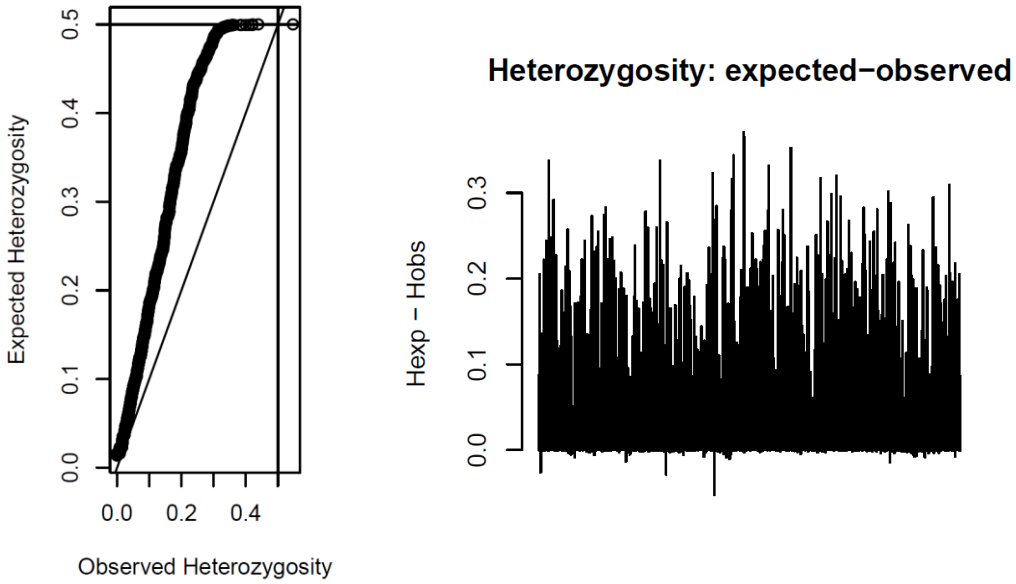


Figure 4.6: Descriptive statistics for dataset 3a-1a outlier loci (1676 SNPs). A) Overall QQ-plot for expected and observed heterozygosity. B) locus specific plot of Expected heterozygosity (Hexp) – Observed heterozygosity (Hobs).

4.11 Clustering analysis

Clustering analysis was then performed in order to define the number of potential groups within the reference sample dataset. This was done using the ADEGENET-DAPC R-tool, which is a combination of Discriminant Analysis (DA, defining groups), based upon a dataset of Principal Components (PC, reducing data to several dimensions) constructed from multi-locus genotypes. Although very preliminary, we found a good clustering of Gulf of Mexico populations, Western and, partially, Eastern Mediterranean populations. The exception is in fact the Eastern Mediterranean Age-0 strata that unexpectedly clustered with the WM reference samples (Figure 4.7).

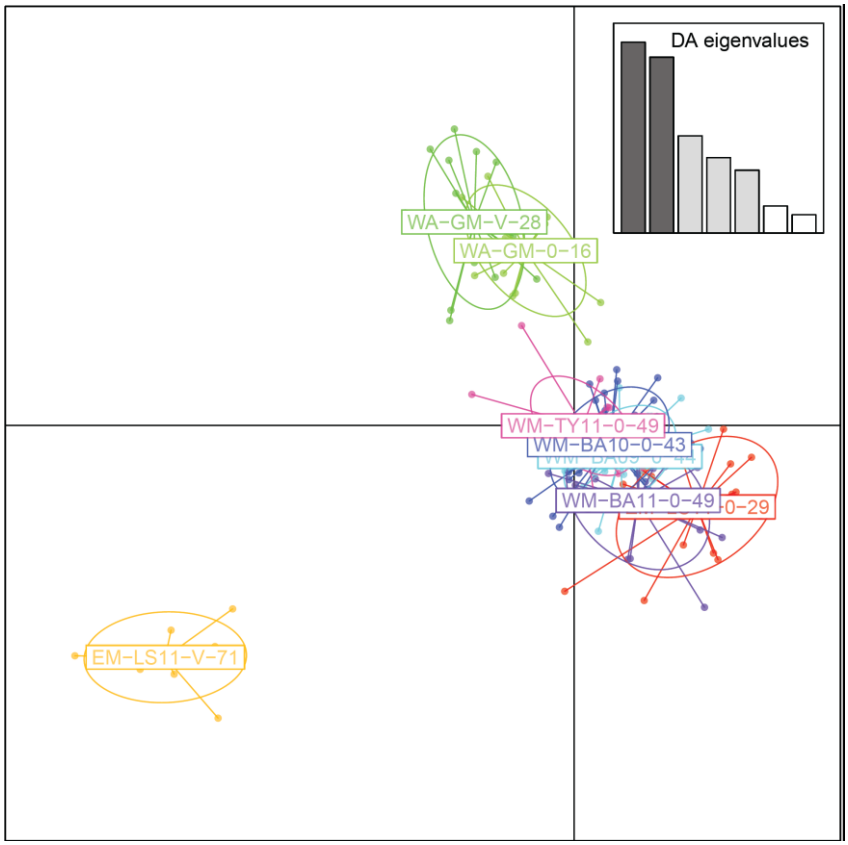


Figure 4.7: Clustering analysis using DAPC based on the eight reference samples. Three clusters can be seen, roughly coinciding to the expected spawning groups. Note the exception of the Eastern Mediterranean Age-0 sample (red samples) that clustered together with the Western Mediterranean reference samples and not with the Eastern Mediterranean Larvae sample.

When performing an assignment of individuals to the three clusters (Figure 4.8), we found a good match between sampling locations and inferred clusters, except for the Eastern Mediterranean Age-0 strata. This population clusters within the Western Mediterranean group. This finding could be due to 1) ecological reasons (Age-0 can display a more migrant behavior than what is so far known, or it could be due to occurrence of sweepstake spawning of one or more itinerant adults) or 2) an underestimated differentiation between larvae and Age-0 strata in the Eastern Mediterranean. Additional analyses are ongoing to define the SNPs defining best both populations within the Eastern Mediterranean.

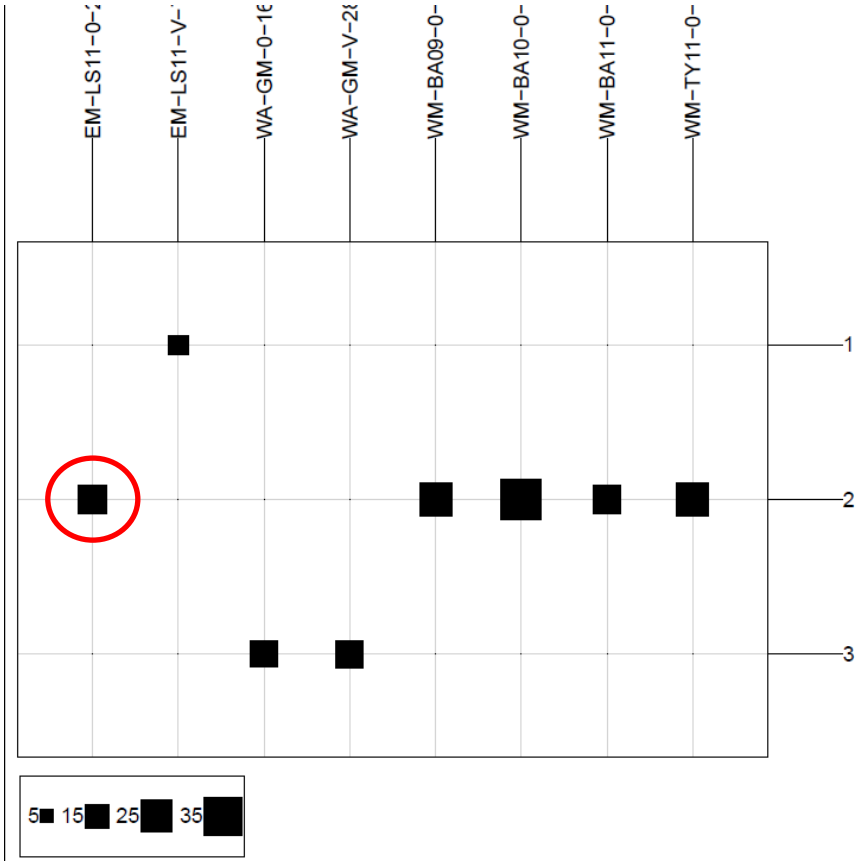


Figure 4.8: Assignment analysis to the three potential clusters (1: EM, 2: WMED, 3: GOM) defined in the DAPC analysis. Curiously, the Eastern Mediterranean Age-0 population (red circle) clusters within the WMED group (group 2).

Further evaluation of the selected markers has been carried out with the aims of i) maximizing discrimination power between the putative geographical spawning areas of Gulf of Mexico, Eastern Mediterranean and Western Mediterranean; ii) minimizing the differentiation within these geographical groups. In order to accomplish this, as well as to address the need for having a reduced number of loci suitable for feasible implementation of traceability controls, we selected a subset of 96 SNPs. When applying the same aforementioned clustering procedure we obtained an improved separation of the Eastern Mediterranean Age-0 sample from the Western Mediterranean reference samples (Figure 4.9), even if still not completely

clustering with the Eastern Mediterranean Larvae samples (potentially due to a bias linked to the lower sampling size of the EM larvae sample). With this fine-tuned panel of 96 loci it is also possible to assess a certain degree of temporal variation within populations.

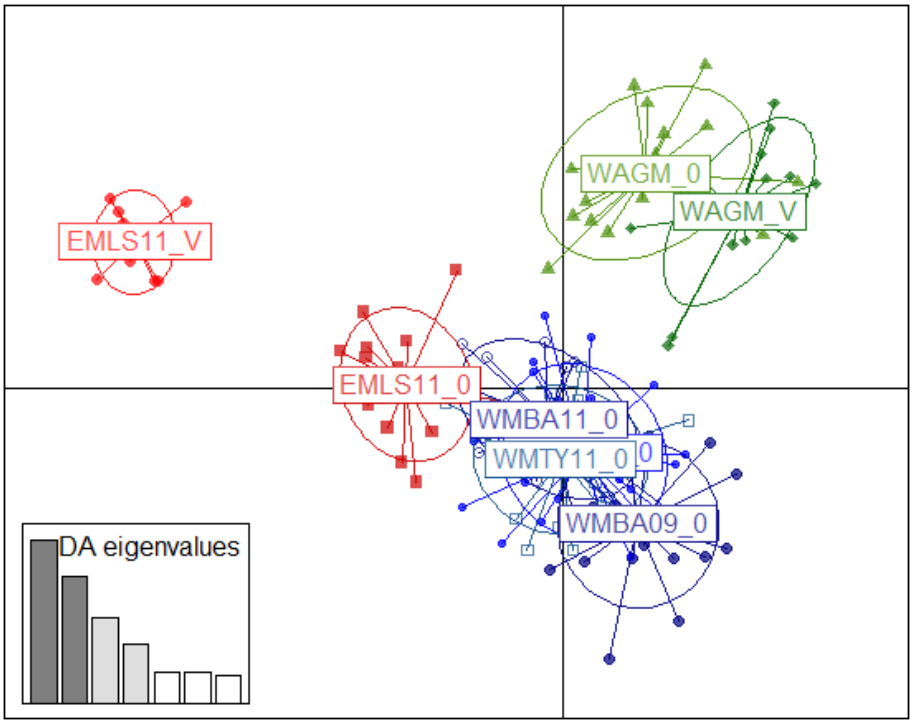


Figure 4.9: Clustering analysis using DAPC based on the eight reference samples and a restricted subpanel of 96 SNP. Three clusters can be seen, roughly coinciding with the expected spawning groups, with an improved separation of the Eastern Mediterranean Age-0 sample from the Western Mediterranean reference samples, even if yet not clustering with the Eastern Mediterranean Larvae sample.

When applying a different clustering technique in order to define the number of potential groups within the reference sample dataset, namely using the Bayesian approach implemented in the software STRUCTURE, the result obtained confirm the clustering inferred from the DAPC. Results are summarized in the membership plot shown in Figure 4.10 where, even if a certain degree of mixing, the same 3 main

clusters can be observed, with the Gulf of Mexico being clearly unique from the other populations and still having an influence on the other populations. The larvae from the Eastern Mediterranean appear to be the least influenced by GOM genetic inputs and display a unique genetic signature. As expected the YOY from the same area appear to be very similar to the Western Mediterranean strata. This may be due to differing spawning events with a few WMED adults visiting the east in 2010. Another hypothesis could be that the YOY are moving between basins, which would suggest that the larvae are the proper analysis target to truly establishing reference populations.

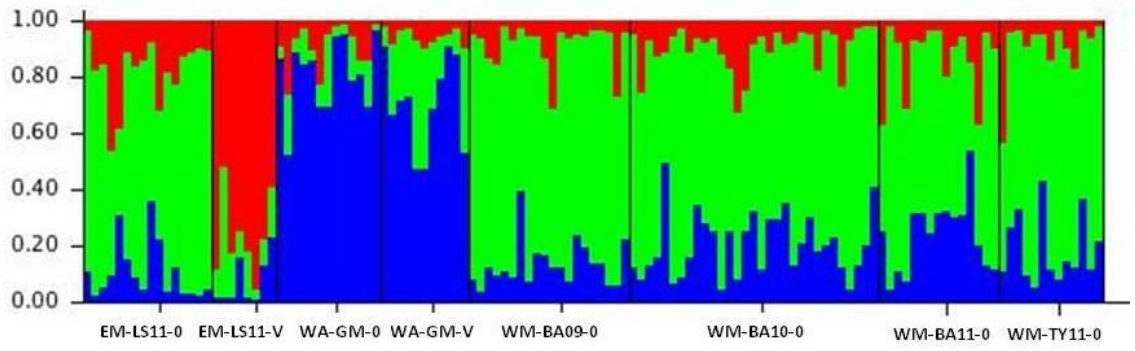


Figure 4.10: Bayesian clustering analysis using STRUCTURE based on the eight reference samples and a restricted subpanel of 96 SNP.

Since RRSG is a technique under development, a standardized and commonly accepted method to evaluate data and results is lacking for the moment and should be done in house (with respect to other more common molecular markers like gene sequences or microsatellite loci). To properly assess the technical reliability of our analyses we carried out parallel tests on more stringent datasets which were derived from the ones illustrated in Table 4.2. We applied more stringent criteria when identifying the SNP markers from the raw sequences processing: a minimum numbers of two and three sequencing reads respectively were set as minimal requirements for a SNP to be considered as such. The data analysis carried out on these higher confidence datasets provided similar results, supporting the

bioinformatic approach followed and the promising although preliminary results obtained.

