SHORT TERM CONTRACT FOR BIOLOGICAL STUDIES (ICCAT-GBYP 06b/2015-2) OF THE ATLANTIC-WIDE RESEARCH PROGRAMME ON BLUEFIN TUNA (Phase 5)

Draft Final Report

for:

ICCAT

Scientific coordinator:

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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 to focus on age dynamics.

During Phase 5, following sampling protocols agreed in earlier Phases, the consortium sampled a total of 1506 bluefin tuna (127 larvae, 196 YOY, 5 juveniles, 142 medium sized fish and 1036 large fish) from different regions (71 from the East Mediterranean, 187 from the Central Mediterranean, 221 from the Western Mediterranean, 15 from the Strait of Gibraltar, 73 from the East Atlantic - West African coast, 48 from the Northeast Atlantic, 26 from the North Sea, 607 from the Central North Atlantic, 30 from the North-Western Atlantic and 228 from the Gulf of Mexico). From these individuals, 2635 biological samples were taken (1275 genetic samples, 919 otoliths and 441 spines).

The structure of the data bank previously agreed with ICCAT Secretariat was revised, a full inventory of biological samples available in the sample bank was conducted and a Shiny web application was developed which will allow better visualizing the existing samples and planning future analyses.

Regarding otolith microchemistry, new carbon and oxygen stable isotope analyses were carried out in 287 otoliths of Atlantic bluefin tuna captured in the central Atlantic Ocean, Canary Islands, Morocco, Portugal and the Bay of Biscay to determine their nursery area. $\delta^{13}C$ and $\delta^{18}O$ values measured in otolith cores indicated substantial mixing in Morocco and the central Atlantic Ocean, specially west of 45ºW. In addition, 1371 individual bluefin were assigned to their natal origin using different classification techniques. Results suggest substantial interannual variability in western contributions to some mixing areas, which warrants year to year monitoring. Assessment of the utility of otolith trace element chemistry along the growth axis of the otolith to reconstruct the spatial movements of adult bluefin tuna along their lifetimes is ongoing. Finally, trace element and stable isotope composition in young-of-the-year (YOY) from different nurseries were analyzed. Result show that, using trace elements, fish born in the eastern Mediterranean can

be distinguished from the ones born in the central and western Mediterranean with 98% discrimination accuracy.

Regarding genetic analyses, the RADSeq analyses have been completed with additional reference samples from the Northwest Atlantic, Western Mediterranean, Central Mediterranean and Eastern Mediterranean. Structure analyses show a clear structure between the Northwest Atlantic and the Mediterranean but no evidences of genetic structuring within the Mediterranean. A set of 192 RAD-seq derived SNPs has been selected and are being validated. Then they will be combined with the best SNPs derived from the GBS panel (Phase 4) and with other SNPs obtained from the literature in order to build a "final, best available SNP panel". Once this panel is technically and biologically validated, it will be ready to assign genetic origin of individuals captured in mixed feeding aggregations.

Regarding otolith shape analyses, otoliths of bluefin from the Gulf of Mexico (N=111) were used to improve the characterisation of the western stock of bluefin tuna using otolith shape. Additional otoliths were obtained from bluefin collected during the 2015 sampling season from several locations in the Mediterranean (Sardinia, Levantine Sea, Balearic Islands and Malta). The future analysis of these otoliths will improve the characterisation of the eastern stock of bluefin tuna using otolith shape. Bluefin from the Canadian fishery with a >80% estimated probability of originating from the Gulf of Mexico spawning grounds based on otolith stable isotope signatures were estimated to be predominantly of western origin based on otolith shape. This indicates that otolith shape is more influenced by environmental history than natal origin.

Regarding the integrated approach to stock discrimination, an integrated stock identification database has been established and is being updated as new material becomes available. Analysis of the integrated database revealed that overall, rates of agreement between methods were reasonably good. Rates of agreement were lowest for fish of potential western origin collected in the Mediterranean and northeast Atlantic and fish of potential eastern origin collected in the western Atlantic. This may reflect the influence of environmental history on phenotypic markers (otolith

shape and chemistry). Otolith shape data, otolith stable isotope data and genetic tissue samples from adult bluefin from the Gulf of Mexico has been obtained through collaboration with NOAA and will facilitate the characterization of the western stock using multiple markers. During the 2015 sampling season a coordinated approach was adopted which ensured the collection of otoliths and tissues from the same fish and representative of the Mediterranean spawning population. Future analysis of this material will facilitate the characterization of the eastern stock using multiple markers. The database, together with the material and data sourced through this task will enable an integrated stock discrimination analysis of Atlantic bluefin tuna.

Regarding the age determination analyses during Phase5, age has been interpreted from 356 calcified structures, 258 otoliths and 98 spines, of which 48 paired structures were obtained from the same specimen. The sample selection aimed to improve the sampling coverage of summer months, the Mediterranean area and purse seine fisheries. Stock identification of aged structures was assured by using samples whose natal origin had already been identified or by implementing preparation procedures for both ageing and for the identification of nursery origin using otolith chemistry and/or shape analysis. Diagnosis of paired age agreement was evaluated by precision indices. The annual, monthly, geographical and by gear stratification of the aged samples was explored for phase 5 and for all phases of the project. Likewise an age length key by calcified structure was built and the average size and its variation by age were examined.

In general, Phase5 was importantly affected by the delay in the contract signature. However, most of the objectives of the Project were met. The analyses already started to provide important information that is relevant for Atlantic bluefin tuna management. As such, project results will feed the upcoming stock assessment and Management Strategy Evaluation (MSE) process.

1. CONTEXT

On June 22nd 2015, the consortium formed by Fundación AZTI-AZTI Fundazioa, Instituto Español de Oceanografía, IFREMER, Universitá di Genova, University of Bologna, IZOR, COMBIOMA, National Research Institute of Far Seas Fisheries, Federation of Maltese Aquaculture Producers, INRH, GMIT, Texas A&M University, IPMA and Istanbul University, with subcontracted parties University of Pau, University of Arizona, SGiker/Ibercron, KaliTuna, Dr. Isik Oray, Dr. Toshihide Kitakado 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 06b/2015-2).

This proposal was awarded by the Secretariat on July 6th 2015. The final contract between ICCAT and the consortium represented by Fundación AZTI-AZTI Fundazioa was signed on July 20th 2015. The contract was amended on January 8, 2016.

According to the terms of the contract, a draft final report (Deliverable nº 4) needs to be submitted to ICCAT by January 31st. The present report was prepared in response to such requirement.

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 under this project is independent from other routine sampling activities for fisheries and fishery resources monitoring (e.g. the Data Collection Framework). Some of the sampling activities included in this report were conducted under other GBYP contracts (i.e. as part of the tagging programs).

2.1 Sampling accomplished

A total of 1506 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 665 individuals (including those to be provided by the tagging cruises), thus the current sampling status represents 226% of the target in terms of number of individuals. By size class, the objectives for larvae, age 0, juveniles, medium and large fish were all accomplished (>100%, 87%, >100%, 284% and 266% respectively, see Table 2.2), except for age 0 where the sampling was slightly below the target.

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

		Larvae	Age 0	Juveniles	Medium	Large	Total		
			<=3 kg	>3 & <=25 kg	>25 & <=100 kg	>100 kg		Target	%
Eastern Mediterranean	Levantine Sea		18		8	45	71	100	71%
Central Mediterranean	Adriatic Sea		50		50		100	100	100%
	Malta					10	10	50	20%
	South Sicily, Strait of Sicily		50				50	50	100%
	Gulf of Syrta				1	26	27	0	>100%
Western Mediterranean	Balearic	80	38				118	100	118%
	Ligurian Sea		25				25	25	100%
	Sardinia			3	66	9	78	50	156%
Strait of Gibraltar	Gibraltar		15				15	0	>100%
East Atlantic - West African coast	Madeira, Canary Islands					23	23	50	46%
	Western coast of Africa					50	50	50	100%
Northeast Atlantic	Bay of Biscay			2	2		4	0	>100%
	Portugal				1	43	44	40	110%
North Sea	Norway					26	26	0	>100%
Central North Atlantic	Central and North				14	593	607	50	1214%
North-Western Atlantic	Gulf of Saint Lawrance					30	30	0	>100%
Gulf of Mexico	Gulf of Mexico, Caribbean Sea	47				181	228	0	>100%
	Total	127	196	5	142	1036	**1506**	665	226%