4.12 Future Activities

So far, 1152 ABFT individuals have been genotyped by RRSG and the re-sequencing of individuals with non-optimal genotyping (< 500,000 reads) is already scheduled in February 2013. At this stage of the GBYP-Phase3 program, we have carried out preliminary data analyses which have shown that the ABFT population genomics strategy (i.e. population sampling and analysis design, RRSG technology and data analysis design) has generated important new significant scientific data, to be used in advice to support ICCAT ABFT management actions in the near future.

Here, we outline the data analysis activities still needed in order to complement the tasks conducted under GBYP-Phase3. Some may be scheduled in future Phases of GBYP to fine tune information content and management applications:

- ✓ To analyze the available additional reference populations once RRSG data has been properly processed
- ✓ To analyze more in depth the outlier loci and define an optimal panel for reference population assignment. An iterative selection procedure is needed to define the best loci per scenario for optimal assignment power
- ✓ To compare datasets to extract the maximum information from less successful individuals
- ✓ To perform a mixed-stock analysis of feeding aggregate samples once all RRSG data will be available
- ✓ To define the best panel for traceability in ABFT and validation with a small scale analysis platform

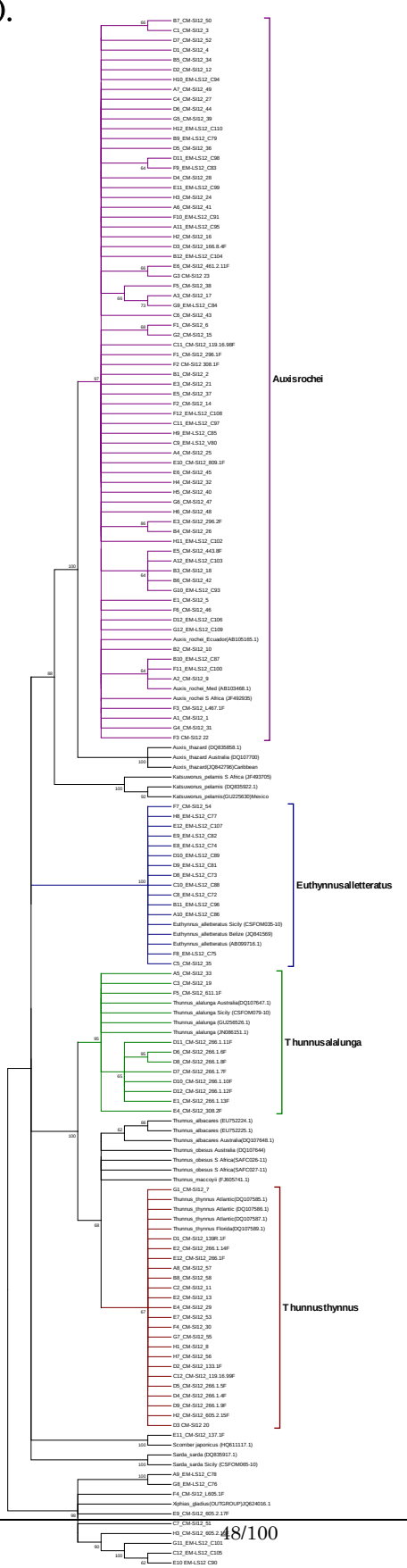
4.13 Conclusions

The preliminary data analyses carried out in GBYP-Phase 3 on a total of 555 ABFT individuals and mainly focusing on the Reference Samples, revealed that high-performing SNP panels can identify and differentiate at least three ABFT spawning populations (GOM, WMED, EMED), that are genetically well clustered (even if the differentiation of the Eastern Mediterranean ABFT Reference Samples should be improved by looking for more efficient SNP loci) and spatially and temporally stable. The assignment of adult ABFTs, potentially with highly-migratory behaviour, to the source spawning populations (not only to those spawning in the Gulf of Mexico or Mediterranean as the isotope markers can do, but also to the Western and Eastern Mediterranean spawning populations) is still to be performed at a later stage.

Due to the complexity and quantity of the RRSG-generated genomic data obtained for the ABFT (i.e. a genomic data-poor non-model fish exhibiting a complex and partially unresolved ecology and biology), various additional analyses are further required to fine tune SNP selection/validation for traceability and management purposes. Providing reliable scientific advice for ABFT tuna management by the end of January 2013 (this report) is as such not possible. Until now, we have completed the full RRSG sequencing and genotyping of more than the 1000 scheduled individuals. In the next months, we plan to analyse more in depth the genomic data generated the last 5 months, rerun failing/bad quality individuals and define a potential traceability panel (48-192 plex) to provide reliable and sound molecular diagnostics based outputs.

4.14 Genetic Appendix

Barcoding results from 130 larvae analyzed from southern coast of Sicily (n=92) and Levantine Sea (n=38).




```
Analysis
  Analysis ---------------------------- Phylogeny Reconstruction
  Scope ------------------------------- All Selected Taxa
  Statistical Method ------------------ Neighbor-joining
Phylogeny Test
  Test of Phylogeny ------------------- Bootstrap method
  No. of Bootstrap Replications ------- 1000
Substitution Model
  Substitutions Type ------------------ Nucleotide
  Model/Method ------------------------ Kimura 2-parameter model
  Substitutions to Include ------------ d: Transitions + Transversions
Rates and Patterns
  Rates among Sites ------------------- Uniform rates
  Pattern among Lineages -------------- Same (Homogeneous)
Data Subset to Use
  Gaps/Missing Data Treatment --------- Pairwise deletion
  Codons Included --------------------- 1st+2nd+3rd+Non-Coding
```

Species	CMED-SI-V	EMED-LS-V
T. thynnus	21	0
Auxis rochei	53	21
E. alletteratus	2	12
T. alalunga	11	0
S. japonicus	1	0
Other	4	5

5. ORIGIN OF ATLANTIC BLUEFIN TUNA IN THE ATLANTIC OCEAN AND MEDITERRANEAN SEA USING δ^{13}C AND δ^{18}O IN OTOLITHS

5.1 Introduction

Several novel tools are currently being used to investigate the natal origin and stock structure of Atlantic bluefin tuna, including electronic tags, molecular genetics, and otolith chemistry. Of the three, chemical markers in otoliths (ear stones) have significant potential for determining natal origin and population connectivity of bluefin tuna (Rooker et al. 2007). This is due to the fact that otoliths precipitate material (primarily calcium carbonate) as a fish grows, and the chemical composition of each newly accreted layer is often associated with physicochemical conditions of the water mass they inhabit. As a result, material deposited in the otolith during the first year of life serves as a natural marker of the individual's nursery or place of origin. Previous studies have demonstrated that trace elements and stable isotopes in otoliths can be used to determine the origin of bluefin tuna from different regions in the Atlantic Ocean and its marginal seas (Mediterranean Sea and Gulf of Mexico; see Rooker et al. 2008a,b, Schloesser et al. 2010). Results from these studies indicate that trans-Atlantic movement is more significant than previously assumed, with a considerable fraction of adolescents in US water originating from spawning/nursery areas in the east (Mediterranean Sea).

5.2 Material and Methods

Here, we investigate the origin of bluefin tuna collected in the central and eastern Atlantic Ocean as well as a variety of locations within the Mediterranean Sea using stable δ^{13}C and δ^{18}O isotopes in otoliths. Samples utilized for this study were

collected under the GBYP and otolith handling followed the protocols previously described in Rooker et al. (2008b). Briefly, following extraction by GBYP participants, sagittal otoliths of bluefin tuna were cleaned of excess tissue with hydrogen peroxide and deionized water. One sagittal otolith from each bluefin tuna specimen was embedded in Struers epoxy resin (EpoFix) and sectioned using a low speed ISOMET saw to obtain 1.5 mm transverse sections that included the core. Following attachment to a sample plate, the portion of the otolith core corresponding to approximately the yearling periods of bluefin tuna was milled from the otolith section using a New Wave Research MicroMill system. A two-vector drill path based upon otolith measurements of several yearling bluefin tuna was created and used as the standard template to isolate core material following Rooker et al. (2008b). The pre-programmed drill path was made using a 500 μm diameter drill bit and 15 passes each at a depth of 50 μm was used to obtain core material from the otolith. Powdered core material was transferred to silver capsules and later analyzed for $\delta^{13}C$ and $\delta^{18}O$ on an automated carbonate preparation device (KIEL-III) coupled to a gas-ratio mass spectrometer (Finnigan MAT 252). Stable $\delta^{13}C$ and $\delta^{18}O$ isotopes are reported relative to the PeeDee belemnite (PDB) scale after comparison to an in-house laboratory standard calibrated to PDB.

Region-specific estimates of nursery origin of bluefin tuna were based on comparing otolith 'cores' (corresponds to otolith material deposited during the first year of life or yearling period) of juvenile and adult bluefin tuna to the baseline or reference samples of yearling bluefin tuna. Estimate of origin for juvenile (3-25 kg) and adult (medium and large (>25-100 kg and > 100 kg, respectively)) bluefin tuna were obtained using the maximum likelihood estimate (MLE) from the mixed-stock program HISEA developed by Millar (1990). Baseline data for mixed-stock analysis was otolith $\delta^{13}C$ and $\delta^{18}O$ of yearling samples collected in the east and west from 2000-2012, with recent samples (e.g. 2009-2011) supplied through GBYP. Otolith cores of juvenile and adult bluefin tuna collected in the Atlantic Ocean and Mediterranean Sea were then used to estimate the origin of these recruits in the bootstrap mode of HISEA, which provided non-parametric estimates of the reliability of predicted contributions from eastern (Mediterranean) and western (Gulf of Mexico) spawning grounds.

During GBYP Phase3, the baseline historically used to estimate mixing proportions was updated and improved (see section below), and both Phase 2 (n=600) and Phase 3 (n=400) samples were re-analyzed using the new baseline. Of the 400 otoliths analysed in Phase3, 297 correspond to mixed areas and the rest correspond to the baseline.

5.3 Results

$\delta^{13}C$ and $\delta^{18}O$ Baseline

Otolith $\delta^{13}C$ and $\delta^{18}O$ in the cores of ***yearling*** bluefin tuna from eastern and western nurseries were distinct (MANOVA, $p < 0.001$, Fig. 1) and used here as baseline data for mixed-stock runs to predict the origin of unknown individuals. Mean otolith $\delta^{13}C$ of yearlings in the updated baseline sample were relatively similar for individuals collected in the east (-8.58 ppt) and west (-8.70 ppt). Conversely, otolith $\delta^{18}O$ of yearlings was markedly different between the east and west, with bluefin tuna of eastern origin having more enriched values (mean -0.77 ppt) relative to yearlings collected in the west (-1.26 ppt). In the current project, we have added significantly to the yearling baseline and developed a new reference set of yearling samples (Fig. 1). The revised baseline only includes samples run at a single lab and all samples were processed using the same milling template for isolating core material. Classification success (based on quadratic discriminant function analysis) of the revised baseline was 90% east and 75% west (overall 83%).

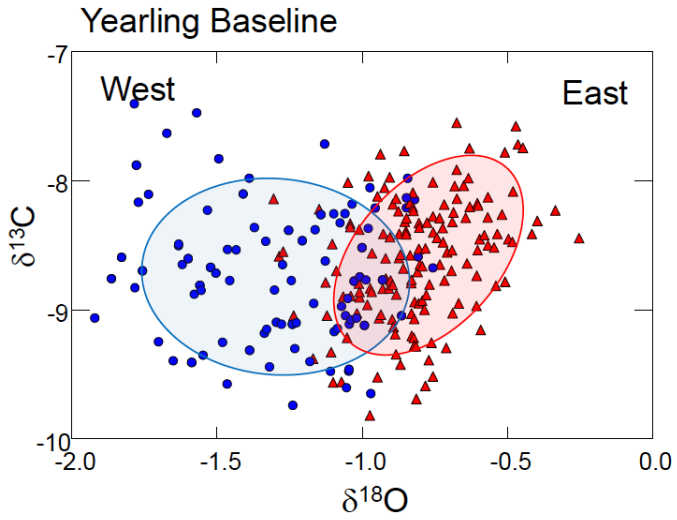


Figure 1. Otolith δ13C and δ18O values for yearling bluefin tuna used as the baseline sample. Samples are for bluefin tuna collected in eastern (n = 150) and western (n = 115) nurseries. All values are based on milled otolith material from 1.5 mm thin sections.

Origin of Bluefin Tuna from the Central and Eastern Atlantic Ocean

$\delta^{13}C$ and $\delta^{18}O$ were measured in the ***otolith cores*** of medium (25-100 kg) and large (>100 kg) bluefin tuna from two locations in the Atlantic Ocean: 1) Central North Atlantic Ocean, 2) Eastern Atlantic Ocean off Africa. Outside the Mediterranean Sea, mixed-stock analysis indicated that mixing of eastern and western stocks occurred (Fig. 2, Table 1). Maximum-likelihood estimates (MLE) of bluefin tuna collected in the Central North Atlantic (n=177) were comprised primarily of individuals from the 'eastern' or Mediterranean nursery (70%). Still, a significant number of 'western' bluefin tuna were present in our sample from the Central North Atlantic (30%), indicating that migrants from both eastern and western populations mix in this region. Standard deviation around estimated percentages was ± 7%. The presence of western migrants in the Eastern Atlantic Ocean off Africa was also notable, with the majority of bluefin samples classified to

the western Atlantic population. There was considerable uncertainty with the HISEA mixed stock run in this region and the predicted SD around estimated percentages was relatively high at ±16%, indicating that the percentages may vary markedly from the estimated values. It should be noted that the samples size from this region was small (n=32) and mixed stock assignment using MLE is quite variable when the unknown sample is mixed and not dominated by a single region. Additional samples are needed from this region to further clarify the degree of mixing, although results to date suggest that western migrants may occur in high numbers off the coast of Africa.

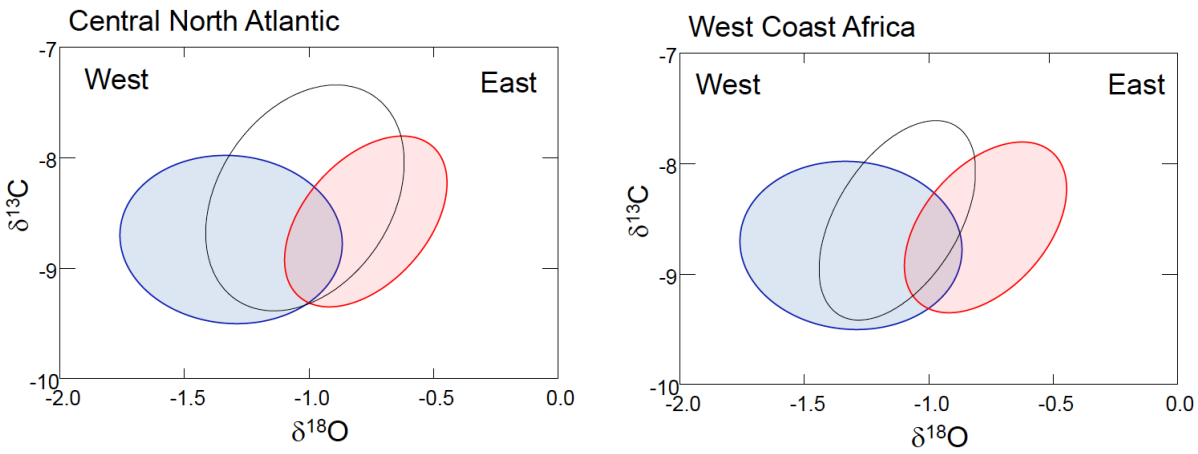


Figure 2. Confidence ellipses (1 SD or ca. 68% of sample) for otolith δ13C and δ18O values of yearling bluefin tuna from the east (red) and west (blue) along with the confidence ellipse (black line) for otolith cores of medium and large bluefin tuna collected in the Central North Atlantic and Eastern Atlantic (Africa).