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
Eastern Mediterranean		18		8	45	71	100	71%
Central Mediterranean		100		51	36	187	200	94%
Western Mediterranean	80	63	3	66	9	221	175	126%
Strait of Gibraltar		15				15	0	>100%
East Atlantic - West African coast					73	73	100	73%
Northeast Atlantic			2	3	43	48	40	120%
North Sea					26	26	0	>100%
Central North Atlantic				14	593	607	50	1214%
North-Western Atlantic					30	30	0	>100%
Gulf of Mexico	47				181	228	0	>100%
TOTAL	127	196	5	142	1036	1506	665	226%
Target	0	225	0	50	390	665		
% wrt target	>100%	87%	>100%	284%	266%	226%		

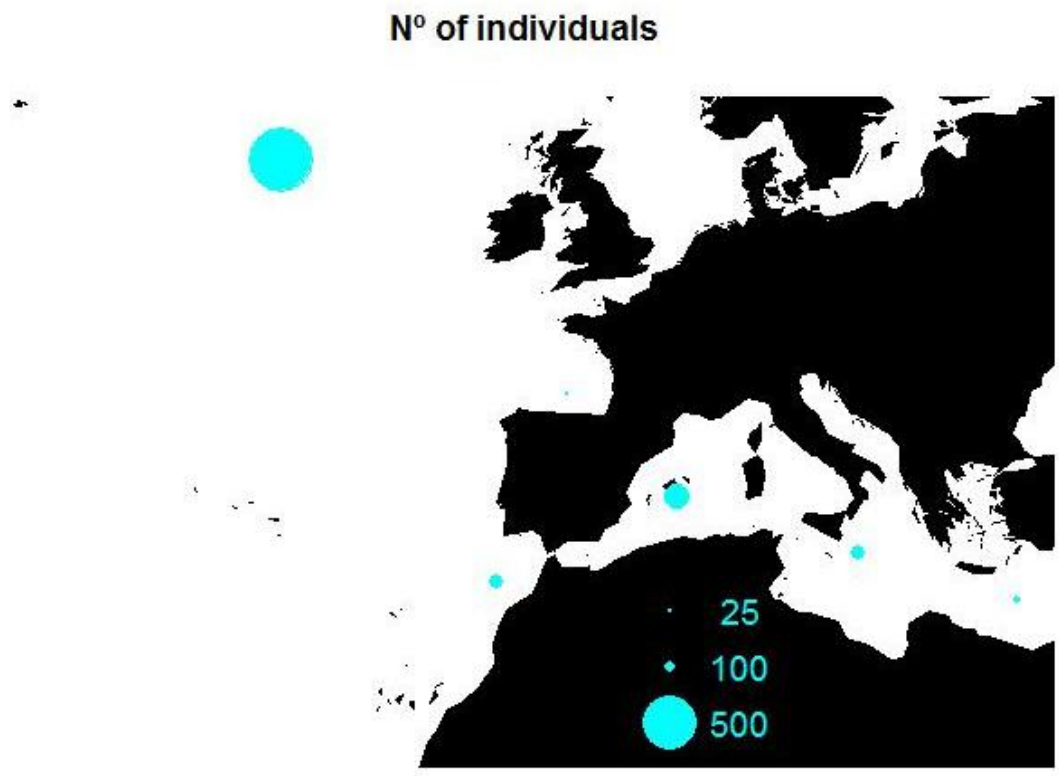


Figure 2.1: Number of individuals sampled in the Northeast Atlantic and Mediterranean, aggregated by main region. Positions of the dots are approximate averages across all samples.

The overall progress of the project was affected by the late award and signature of the contract, that came after many fisheries had already started or were already closed. Although members of the consortium tried to keep up with their tasks, the late signature of the contract affected mainly in those cases where travel, purchase and/or subcontracting costs were needed to accomplish the tasks. Yet, the sampling objectives were generally met.

In the Eastern Mediterranean, 71% of the target number of individuals (YOY and adults) has been sampled. The sampling for YOY in the Levantine Sea was below the original plan, with only 18 individuals sampled, mostly in the area near the Turkish-Syrian border. The lower number of YOYs sampled this year is due to the bad weather conditions and the prevailing disputes in Syria.

In the Central Mediterranean, 94% of the target number of individuals has been sampled so far (YOY and adults). Sampling of wild adult fish in Malta was complemented with sampling of farmed fish from the Gulf of Syrta. The sampling during harvest of Adriatic farms (by IZOR and Kalituna) was also completed in early 2016.