Table 1. Maximum-likelihood predictions of the origin of medium (25-100 kg) and large (>100 kg) bluefin tuna collected from Central North Atlantic and Eastern Atlantic Ocean (North West Africa). Estimates are given as percentages and the mixed-stock analysis (HISEA program) was run under bootstrap mode with 1000 runs to obtain standard deviations (~error) around estimated percentages (± %).

		Predicted Origin		
Region	**N**	**% East**	**% West**	**% SD**
Central North Atlantic	177	70	30	± 6.5
NW Africa	32	27	73	+15.6

Origin of Bluefin Tuna from the Bay of Biscay

$\delta^{13}C$ and $\delta^{18}O$ measured in the otolith cores of juvenile (3-25 kg) and medium (25-100 kg) bluefin tuna from the Bay of Biscay were markedly different from medium to large bluefin tuna from other areas outside the Mediterranean Sea (Fig. 3). Mixed-stock analysis indicated that mixing of young bluefin tuna from eastern and western nurseries in the Bay of Biscay was negligible (1%; Table 2). Maximum-likelihood estimates (MLE) indicated that bluefin tuna collected in the Bay of Biscay (n=262) were almost exclusively individuals of 'eastern' or Mediterranean origin (99%). Standard deviation around estimated percentages was $\pm$ 1%, indicating the degree of confidence in our predicted assignment was high.

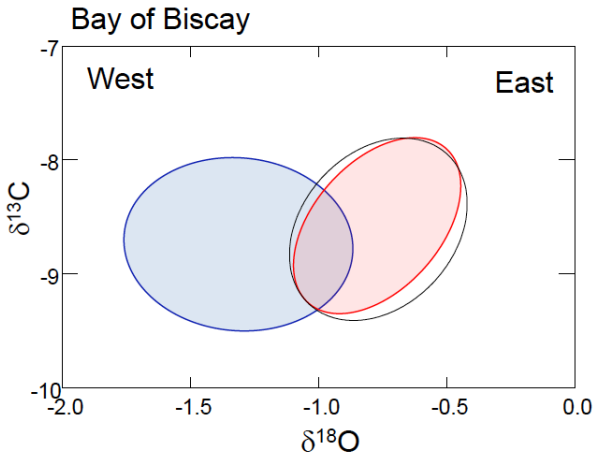


Figure 3. Confidence ellipses (1 SD or ca. 68% of sample) for otolith δ13C and δ18O values of yearling bluefin tuna from the east (red) and west (blue) along with the confidence ellipse (black line) for otolith cores of juvenile to medium bluefin tuna collected from the Bay of Biscay.

Table 2. Maximum-likelihood predictions of the origin of juvenile (3-25 kg) and medium (25-100 kg) bluefin tuna collected from the Bay of Biscay. Estimates are given as percentages and the mixed-stock analysis (HISEA program) was run under bootstrap mode with 1000 runs to obtain standard deviations (~error) around estimated percentages (± %).

		Predicted Origin		
Region	**N**	**% East**	**% West**	**% SD**
Bay of Biscay	262	99	1	+ 1.0

Origin of bluefin tuna in the Mediterranean Sea (Strait of Gibraltar to Turkey)

At the point of entry/exit into/from the Mediterranean Sea (Strait of Gibraltar), the occurrence of western migrants was not observed, with 100% of the medium and large bluefin tuna predicted to be of eastern origin (Fig. 4, Table 3). This trend continued within the Mediterranean Sea, and the predicted origin of medium and large bluefin tuna from Balearics, Sardinia and Malta, as well as of juvenile Bluefin from the Adriatic was 100% 'eastern' fish. Our MLE for bluefin tuna sampled in the eastern Mediterranean (Turkey) was 99% 'eastern' fish and the standard deviation around percentage from this region was ± 3%, indicating this region may also be comprised exclusively of 'eastern' bluefin tuna.

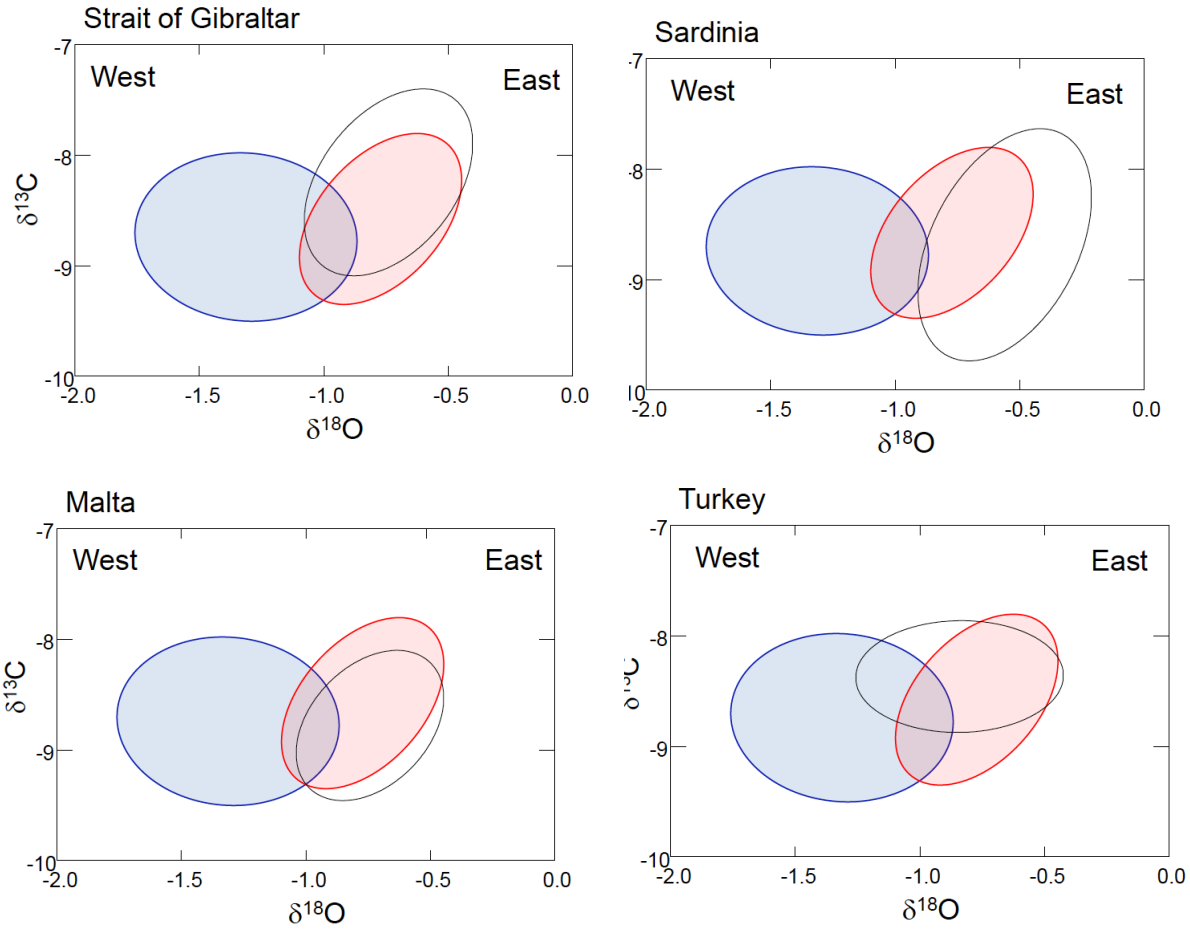


Figure 4. Confidence ellipses (1 SD or ca. 68% of sample) for otolith δ13C and δ18O values of yearling bluefin tuna from the east (red) and west (blue) along with the confidence ellipse (black line) for otolith cores of medium to large bluefin tuna collected from Gibraltar, Malta, Sardinia, and Turkey.

Table 3. Maximum-likelihood predictions of the origin of medium and large bluefin bluefin tuna collected from six regions within the Mediterranean Sea: Strait of Gibraltar, Balearic Sea, Malta, Sardinia, Adriatic Sea and Turkey. Estimates are given as percentages and the mixed-stock analysis (HISEA program) was run under bootstrap mode with 1000 runs to obtain standard deviations around estimated percentages (± %).

		Predicted Origin		
Region	**N**	**% East**	**% West**	**% Error**
Strait of Gibraltar	190	100	0	± 0.0
Balearic Sea	39	100	0	± 0.0
Malta	82	100	0	± 0.0
Sardinia	20	100	0	± 0.0
Adriatic Sea	47	100	0	± 0.0
Turkey	48	99	1	+ 2.9

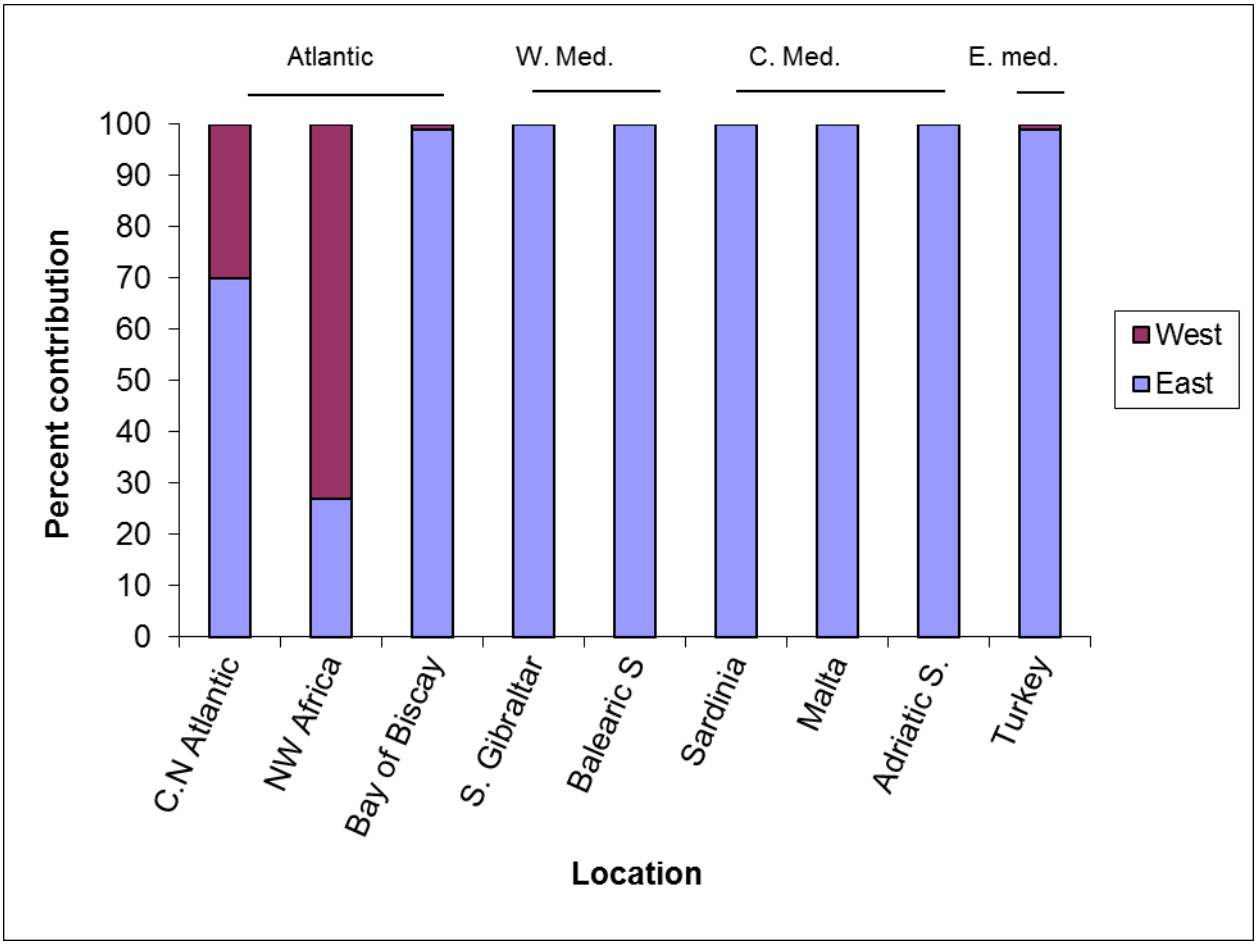


Figure 5. Summary of predicted origin of medium (25-100 kg) and large (>100 kg) bluefin tuna from the Atlantic Ocean and Mediterranean Sea. Sample size provided for each region.

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6. AGE DETERMINATION ANALYSES

Task Leader: Enrique Rodriguez-Marín (IEO)

Participants

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6.1 Introduction

Biological studies on age and growth of fish are crucial components for describing their life cycle (age at maturity, age at recruitment, longevity, etc.). Age determination is an essential feature in fish stock assessment to estimate the rates of mortalities and growth. Assessment of Atlantic bluefin tuna (*Thunnus thynnus*, ABFT) using age structured models has proved useful in establishing a diagnosis of stock status.

The biological sampling of this project includes information on age composition of the samples carried out for population structure purpose, and at the same time, for obtaining information about the age composition of the bluefin tuna catches. To estimate the age of the catch the following approach was selected: to measure the length of a representative sample of the catch (i.e. through random sampling) and applying an age-length-key (i.e. through length-stratified sampling) to convert size into age distributions. This approach has been also applied for estimating southern bluefin tuna (*Thunnus thynnus*) age composition (Anon., 2002).

We used two calcified structures for the age interpretation of ABFT: sagittal otolith and first dorsal fin spine (hereby spine). This procedure was employed because sampling of either structure depends on dockside practices. Otoliths represent an advantage in relation to other calcified structures because all ages can be interpreted since there is no nucleus resorption. Otoliths can be used to age giant ABFT, while other hard parts are difficult to interpret from 10 years upwards (Rodriguez-Marin et al., 2007). Conversely, spines are easier to collect and prepare than otoliths. In this Project we attempted to provide a preliminary age-length key based on otoliths and another one based on spines.