In the Western Mediterranean, 126% of the target number of individuals was sampled, including fish from all sizes. The collaborations with the tagging teams in Sardinia (COMBIOMA) went very well and the target number of samples was reached in both cases, although in the case of Sardinia, the low sampling of large fish was compensated with medium size fish. IEO provided a Balearic larval sample from previous campaigns that was helpful for the genetic analyses as temporal replicate. Agreements were reached with the Balfego Group to access large fish from the Balearics and samples will be made available by IEO after Phase 5.

In the East Atlantic-West African coast, 73% of the target number of individuals was sampled. Sampling in Canary Islands was below the original expectation. In Morocco, the collaboration with tagging teams (INRH) went well and sampling of adult fish was conducted. The samples of Morocco and the Canary Islands provide opportunities to further inspect mixing in this area.

In Portugal, IPMA, in collaboration with observers and Tunipex trap fishermen, conducted the sampling. Regarding spines, there were some difficulties in getting the condyle completely without damaging the tuna, and the best possible efforts were made to include as much condyle as possible with each spine. Regarding otoliths, several where broken by the bullet used to euthanize the fish.

Furthermore, unexpected samples from Norway were obtained for the first time (provided by IMR). In the Central Atlantic, the number of samples available is well beyond the original expectation, which will potentially allow for interesting insights into mixing of stocks.

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

		Otolith	Spine	Muscle/Fin	Sampler
Eastern Mediterranean	Levantine Sea	62	71	71	ISTA/AZTI (Oray)
Central Mediterranean	Adriatic Sea	100	100	100	IZOR/UNIBO
	Malta	10		10	FMAP
	South Sicily, Strait of Sicily	50	50	50	UNIBO
	Gulf of Syrta	27	27	27	FMAP
Western Mediterranean	Balearic	38	38	118	IEO
	Ligurian Sea	25	25	25	UNIGE
	Sardinia	43	78	78	UNICA
Strait of Gibraltar	Gibraltar	15	15	15	IEO
East Atlantic - West African coast	Madeira, Canary Islands	23		23	IEO
	Western coast of Africa	50		50	INRH
Northeast Atlantic	Bay of Biscay	4		4	AZTI
	Portugal	40	36	44	IPMA
North Sea	Norway		1	24	IMR
Central North Atlantic	Central and North	402		408	NRIFSF
North-Western Atlantic	Gulf of Saint Lawrance	30			DFO
Gulf of Mexico	Gulf of Mexico, Caribbean Sea			228	NOAA/AZTI
	Total	**919**	**441**	**1275**	
			2635		

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

	Otolith	Spine	Muscle/Fin	TOTAL
Eastern Mediterranean	62	71	71	204
Central Mediterranean	187	177	187	551
Western Mediterranean	106	141	221	468
Strait of Gibraltar	15	15	15	45
East Atlantic - West African coast	73		73	146
Northeast Atlantic	44	36	48	128
North Sea		1	24	25
Central North Atlantic	402		408	810
North-Western Atlantic	30			30
Gulf of Mexico			228	228
TOTAL	**919**	**441**	**1275**	**2635**
Target	665	565	665	1895
% wrt target	138%	78%	192%	139%

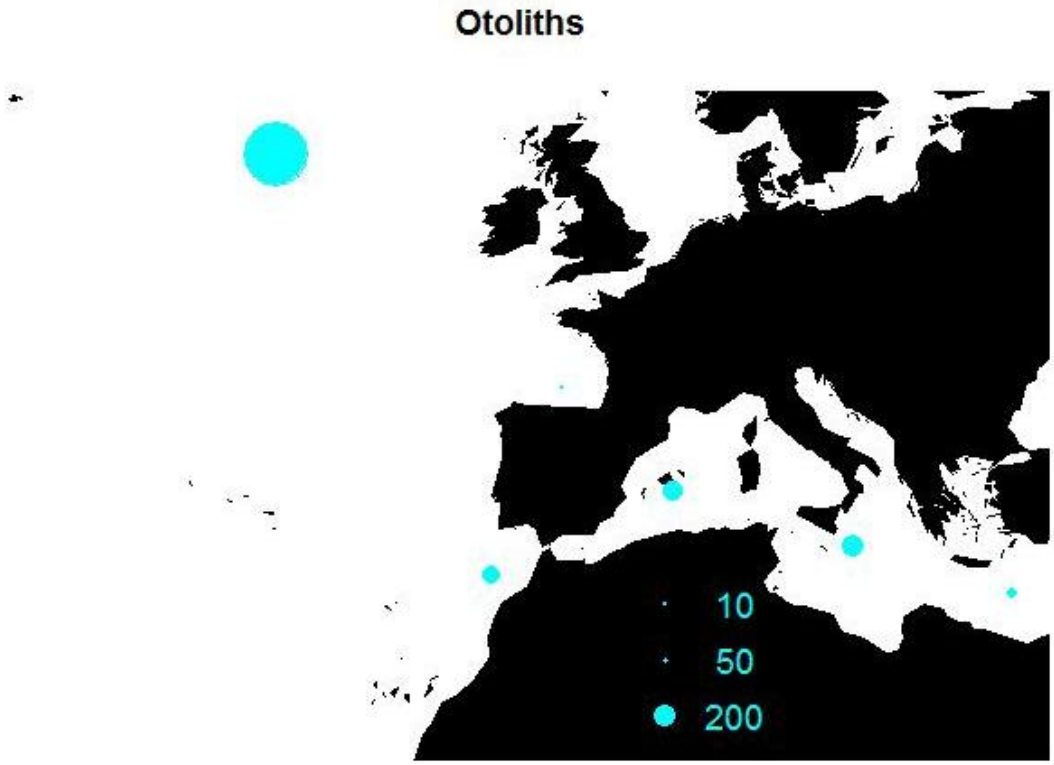


Figure 2.2: Number of individuals with otolith sampling in the Northeast Atlantic and Mediterranean, aggregated by main region. Positions of the dots are approximate averages across all samples.

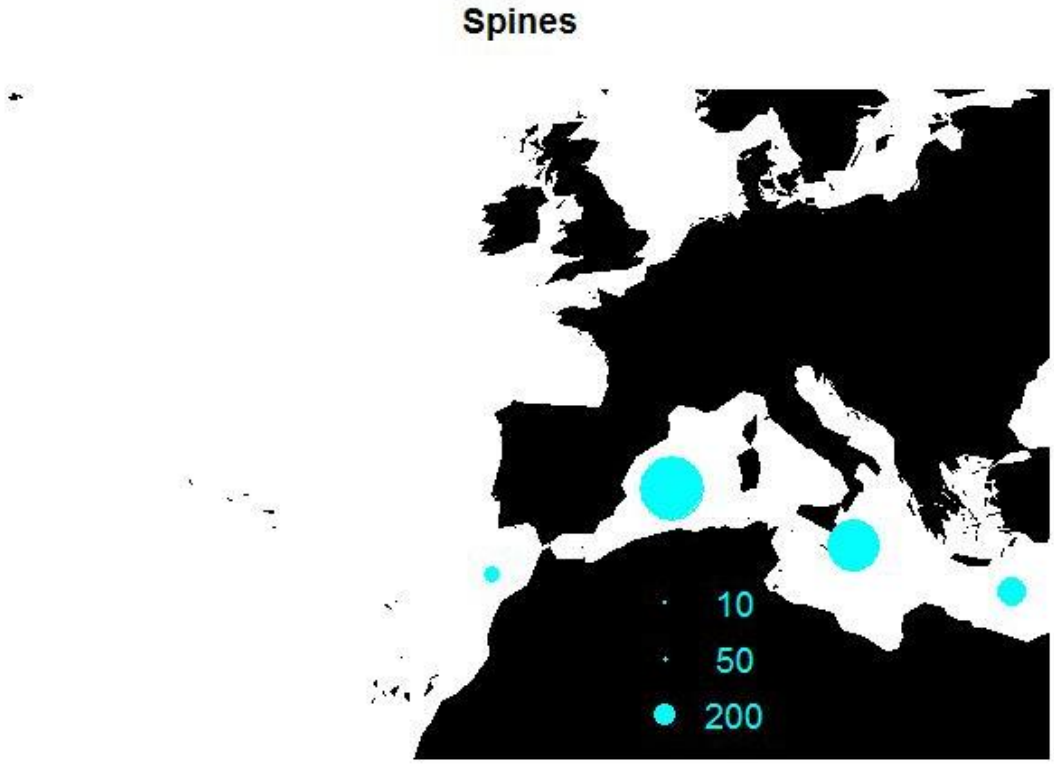


Figure 2.3: Number of spines collected in the Northeast Atlantic and Mediterranean, aggregated by main region. Positions of the dots are approximate averages across all samples.