Direct ageing techniques using otoliths were verified for ABFT in 2008 by Neilson and Campana (2008), but analogous validation studies are not yet available for spines. Thus, in this study we have put special stress upon the comparison of the age interpretation from spines and otoliths from the same specimen. Another aspect that has also been given special care, is the consensus on the methodology of preparation and reading of otoliths with other research centers in countries like the U.S. and

Canada who also conduct age estimates from otoliths (Center for Environmental Science of the University of Maryland, Panama City Laboratory of the National Marine Fisheries Service, Gulf of Maine Research Institute and Fisheries and Oceans Canada). In this respect, the IEO scientists have participated, together with scientists from other laboratories, in ageing workshops in 2011, 2012 and the present year, in order to standardize important areas of methodological concern that may influence age estimates of ABFT using otoliths. Direct ageing using spines have also been comprehensively reviewed in a paper that is been actually under revision (Luque et al., 2013).

During the 2012 ICCAT-GBYP operational meeting it was stressed the need to take into account the seasonal growth and thus to have an age length key (ALK) with an adequate sampling throughout the year covering the whole size range. Given these requirements, and attending to the seasonality of the fisheries that take place mainly between May and November, we aimed at splitting the year in two and getting two ALKs, one per semester and calcified structure, i.e. otoliths and spines.

6.2 Material and Methods

Sampling

In this phase 3 of the GBYP project a total of 157 otoliths and 158 spines were used for age interpretation of ABFT. Table 6.1 and Table 6.2 show the number of samples from both phases of the project, including the number of samples obtained in 2011 and the ones from 2012, which were used to improve month and size coverage of the sampling for both otoliths and spines, respectively. Samples were collected from May to November in 2011 and from January to November in 2012. Specimens were caught in the eastern, central and western Mediterranean Sea, and in the north-eastern Atlantic in offshore waters of the Iberian Peninsula. Bluefin tuna juveniles were caught by bait boats and adults by longliners, hand line, purse seiners and traps. Fin spine and sagittal otoliths extraction and conservation were carried out following the present project sampling protocols. There is a small difference in the GBYP phase 2 number of otolith samples from the present and the previous

(Phase2) report, and it is due to the elimination of a six samples whose lengths were not properly reported.

The number of paired samples, otoliths and spines, coming from the same specimen is shown by length range in Table 6.3.

Table 6.1. Summary of bluefin tuna otoliths used for age interpretation by length range. ABFT length was measured as straight fork length (SFL) in cm.

Otolith samples									
	GBYP-Phase 2 (samples from 2011)			GBYP-Phase 3 (samples from 2011 & 2012)					Total
SFL (cm)	1st semester	2nd semester	Total	1st semester	2nd semester	2011	2012	Total	Phase 2 & 3
20-30		10	10						10
30-40		10	10						10
40-50		6	6						6
50-60	2	7	9						9
60-70	2	9	11						11
70-80	4	9	13	8	1	9		9	22
80-90	5	16	21		1	1		1	22
90-100	7	5	12	5		5		5	17
100-110	2	17	19	5	2	6	1	7	26
110-120	2	28	30	10	10	17	3	20	50
120-130	4	12	16	1	3	3	1	4	20
130-140	11	13	24		2	2		2	26
140-150	6	11	17		4	3	1	4	21
150-160	6	7	13	4	6	6	4	10	23
160-170	4	8	12	3	9	3	9	12	24
170-180	5	3	8	3	2		5	5	13
180-190	18	3	21	5	1	1	5	6	27
190-200	17	4	21	6	1		7	7	28
200-210	17	3	20	11	10	2	19	21	41
210-220	17	4	21	6	12		18	18	39
220-230	17	3	20	6	4		10	10	30
230-240	11	9	20	3	3		6	6	26
240-250	6	4	10	6	1		7	7	17
250-260		3	3	1			1	1	4
260-270				1	1		2	2	2
270-280		1	1						1
Total	163	205	368	84	73	58	99	157	525

Table 6.2. Summary of bluefin tuna spines used for age interpretation by length range. ABFT length was measured as straight fork length (SFL) in cm.

Spine samples									
	GBYP-Phase 2 (samples from 2011)			GBYP-Phase 3 (samples from 2011 & 2012)					Total
SFL (cm)	1st semester	2nd semester	Total	1st semester	2nd semester	2011	2012	Total	Phase 2 & 3
20-30		10	10						10
30-40		10	10						10
40-50		6	6						6
50-60	2	8	10						10
60-70	1	5	6		5		5	5	11
70-80	4	8	12	11	4	15		15	27
80-90	2	32	34		2	2		2	36
90-100	7	3	10	5		5		5	15
100-110		16	16	4	2	5	1	6	22
110-120	6	31	37	9	8	14	3	17	54
120-130	10	19	29		3	3		3	32
130-140	16	12	28		2	2		2	30
140-150	12	16	28		5	5		5	33
150-160	10	9	19	4	3	3	4	7	26
160-170	4	8	12	2	3	4	1	5	17
170-180	3	1	4	3	2	3	2	5	9
180-190	20		20	5	1	1	5	6	26
190-200	15	2	17	8	2	2	8	10	27
200-210	14	1	15	11	5	5	11	16	31
210-220	14		14	7	5	5	7	12	26
220-230	15		15	6	7	7	6	13	28
230-240	12	3	15	4	7	7	4	11	26
240-250	1	1	2	6	1	1	6	7	9
250-260	3	3	6	1			1	1	7
260-270				1	3	3	1	4	4
270-280									
280-290					1	1		1	1
Total	171	204	375	87	70	93	65	158	533

Table 6.3. Summary of bluefin tuna paired structures, otoliths and spines, coming from the same specimen by length range. ABFT length was measured as straight fork length (SFL) in cm.

Otoliths and spines paired samples						
	GBYP-Phase 2		GBYP-Phase 3			Total
SFL (cm)	2011	Total	2011	2012	Total	Phase 2 & 3
20-30	7	7				7
30-40	10	10				10
40-50	6	6				6
50-60	7	7				7
60-70	5	5				5
70-80	9	9	9		9	18
80-90	10	10	1		1	11
90-100	10	10	5		5	15
100-110	13	13	5	1	6	19
110-120	25	25	14	3	17	42
120-130	9	9	3		3	12
130-140	9	9	2		2	11
140-150	9	9	2		2	11
150-160	8	8	3	4	7	15
160-170	9	9		1	1	10
170-180	4	4		1	1	5
180-190	11	11		5	5	16
190-200	10	10		6	6	16
200-210	10	10		11	11	21
210-220	8	8		6	6	14
220-230	10	10		6	6	16
230-240	8	8		3	3	11
240-250	2	2		5	5	7
250-260	3	3		1	1	4
260-270				1	1	1
Total	212	212	44	54	98	310

Preparation of calcified structures and age interpretation

Spine preparation and age interpretation criteria were performed according to Rodriguez-Marin et al. (2012). Spine section location was established at 1.5 times the condyle base width. Sections were obtained using a precision rotating diamond saw and mounted on glass slides. It is easy to identify the translucent and opaque bands formed on the spine of young individuals. However, in fish over two years old, the central area of the spine begins to reabsorb and the bands consequently disappear. To overcome the problem of nucleus reabsorption with age, the translucent band diameters measured from spines without reabsorption (i.e. spines from young specimens) had to be used to assign an age to the first inner visible

translucent band in reabsorbed spines (Figure 6.1). Age was estimated by counting the translucent bands which are deposited annually between November and April (Luque et al., 2013). For the interpretation of the border of the spine section we followed Rodriguez-Marin et al. (2007) criterion, in which a bluefin tuna with a translucent band formed at the edge of the spine section and caught at the beginning of the year was interpreted as having one year more, although there were still five or six months before its true date of birth (Rooker et al., 2007), whereas when the fish was caught in autumn, this band was not considered as one year more.

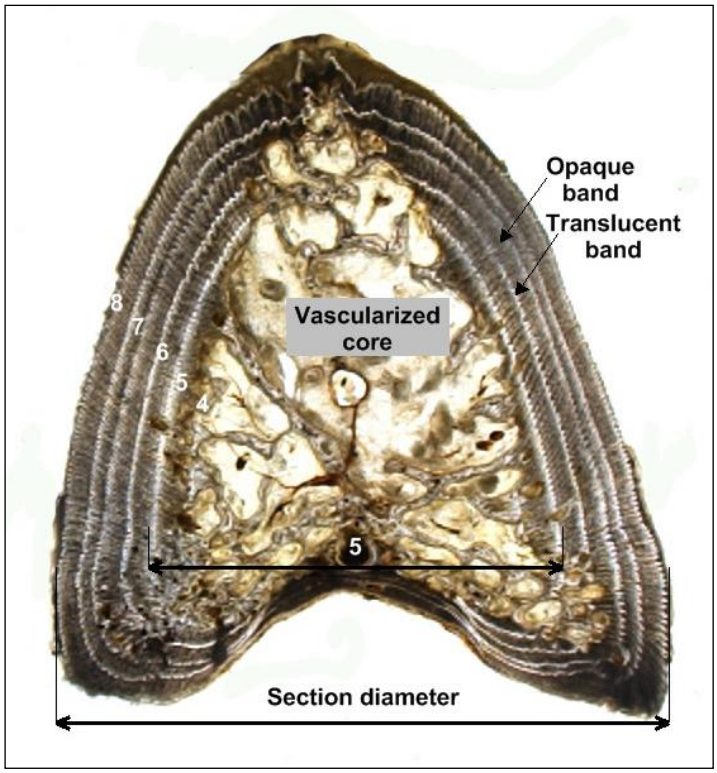

Figure 6.1. Spine section image of an Atlantic bluefin tuna aged 8 years old. Numbers reflect translucent bands considered annually formed. A false translucent band appears after the 6th band.

Spines direct ageing was carried upon digital images that were captured using a binocular lens magnifier connected by digital camera NIKON. An image analyzer (Nis-elements D 3.0 Nikon software) was used to measure the maximum spine diameter as well as diameter for successive growth bands. Spines sections were read by two independent readers. For those spines that there was a disagreement

between readers, an additional reading was achieved and the final estimated age assigned was the consensus among readers.

Otoliths were sectioned by embedding them in a matrix resin within a mould. Three sections of 300-400 μm were obtained in the core area of each otolith (Figure 6.2), using a low-speed diamond cutting saw (Isomet 1000) equipped with four 0.3mm wide diamond impregnated blades with spacer at 0.3-0.4 mm. Encased otolith sections were mounted on glass slides using Eukitt, and then polished using 240-600 grit sandpaper with 0.3 micron polishing compound to improve the contrast of bands before imaging. Polished sections were placed in Petri dish and covered with ethanol to improve the contrast of bands. Otolith images were taken using reflected light on a black background and the same procedure described for spines was used to obtain digital images of otoliths. Age interpretation was performed on digitally enhanced images using Adobe Photoshop and annulus counts were made along the longest (ventral) arm of the sectioned sagittae otolith. Age was estimated by counting the translucent bands.

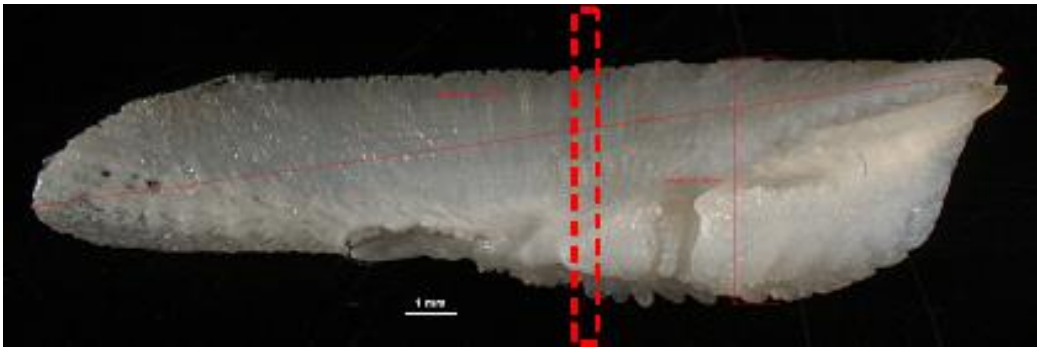

Figure 6.2. Right sagittal otolith of a bluefin tuna showing location of optimal section (discontinous red line) and whole otolith measurements. The anterior part of the otolith is narrower and is called rostrum (left) and the rear part is wider and is called postrostrum (right). The protuberance in this side of the otolith is called antirostrum, and is used as guide for the sectioning location.

Quality in terms of readability for both calcified structures was annotated. Samples from different months and geographic areas were combined for constructing the age-length keys.

Calcified structures biometry

Several biometric measures were recorded for each structure in order to analyze the relationship between the growth of the hard part and the specimen sampled. Spine diameter and total spine length were measured. For otoliths the longest and widest axes of the sagittal otolith were measured by placing the whole sagittal otolith sulcus side down and using reflected light on a black background (Figure 6.2). Weight was also recorded. Incomplete otoliths were not used for this biometric analysis, but the number of otoliths used in biometrics (n = 569) was superior to those used in interpreting their age. Linear and power regression functions were tested for the relationships mentioned above, using the coefficient of determination (r^2) as a goodness index.

Comparing age estimates between calcified structures: precision and relative accuracy

Comparison of age estimates between different calcified structures coming from the same specimen was carried out. Two indices were used to estimate precision and relative accuracy of spine age interpretations in relation to otolith age interpretations. Precision was calculated by the Percentage Agreement (PA), which indicates spines agreement with respect to otoliths age. The accuracy represents the closeness of a measured value to its true value. In this context, due to the absence of known age specimens, the relative accuracy was estimated by the relative bias, which represents over or underestimation of spine readings compared to otolith readings. Both indices were estimated by the Eltink's workbook (Eltink *et al.*, 2000).

6.3 Results and Discussion

Relationship between otolith and spine size and fish length

Biometric relationships for otoliths are described in Figure 6.3. The goodness of fit was high, despite increasing variation in data in all size-length relationships for fish over 180 cm SFL. Regression functions showed high determination coefficients (r^2), with better potential than linear relationship, between otolith size (length, height and weight) and fish length.

A total of 468 spine samples were used for the biometric analysis. Both linear and power equations fit adequately the spine length and diameter versus fish length relationship (Figure 6.4). The goodness of fit between the spine diameter and SFL showed that the fish body length and the size of the calcified structure were closely related.

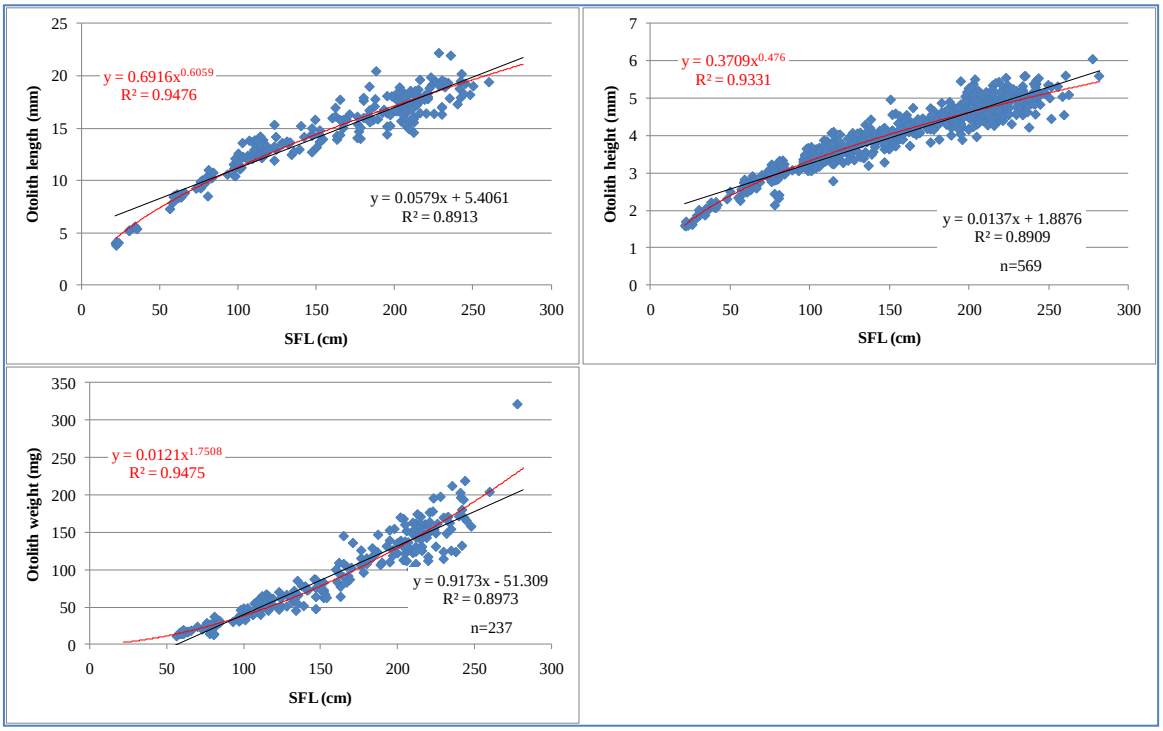

Figure 6.3. Biometric relationships between otolith measurements and Atlantic bluefin tuna straight fork length (SFL).

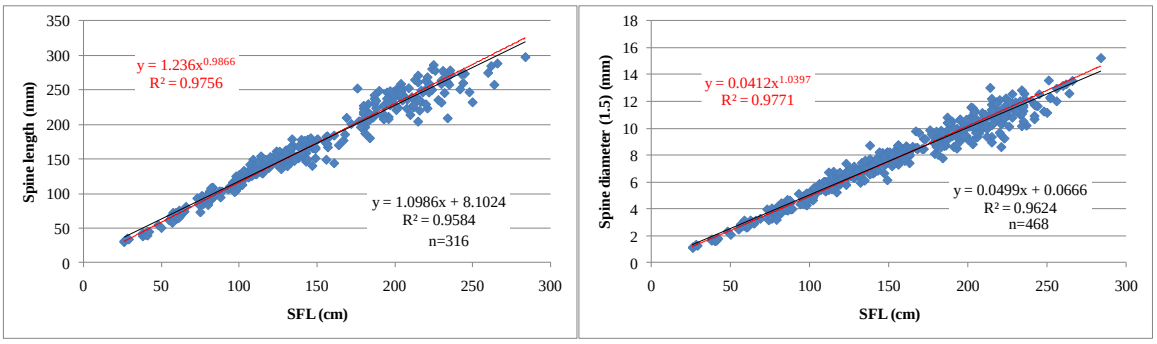

Figure 6.4. Biometric relationships between fin spine length and diameter versus Atlantic bluefin tuna fork length (SFL).

Age estimates

Otoliths and spines age length keys (ALKs) by semester are displayed in Table 6.4 and Table 6.5, respectively. The target objective for sampling 10 specimens by 10 cm length range was nearly achieved, but there are still some gaps due to the wide length range of this species. Number of samples for both calcified structures and semester ALKs is sufficiently represented up to age 11 and first semester comprises mainly the months of May and June and second semester comprises the months from July to November. The ages covered in this ALK are remarkable, since young ages, mainly from 1 to 6 years old otolith samples, are difficult to interpret in bluefin tuna (Rodriguez-Marin et al., 2007). This difficulty in interpreting the age of young specimens also occurs in southern bluefin tuna (*Thunnus maccoyii*) (Anon., 2002).

ABFT mean lengths at age were estimated from ALKs (Table 6.6). It can be appreciated an increase in the average size by age of the second semester in relation to the first one for spines ALKs, especially after age 6. In contrast, there were no major differences in mean length at age obtained from otolith ALKs of both semesters. Mean length at age from ALKs were also compared with currently adopted growth curves for the East and West Atlantic stocks of this species (Cort, 1991; Restrepo et al., 2010). For otoliths, there was no difference exceeding one year between present ALKs mean length at age and the growth curve from Restrepo et al. (2010). The mean lengths of ALKs obtained from spine interpretations do not present any difference with Cort's growth curve except for ages greater than 6 year old, especially for the second semester ALK, where there are differences of up to two years between the two data sets. There are several explanations for the differences between the results of present ALKs and the growth curves previously cited, but the sampling can be the major cause of this small disagreement.

Table 6.4. ALKs by semester based in age interpretation from Atlantic bluefin tuna otolith sections. Numbers represent percent by number by length class (SFL, cm).

First semester

Length class	Age class																				n
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
20-30																					
30-40																					
40-50																	0-20%				
50-60	50	50															20-50%				2
60-70		100															50-100%				2
70-80			100																		12
80-90		40	60																		5
90-100			8	83	8																12
100-110			29	71																	7
110-120			8	25	58	8															12
120-130				20	60		20														5
130-140					45	45	9														11
140-150					50	33	17														6
150-160							50	30	20												10
160-170							14	57	29												7
170-180						13		13	50	25											8
180-190								17	48	26		9									23
190-200								9	30	35	22	4									23
200-210									11	36	43	7	4								28
210-220										13	48	35	4								23
220-230									13	13	22	30	22								23
230-240											29	29	29	14							14
240-250									8		17	25	17	25	8						12
250-260																				100	1
260-270																100					1
270-280																					
Total	1	5	19	19	19	9	9	14	33	32	39	27	13	5	1	1				1	247

Second semester

Length class	Age class																				n
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
20-30	100																				10
30-40	100																				10
40-50	100																0-20%				6
50-60	29	71															20-50%				7
60-70		100															50-100%				9
70-80		70	30																		10
80-90		35	53	12																	17
90-100			40	60																	5
100-110			16	58	21	5															19
110-120				13	47	37	3														38
120-130					47	53															15
130-140					33	40	20	7													15
140-150					7	20	60	13													15
150-160				8		23	31	23	15												13
160-170					6	6	6	41	12	12	12	6									17
170-180								60	20	20											5
180-190								25	25	50											4
190-200									40		60										5
200-210									23	15	38	8	15								13
210-220							6			38	38	13	6								16
220-230								14		14	29	14		14	14						7
230-240										8	8	67	8	8							12
240-250											20	40		20			20				5
250-260												100									3
260-270																			100		1
270-280																				100	1
Total	28	27	17	22	36	36	19	18	11	15	20	18	4	3	1		1		1	1	278

Table 6.5. Age-length key based in age interpretation from Atlantic bluefin tuna spine sections. Numbers represent percent by number by length class (SFL, cm).

First semester

Length class	Age class																		
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	n
20-30																			
30-40																			
40-50																			
50-60		100																	2
60-70		100																	1
70-80		7	87	7															15
80-90			100																2
90-100				100															12
100-110				75	25														4
110-120					67	33													15
120-130					10	90													10
130-140					6	81	13												16
140-150					8	50	25	17											12
150-160						14	57	21	7										14
160-170								83	17										6
170-180								17	50	33									6
180-190							8	24	48	20									25
190-200								17	35	39	9								23
200-210								4	16	48	24	8							25
210-220										24	57	10	5	5					21
220-230										29	29	33	5	5					21
230-240											31	56	13						16
240-250												71		29					7
250-260											25	25	25					25	4
260-270															100				1
270-280																			
280-290																			
Total		4	15	16	14	35	15	22	29	39	32	26	5	4	1			1	258

0-20%
20-50%
50-100%

Second semester

Length class	Age class																		
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	n
20-30	100																		10
30-40	100																		10
40-50	100																		6
50-60	13	88																	8
60-70		90	10																10
70-80		8	92																12
80-90			85	15															34
90-100				100															3
100-110				44	50	6													18
110-120				13	67	21													39
120-130					41	55	5												22
130-140						79	21												14
140-150					5	48	48												21
150-160						50	50												12
160-170						18	45	27	9										11
170-180							100												3
180-190								100											1
190-200								25	75										4
200-210								17	50	17		17							6
210-220								40	20	40									5
220-230								29	14	14	43								7
230-240									20	20	40	10	10						10
240-250										50	50								2
250-260											33	67							3
260-270													67	33					3
270-280																			
280-290																	100		1
Total	27	17	41	21	45	50	28	10	11	7	9	4	3	1			1		275

Table 6.6. ABFT mean length at age per semester and by calcified structure. Length measured as SFL (cm).

Mean length (SFL) at age from ALKs				
	ALK 1st semester		ALK 2nd semester	
Age	Otoliths	Spines	Otoliths	Spines
0	50.0		34.0	34.5
1	68.4	60.5	69.8	61.5
2	83.6	77.2	86.8	81.1
3	102.7	96.6	105.4	100.0
4	125.0	118.9	120.2	115.3
5	139.4	131.4	126.6	133.2
6	149.8	154.7	147.1	151.6
7	173.0	173.5	165.2	194.2
8	189.2	187.4	180.9	206.9
9	199.4	202.7	202.9	223.9
10	213.7	217.5	207.2	233.4
11	219.6	230.2	229.9	237.8
12	228.2	232.8	216.8	255.0
13	239.6	230.8	232.0	262.0
14	243.0	260.0	221.0	
15	260.0			
16			240.0	284.0
17		251.0		
18			263.0	
19	251.0		278.0	

Comparison between age estimates from different calcified structures coming from the same specimen.

The mean age estimates between spines and otoliths from samples collected from the same specimen, plotted as a function of the otoliths-based age are shown in Figure 6.5. The comparison of otoliths and spines age interpretation showed a good fit to a linear relationship between both age estimations up to 10 years, and from this age it is observed that the spine age interpretations are lower than those of the corresponding otoliths. However, there are few individuals over 12 years old and it is necessary to expand the age range to have conclusive results.

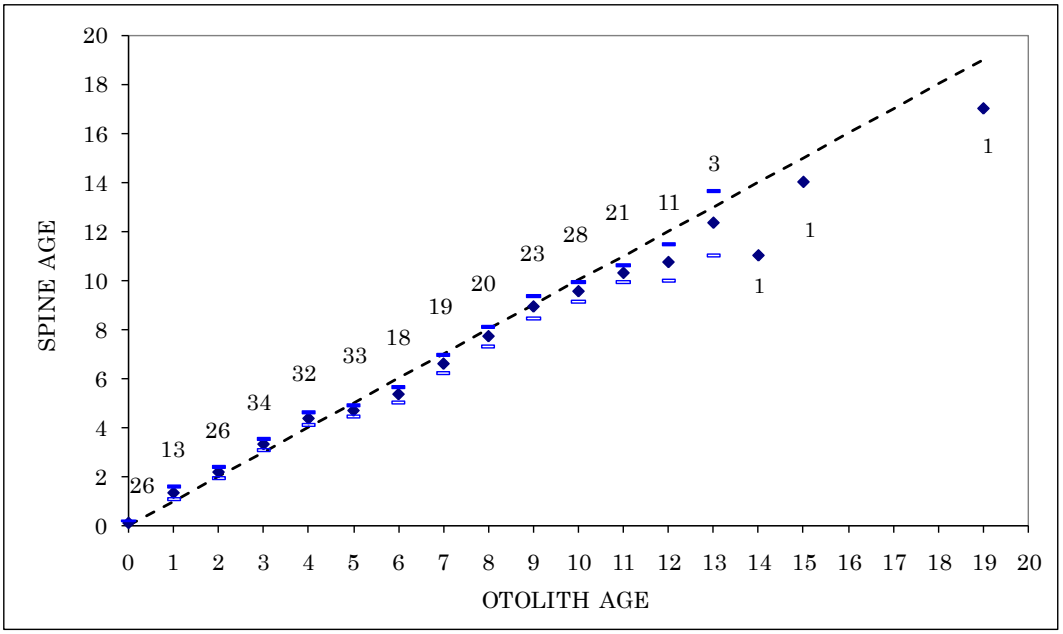

Figure 6.5. Bias comparison between otolith and spine age interpretations. Spines age readings are presented as the mean age and 95% confidence interval corresponding to otolith age readings (numbers above values represent number of calcified structures used, total number: 310).

The mean percent agreement of spine readings with respect to otoliths age interpretations was 55.3% for overall age classes. Highlighting, the high agreement obtained for early years such as age "0" (~ 90%), slightly lower for ages 1 to 3 (70-80%) and from age 4 to 11 around 45% agreement (Table 6.7).

Overall, a low relative bias was estimated for all age classes, with mean relative bias between 0.08 to 0.3 for age classes 0 to 4 years old, whereas negative relative biases values (-0.1 to -0.7) were showed for older age classes, from 5 up to 11, indicating an underestimation of age in spines in relation to otoliths of less than one year (Table 6.7.). So, there appeared to be no relative bias in most of the ages due to the age reading errors were normally distributed, except for ages 12, which showed an underestimation of up -1.3 annulus in spines with respect to otolith. The overall results indicated a low discrepancy between spines and otolith readings for ages 0 to 10 years old.

Table 6.7. Comparison between otolith and spine age interpretations. Precision and relative accuracy of spine age interpretations in relation to otolith age interpretations.

Age	0	1	2	3	4	5	6	7	8	9	10	11	12
Number samples	26	13	26	34	32	33	18	19	20	23	28	21	11
Precision: Percent agreement	92.3	69.2	80.8	64.7	59.4	57.6	44.4	31.6	55.0	43.5	42.9	33.3	9.1
Relative accuracy: Relative bias	0.08	0.31	0.15	0.29	0.34	-0.33	-0.67	-0.42	-0.30	-0.09	-0.46	-0.71	-1.27

6.4 Conclusions

To obtain age composition of ABFT catches by direct ageing it is needed a significant sampling effort. The difficulty of sampling calcified structures in this species due to its high market value, the seasonality of fisheries targeting age fractions of the population and its wide length range makes advisable to sample a large number of calcified structures in order to obtain a representative ALKs trough the year. This effort can be done in one or various years, obtaining annual ALKs or multiannual ALKs, respectively.

None of these two calcified structures can be excluded for routine direct ageing because in certain fisheries, fish processing or fish market practices would hinder the sampling of either structure. This means that more ageing comparison studies are needed on the calcified structures of the same specimen, increasing the sampling of specimens larger than 250 cm SFL.

The good age agreement between age estimations from spines and otoliths from the same fish indicates that both structures could be used for age determination of Atlantic bluefin tuna for ages up to 10 years old. This is useful since most of ABFT catches of the Eastern stock unit are constituted by specimens up to 10 years and in

the past the age 10+ group has been used in assessments of this stock. The present results open the possibility of joining the age interpretations of both structures for combined ALKs. However it would be desirable to extend the comparison studies between otoliths and spines of the same specimen to extend the age for the indistinct use of both structures.

There are some laboratories from different countries involved in direct ageing standardization, but it is necessary to increase the number of laboratories involved in this task, especially in the eastern side of the Atlantic.

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7. HISTOLOGICAL ANALYSES OF GONADS OF BLUEFIN TUNA

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7.1 Introduction

The study of ovary maturation has become increasingly important in the study of fish population dynamics, especially in the case of a species spawning in constrained areas and periods, such as bluefin tuna. Microscopic analyses of bluefin tuna gonads can be used for a variety of purposes, e.g. focused on identification of spawning grounds along the Mediterranean Sea, determination of maturation stages – and of their variability within the Mediterranean –, and fecundity estimates.

The present report summarizes the histological analyses conducted under this project both for females and males gonads in 2012.

7.2 Material and Methods

Several hundreds of samples were sampled during GBYP Phase 3 (see section 2.1). The final selection of samples to analyze histologically was constrained by sample availability. As a result, a histological analysis was conducted on 158 gonads, 95 ovaries and 63 testes, to determine their reproductive status (Table 5.1).

Table 5.1: Number of gonads sampled in the Western Mediterranean and in the Eastern Atlantic, and number of gonads analyzed.

Partner code	Area	Fishing gear	Nº of gonads histologically analyzed	
			Females	Males
IEO	Balearics	Longline	22	15
IEO	Gibraltar	Baitboat, handline, longline	24	19
IEO	Gibraltar	Trap	21	29
UNICA	Sardinia	Trap	28	
TOTAL			**95**	**63**

7.3 Results

The classification schemes used in the present report (modified from Schaefer, 1996) are shown in Tables 5.2 and 5.3 for females and males, respectively. Depending on the most advanced cohorts of oocytes encountered in the ovary (MACOs), bluefin females can be classed into one of five maturation stages. The ovaries of resting (quiescent) or pre-maturing (early vitellogenic) females, which contain previtellogenic or small vitellogenic oocytes as the MACOs, are reproductively inactive. The ovaries of active non-spawning females contain advanced vitellogenic oocytes and minor, if any, α atresia. Active females are classified as active spawning if the ovaries show additional evidence of either recent spawning (postovulatory follicles are present) or imminent spawning (migratory-nucleus or hydrated oocytes can be identified in the ovarian parenchyma). Females are considered inactive mature when they have entered into regression following a phase of reproductive activity, in which case the ovary encloses either previtellogenic or early yolked oocytes plus α and/or β atresia, or advanced yolked oocytes plus major atresia (Table 5.2).

Male tuna are considered mature when a significant amount of spermatozoa are present in the sperm duct (Table 5.3).

Table 5.2.Classification of ovaries and reproductive state of female bluefin tuna.

Stage	Histological description (females)	Physiological state
1 - Quiescence	Perinucleolar oocytes Lipid stage oocytes	Inactive
2 – Early vitellogenesis	Early vitellogenesis	Inactive
3 - Late vitellogenesis	Late vitellogenesis	Active, non-spawning
4 - Spawning	Migratory nucleus, GV breakdown and/or Hydrated oocytes and/or Post-ovulatory follicles	Active, spawning
5 - Spent	α atresia (≥50%) of late vitellogenic oocytes	Inactive

Table 5.3.Classification of testes and reproductive state of male bluefin tuna.

Stage	Histological description (males)	Physiological state
1 - Quiescence	Germinal cysts containing spermatogonia, few spermatocytes, and rare spermatids and spermatozoa	Inactive
2 - Early spermatogenesis	All stages: spermatogonia; increase of spermatocytes and spermatids; few spermatozoa	Inactive
3 - Late spermatogenesis	Many spermatid cysts; abundance of spermatozoa; sperm in lumina	Active
4 – Mature/Spawning	Lumen of seminiferous tubules and main sperm duct filled with spermatozoa	Active
5 - Spent	Residual spermatozoa in lumina	Inactive

Specimens from Balearic Sea. Longline fishery.

A total number of 49 bluefin tuna (27 females, 21 males and 1 indeterminate) were sampled but only 22 ovaries and 15 testes were histologically analyzed from the Spanish Longline fishery in the Balearic Sea between the 2ndof April and the 24thof September 2012. The size of the sampled individuals ranged from 48 cm FL to 143 cm.

Females

17 out of 22 ovaries analyzed (77.3%) showed previtellogenic oocytes as the MACOs without presence of vitellogenic oocites or atresia. Therefore, these female could be considered immature or quiescent female, and in consequence reproductively inactive (Fig 5.1 a, b). Taking into account that these female were mainly caught in the reproductive season and area, and due to the size of the specimens (around the size of first maturity) these female could be probably classified as immature ones.

One of the analyzed ovaries (4.5%) showed early vitellogenic oocytes as the most advanced oocytes without presence of postvitellogenic oocytes or postovulatory follicles, whereas the amount of atretic follicles was anecdotic (Fig 5.1c). Therefore, this individual was classified as a reproductively inactive female. Another ovary analyzed (4.5%) showed late vitellogenic oocytes as the most advanced oocytes without presence of postvitellogenic oocytes or postovulatory follicles, whereas the amount of atretic follicles was very low (Fig 5.1d). This individual can be considered a reproductively active but non-spawning female, a reproductive state that may reflect the natural condition of prespawner bluefin tuna (Medina et al., 2002).

The other three ovaries analyzed (corresponding to around 13.64 %) were considered active females because the ovaries show evidences of recent spawning (postovulatory follicles are present). Those individuals are considered to be spawning during the sampling period in the area (Figure 5.1e).

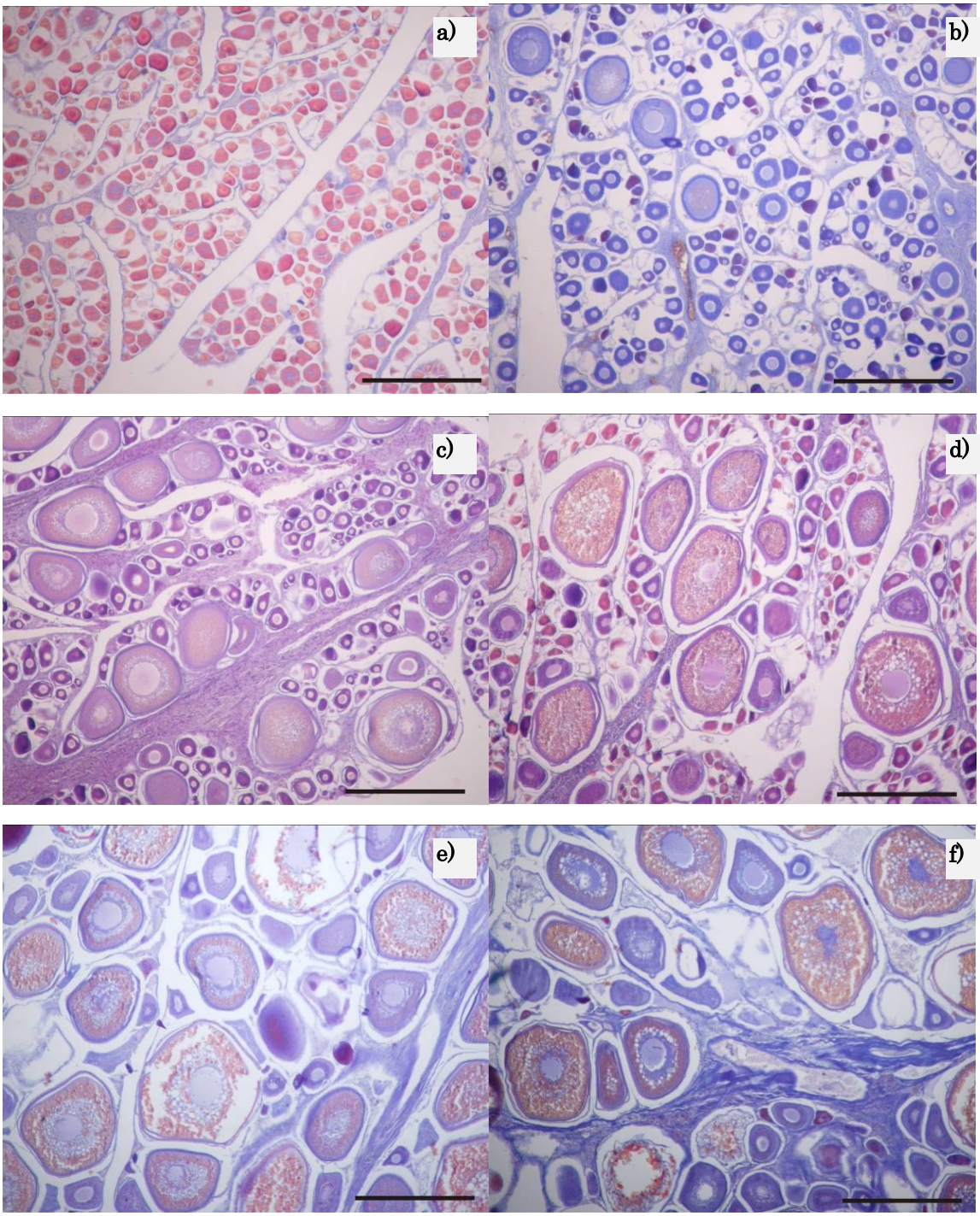

Figura 5.1. Atlantic Bluefin tuna T. thynnus ovaries sampled from the longline fishery in the Balearic Sea. Histological sections were stained with Mallory' trichrome. (a) quiescence ovary showing only perinucleolar oocytes, (b) quiescence ovary showing perinucleolar and lipid oocytes, (c) early vitellogenic ovary, (d) ovary in non spawning late vitellogenic stage, (e) Spawning ovary with POFs and some

atretic oocytes, and (f) spawning ovary with abundant atretic oocytes, Scale bar = 500 μm.

Males

Seven (46.6%) out of the 15 testes examined were quiescence, physiologically inactive, showing only spermatogonias at the cortical region (figure5.2.a). In all cases, the lumina of the testicular lobules, the secondary duct system and the main sperm duct were empty of sperm masses. Some of these specimens were caught in the spawning season and can be considered immature male due to their small size (80, 96 and 100 cm FL). The rest were caught in September, so it is not possible to determine if these individuals were resting or immature.

Three testes (20%) were in early spermatogenesis showing all developing stages of the male germ cell line (from spermatogonia to late spermatids and few spermatozoa) at the cortical region (proliferative region). These fish appeared to be in the middle of the spermatogenetic process, showing abundant spermatocyte cysts as well as cysts containing all spermatid stages, but little amount of spermatozoa and the lumina were empty (figure 5.2.b). These male were also considered to be physiologically inactive.

Four of the analyzed testes (26.7%) were apparently in advanced spermatogenesis (late spermatogenesis, physiologically active) as the flagellate spermatid cysts were clearly predominant over earlier spermatogenic stages (figure 5.2c). In all cases the lumina of the testicular lobules, the secondary duct system and the main sperm duct were fully packed with sperm masses. The histological structure of these testes is similar to that described previously in male ABFT spawners captured in traps as they enter the Mediterranean Sea to spawn.

Only one (6.6%) of the male analyzed was inactive (spent), displaying lumina of testicular lobules and ducts containing only residual spermatozoa (figure 5.2d). No active germ cell cysts were found in the peripheral region of the testes. The presence of residual sperm in the testicular ducts along with the relatively large size of the

sampled specimen (143 cm FL) lead us think that this fish was in postspawning stage.

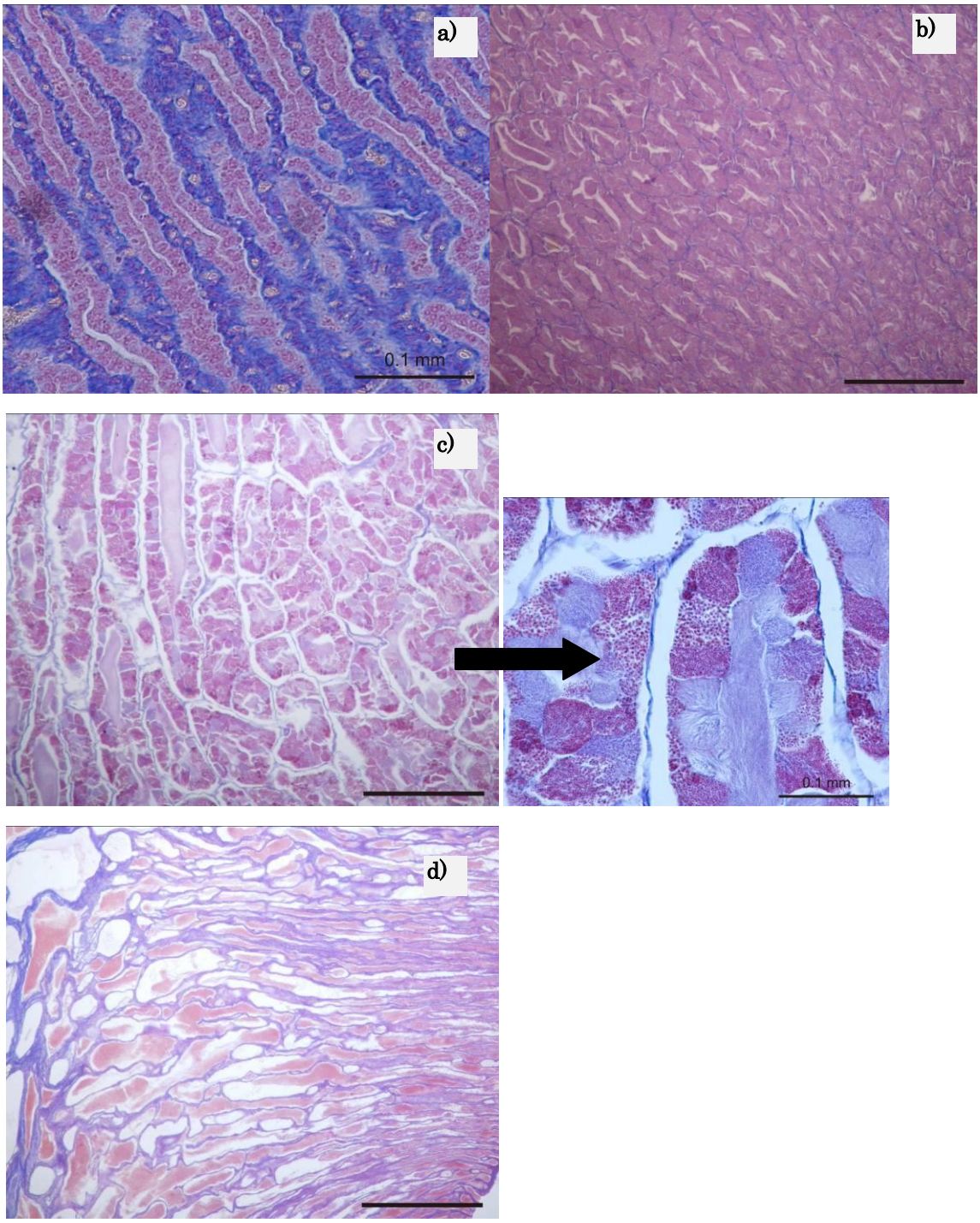

Figura 5.2. Bluefin tuna testes sampled from the longline fishery in Balearic Sea. Histological sections were stained with Mallory' trichrome. (a) quiescence testis, only spermatogonias can be found in the proliferative region (scale bar = 100 µm.), (b)

early spermatogenesis stage testis (Scale bar = 500 µm.), (c) testis in late spermatogenesis stage (Scale bar = 500 µm), the micro-photography on the right shows a detail of the proliferative region with spematid cist, spermatozoa and sperm in lumina (Scale bar = 100 µm.), and (d) spent testis, only residual sperm can be found in the lumina (Scale bar = 500 µm).

Specimens from Strait of Gibraltar.Bait boat fishery.

A total number of 43 individuals (19 males and 24 females) from the Bait boat fishery in the Strait of Gibraltar were analyzed. The specimens were caught in January, October and November, 2012.

Females

All the ovaries analyzed (n = 24, ranging from 94 to 189 cm FL) were quiescent (thus inactive), containing only previtelogenic (perinucleolar stage) oocytes (Figure 5.3a). The mere histological assessment does not allow us to determine whether the reproductively inactive state observed in all females is due to lack of maturity (young age) or wether it just reflects a physiological state of quiescence, since the size range of the majority of sampled specimens is around the size at first-maturity established for eastern ABFT (Corriero et al., 2005).

Males

All the males examined (n = 19) were inactive (spent or immature), displaying lumina of testicular lobules and ducts completely empty (figure 5.3b). No active germ cell cysts were found in the peripheral region of the testes. The absence of residual sperm in the testicular ducts along with the size range of the sampled specimens (from 77 to 188 cm FL) does not allow to determine whether the reproductively inactive state observed in those male of smaller size is due to lack of maturity (young age) or wether it just reflects a physiological state of spent.

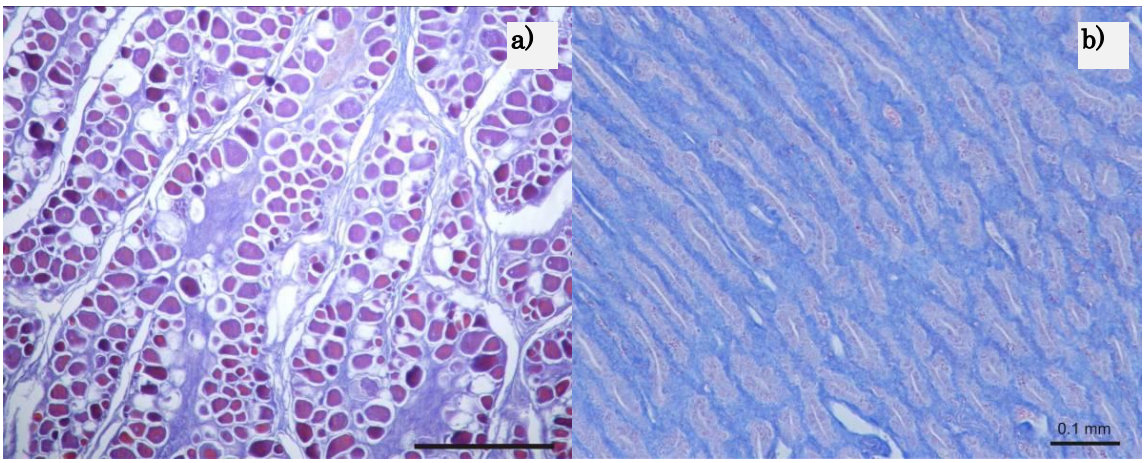

Figura 5.3.Bluefin tuna gonads sampled from the bait boat fishery in the Strait of Gibraltar. Histological sections were stained with Mallory' trichrome. (a) micro-photography showing a quiescence ovary, without vitellogenic oocytes (Scale bar = 500 µm), and (b) Quiescence testis (Scale bar = 100 µm).

Specimens from the Strait of Gibraltar Trap fishery

Samples from migrant ABFT in the area of the Strait of Gibraltar (eastward run: tuna swimming from the Atlantic towards Mediterranean spawning grounds) were collected from trap catches in the Strait of Gibraltar from the 3th to the 6th of June, 2012. A total number of 50 ABFT (29 males and 21 females) were analyzed.

Females

One out of the 21 ovaries examined (4.8%) was quiescent (thus inactive), containing only previtelogenic (perinucleolar stage) oocytes (figure 5.4a). Due to the large size of this specimen (221 cm FL), we classified it as a likely quiescencent female instead of an immature one.

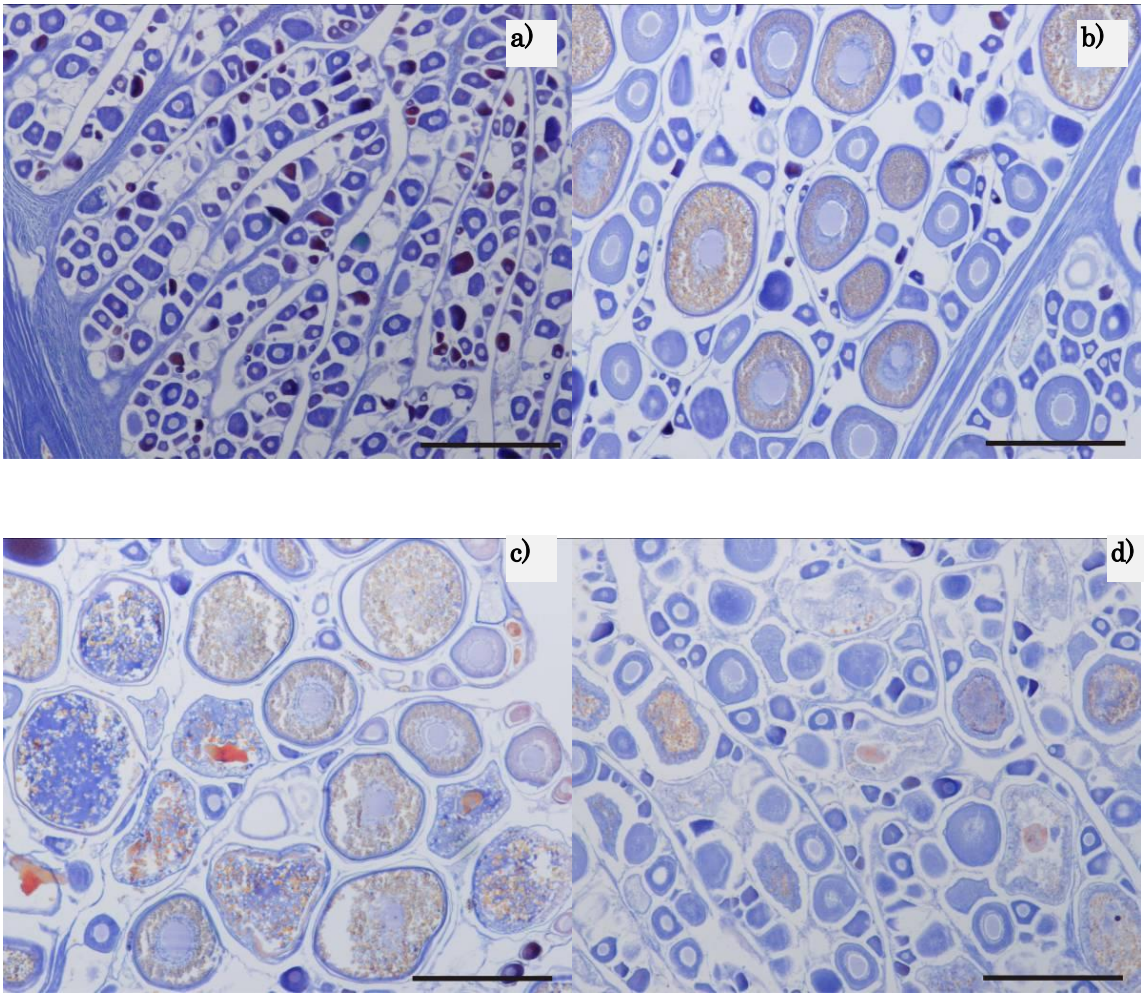

Figura 5.4.Michro-photographs of Bluefin tuna ovaries sampled from the trap fishery in the Strait of Gibraltar. Histological sections were stained with Mallory' trichrome. (a) quiescence ovary, (b) ovary in late vitellogenic stage, (c) spent ovary showing a atresia in the 57% of vitellogenic oocytes, and (d) spent ovary showing a atresia in the 100% of their vitellogenic oocytes. Scale bar = 500 µm.

Eight of these specimens (38%) showed late vitellogenic oocytes and lacked postvitellogenic oocytes (nuclear migration or hydrated oocytes) or postovulatory follicles (figure 5.4b), whereas the amount of atretic follicles was moderate (from 23% to 49%, average of 39%). Therefore, these individuals were classified as reproductively active but non-spawning, a reproductive state that reflects the natural condition of migrant ABFT spawners as they pass through the Strait of Gibraltar (Medina et al., 2002).

The twelve other individuals examined (57%) were found to be inactive (spent), as their ovaries contained abundant α & β -atresia (≥50%) of large yolked oocytes, indicating that they were entering regression (figure 5.4c). It is impossible from the histological evaluation to ascertain whether these specimens would be capable to resume ovarian maturation and eventually spawn later on through the season. Nevertheless the high levels of atresia of vitellogenic oocytes (from 50% to 100%, average 63%) suggest that likely some of these specimens could skip the spawning season (figure 5.4d).

Males

Two testes out of the 29 male gonads analyzed (6.9%) were in early spermatogenesis showing all developing stages of the male germ cell line (from spermatogonia to late spermatids and few spermatozoa) at the proliferative region. These fish appeared to be in the middle of the spermatogenetic process, showing abundant spermatocyte cysts as well as cysts containing all spermatid stages, but few amount of spermatozoa and the lumina were empty (figure 5.5a). These male were considered physiologically inactive.

13 out of 29 testes examined (44.8%) were in late spermatogenesis stage, so histologically active, showing all developing stages of the male germ cell line (from spermatogonia to late spermatids and spermatozoa) at the cortical region (figure 5.5b). These fish appeared to be in the late spermatogenetic process as the flagellate spermatid cysts were clearly predominant over earlier spermatogenic stages. In all cases the lumina of the testicular lobules, the secondary duct system and the main sperm duct were fully packed with sperm masses. The histological structure of these testes is similar to that described previously in male ABFT spawners captured in traps as they enter the Mediterranean Sea to spawn.

The remaining fourteen testes analyzed (48.3%) were in mature spawning stage with Lumen of seminiferous tubules and main sperm duct filled with spermatozoa (figure5.5c).

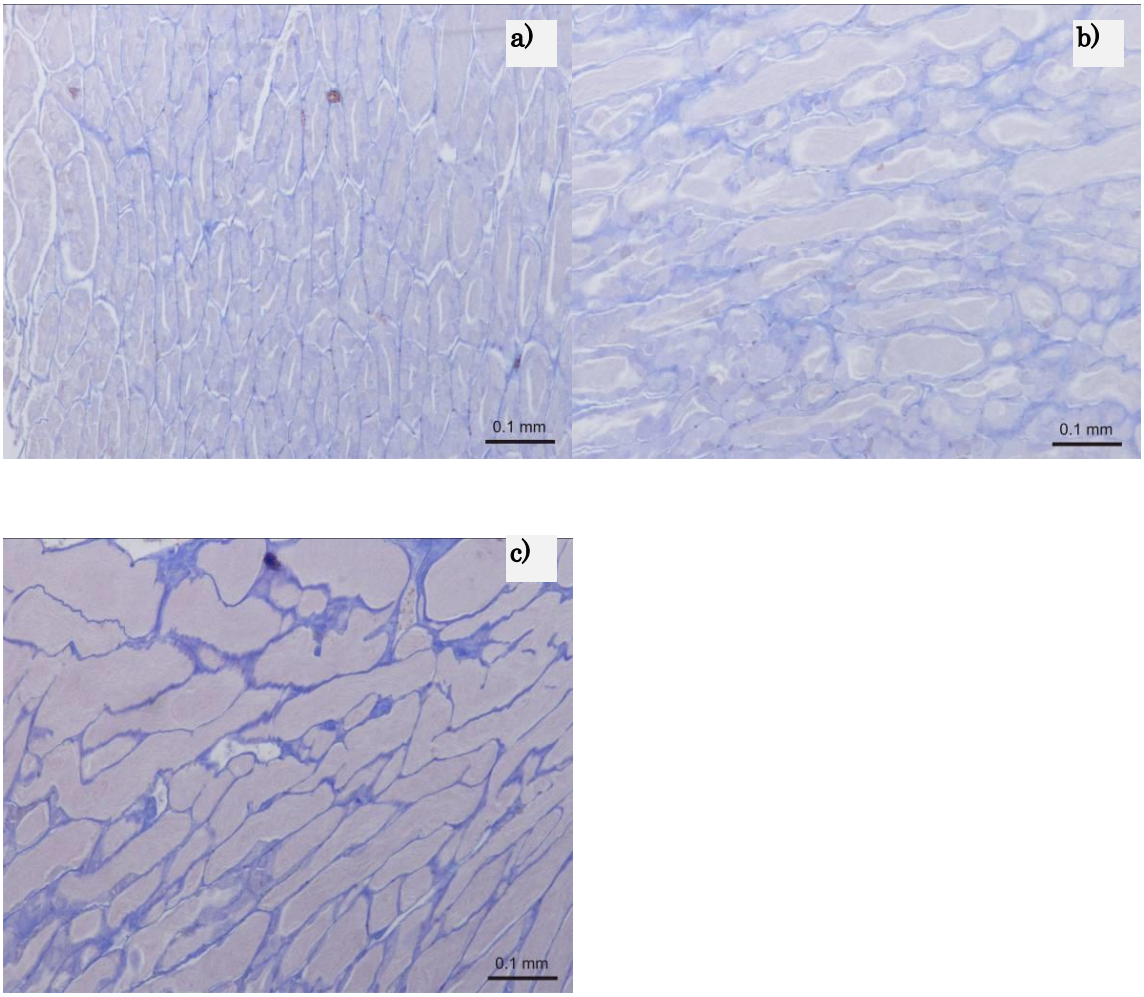

Figura 5.5.Michro-photographs of Bluefin tuna testes sampled from the trap fishery in the Strait of Gibraltar. Histological sections were stained with Mallory' trichrome. (a) inactive testis in early spermatogenesis stage, (b) late spermatogenesis testis (active) , and (c) active testis in mature spawning stage.Scale bar = 100 µm.

Specimens from Sardinia. Trap fishery.

A total number of 51 bluefin tuna (28 females and 23 males) were sampled but only female ovaries were histologically analyzed from the Italian Trap fishery in the Sardinian Sea between the 9thof May and the 11thof June 2012. The size range of the sampled individuals ranged between 127 and 241 cm-s. On these samples, only females were analyzed.

Females

20 out of 28 ovaries analysed (71.4%) showed late vitellogenic oocytes as the most advanced oocytes without presence of matured oocytes or postovulatory follicles, whereas the amount of atretic follicles was low or moderate (less than <50 % of atresia in 15 individuals and > 50 % in 5) (Fig 5.6a, b). Therefore, the individuals classified with low level of atresia can be considered reproductively active but non-spawning females, a reproductive state that may reflect the natural condition of migrating Bluefin tuna spawners (Medina et al., 2002) before spawning or the progress to a postspawning condition (i.e. the atresia levels will increase as time progressed). However, the individuals in late vitellogenesis with high levels of α-atresia (≥50%) can be considered to be inactive mature females (spent) in a recovery stage, as their ovaries contained abundant large yolked atretic oocytes, indicating that they were entering regression (Figure 5.6b). In the former case, it is assumed from a histological evaluation that those specimens would be capable to eventually spawn later on through the season.

The rest of the ovaries analyzed (n = 8 corresponding to around 15 %) were considered active females because the ovaries show evidences of either recent spawning (postovulatory follicles are present) or imminent spawning (migratory-nucleus are identified in the ovarian parenchyma). Those individuals are considered to be spawning during the sampling period in the area (Figure 5.6c).

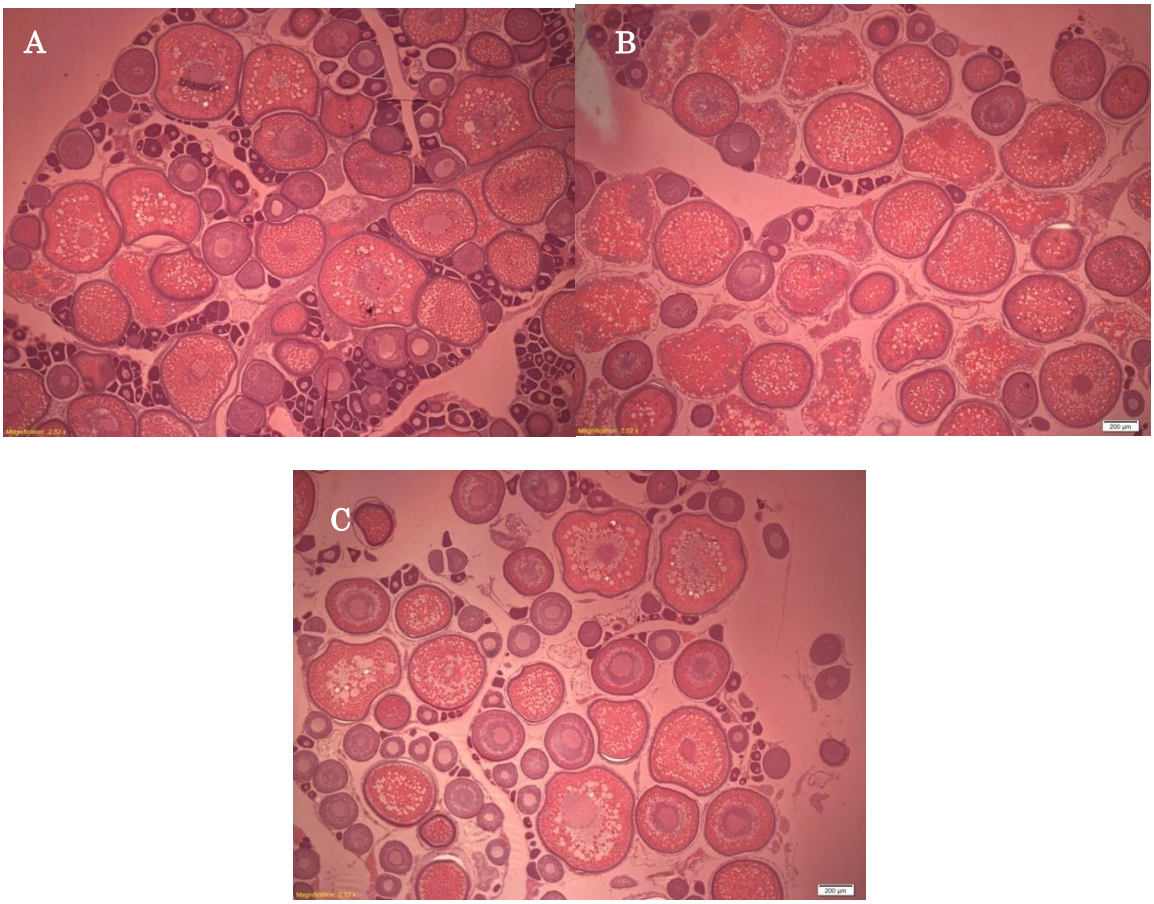

Figure 5.6.- (A) bluefin tuna gonad presenting late vitellogenic oocytes as the most advanced oocytes with atretic levels <50 %, (B) bluefin tuna gonad presenting late vitellogenic oocytes as the most advanced oocytes with atretic levels > 50 %, and (C) Bluefin tuna gonad presenting migratory nucleus oocytes as the most advanced oocyte stage.

7.4 Discussion

The sampling on ABFT in the Balearic Islands was conducted aiming at characterizing and assessing first maturity of ABFT eastern populations, so young specimens were sampled in the spawning area and season (April to September). Only five females showed vitellogenic oocytes in their ovaries. Our results indicate that young ABFT enter in active stages late in the season: late vitellogenesis (only one female) at the end of May and spawning females were found only since middle of June (three specimens).We suggest that probably these young specimens make a poor contribution to the total egg production of the population.

The ABFT captured by bait boat fishery in the Strait of Gibraltar were quiescent. These fish are assumed to have spawned in the reproductive season (June-July), entered into regression in late July-August, and finally reached the resting stage by August-September. All the specimens sampled were caught in January, October and November so all of them were in the expected inactive physiological stage.

As the results obtained in 2011, the present results from the histological analysis of ABFT caught by trap as they enter the Mediterranean Sea to spawn (eastward run) are quite unexpected. Albeit the male reproductive organs were apparently active and similar in structure to what has been reported earlier (Abascal et al., 2004), apparent signs of reproductive impairment were present in most of the sampled ovaries. An extremely high (57%) percentage of females were sexually mature but inactive, showing ovaries that had entered into regression, reabsorbing numerous vitellogenic oocytes through atretic processes. These observations are inconsistent with others made on eastward migrant ABFT caught in the Strait of Gibraltar by trap (Medina et al., 2002). The most likely reason for the present results is that a great number of the tuna captured in 2012 (well in excess of the assigned TAC) were retained for days to weeks in the trap facilities in order to negotiate TAC reallocation. Stressful conditions of captivity along with the interception of the natural migratory route could probably result in the interruption of gametogenesis (mostly oogenesis) and further resorption of oocytes. Under these circumstances, the fish sampled in 2011-2012 seasons should not be considered as representative of the normal reproductive population.

The bluefin tuna captured by the trap fishery in the Sardinian Sea in May and June were mostly in advanced vitellogenic stages. Although some of them showed high level of atresia (postspawning and resting condition), most of them (42 % of the total sampled females) showed low levels of atresia which could be interpreted as fishes in mature active condition and capable of spawning during the current season (July). Those samples could be used to give some information about the reproductive biology of bluefin tuna.

Histological samples of gonads from several areas have not been obtained because some of the fisheries were already closed at the onset of the project, or quotas were transferred in some key fisheries, making the sampling impossible or yielding gonad samples out of the reproductive season. Due to the spatially and temporally constrained reproduction of the bluefin tuna, there are specific areas of its broad distribution range that prove to be essential for the understanding of the species' reproductive biology and the determination of the reproductive potential of the stocks and the age of first maturity. Of particular interest are the spawning grounds (Balearic Archipelago, South Tyrrhenian Sea-Malta, and Levantine Sea), where the preferred sampling gear is the purse seine, which specifically targets schools of breeders at their reproductive peak. Specimens captured by longline shortly before, during and shortly after the reproductive season can be very useful as well. Further efforts should be made to allow scientifically meaningful sampling onboard purse-seine and longline boats.

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8. SUMMARY AND RECOMMENDATIONS

This section summarizes the main achievements of the project, as well as the main difficulties encountered and some future prospects and recommendations.

The consortium aimed to sample 1750 individuals and get 4500 biological samples (1550 genetic samples, 1450 otoliths, 1250 spines and 250 gonads). At the end of the project, the consortium had sampled a total of 2813 bluefin tuna (71 larvae, 604 YOY, 701 juveniles, 598 medium size fish, and 839 large fish) from different regions (427 from the East Mediterranean, 286 from the Central Mediterranean, 723 from the Western Mediterranean, 928 from the Northeast Atlantic, 399 from the Central North Atlantic and 50 from the Western Atlantic). From these individuals, 6256 biological samples were taken (2733 genetic samples, 1759 otoliths, 1413 spines and 351 gonads).

The consortium aimed to perform 1000 genetic analyses, 400 microchemical analyses, 250 aging analyses and 60 histological analyses. By the end of the project, the consortium genotyped 1152 individuals, completed 400 michochemical analyses on otoliths, analyzed 315 hard parts for aging purposes and conducted 158 histological analyses on gonads.

Some preliminary genetic data analyses were carried out on a subset (n=555) of the total number of individuals genotyped, mainly focusing on the Reference Samples. The results were much more encouraging than those of Phase 2, and revealed that high-performing SNP panels can identify and differentiate at least three ABFT spawning populations (GOM, WMED, EMED), that are genetically well clustered (even if the differentiation of the Eastern Mediterranean ABFT Reference Samples should be improved by looking for more efficient SNP loci). However, due to the complexity and quantity of the RRSG-generated genomic data obtained for the ABFT, the genomic data needs to be analysed more in depth in the future and various additional analyses are further required to fine tune SNP selection/validation for traceability and management purposes.

Regarding otolith michochemistry, the baseline of yearling fish from known eastern and western origin that is used to assign origin to mixed populations has been revised. The revised baseline includes new individuals analysed during Phase 3. It only includes samples run at a single lab and all samples were processed using the same milling template for isolating core material. Classification success (based on quadratic discriminant function analysis) of the revised baseline was 90% east and 75% west (overall 83%). Results of mixed stock analyses using the revised baseline suggest >99% eastern origin fish in all studied areas (including the Bay of Biscay, Gibraltar and several Mediterranean locations), except in the Central North Atlantic (70%) and the Atlantic coast of Morocco (27%). However, the sample size was small in the latter case, with very uncertain estimates, and additional analyses are suggested to verify the origin of fish caught in this area.

Regarding age determination analyses, 157 otoliths and 158 spines were interpreted. Inter-reader agreement was high and age-lenth-keys were generated for both semesters, using both spines and otoliths. The comparison between ages estimated from different structures of the same specimen showed a good age agreement. This indicates that both structures may be used indistinctly for age determination of Atlantic Bluefin tuna for ages up to 10 years old.

A histological analysis was conducted on 158 individuals from the Strait of Gibraltar, Balearics and Sardinia. Results were more promising than in Phase2, specially on those samples that were collected during the reproductive season. Although samples on some areas (e.g. Strait of Gibraltar) did not provide much insight on reproductive activity of bluefin tuna, samples from the Balearics and Sardinia showed active reproductive condition for some individuals, and could be used to gather some information about the reproductive biology of bluefin tuna.

When accomplishing the tasks of the project, the main difficulty came from the late signature of the contract (not only, but mainly due to the inability of some institutions to provide administrative documents in a timely manner while trying to become part of the consortium), which is linked to the first payment. By the time the contract was signed, several fisheries where closed due to having reached their quotas or due to time area closures (see Section 2.3). In the meantime, some

partners were able to accomplish an important fraction of the proposed sampling, but in some cases it was difficult to allocate all necessary resources to accomplish this. Special sampling efforts were made to try to overcome this to the extent possible, and finally the total number of samples exceeded the target.

The late start of the project also affected most downstream analyses. Since these take considerable time (especially the optimization of genetic markers and assay ordering to third party companies), they needed to start (in general) before the sampling was finished, thus the samples to be analyzed necessarily were selected among those that were available.

Overall, and in spite of the difficulties faced during this first year, the project already started to provide some results, and it is expected to provide important information on population structure, catch composition, age structure and reproductive ecology.

For subsequent years, if this program continues, it is recommended to start several months prior to the start of the fishing season (e.g. February) so as to be able to appropriately organize the time for a general meeting of the whole consortium, improve coordination within the consortium, and avoid problems derived from late signature of the contract. In case of difficulty to start earlier, it would be desirable to be able to extend the deadline for the final report so as to be able to properly analyze the samples obtained.

On top of this, and because the sampling needs to go beyond the fraction of the population that is allowed to catch under the current management regime, it is fortunate that ICCAT has put in place the Research Mortality Allowance (RMA), that allows to access the non-catchable fraction of the population (e.g. reference samples such as larvae and YOY individuals, as well as juveniles). In this scenario, it would be desirable if the administrations could facilitate the process of getting scientific permits for the project team to make use of the RMA.

9. APPENDICES

9.1 Appendix 1: Sampling Protocols