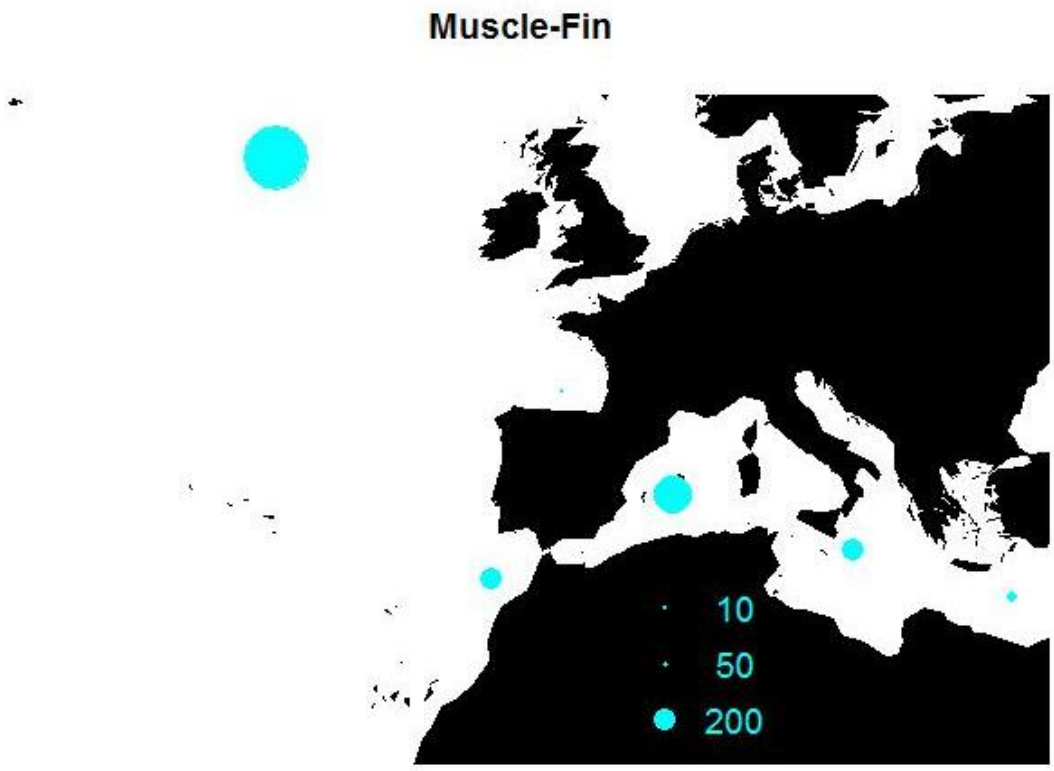


Figure 2.4: Number of muscle or fin tissue samples collected in the Northeast Atlantic and Mediterranean, aggregated by main region. Positions of the dots are approximate averages across all samples.

2.2 Sample bank and database

Most (but not all) of these samples have been sent to AZTI, following the protocols. 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.

During Phase 5, several modifications were performed in the database:

1.- A slightly new structure for age-weight information is adopted, that allows discerning between real measurements and estimates (e.g. using conversion factors). The information is classified into the following variables:

- Length [cm]: measured length
- Type of length: SFL, LD1, etc.
- Estimated Straight Fork Length [cm]: estimate using conversion factors
- Weight [kg]: measured weight
- Type of weight: GG, TW, etc.
- Estimated Round weight [kg]: estimate using conversion factors
- Notes for length and weight: conversion factors and equations used, and/or any other note specific for length/weight information.

Only direct measurements of length and weight are noted under “length”, “type of length”, “weight” and “type of weight”. Whenever length or weight are estimated (e.g. using conversion factors), this is noted under “estimated straight fork length” or “estimated straight fork weight”, and the specific conversion factors that were used are noted under “notes for length and weight”.

2.- The levels within each variable are updated, following the new area and gear strata defined by GBYP,

3.- The data entry formularies used by consortium partners have been modified to include predefined lists with the levels defined within each variable. These lists are used to select the value for each cell when filling the sampling form, reducing the number of errors that end up in the database (compared to previous practice where free entries were allowed).

In addition, a special effort was conducted to make an inventory of all available samples in the data bank held at AZTI. The inventory included samples from all previous GBYP

Phases, and included information (e.g. number of broken otoliths, genetic replicates…) that is not contained in the general database. The new inventory, that informs about which biological samples (otoliths, spines, genetic samples and gonads) are physically stored in AZTI, is linked to the general database, which allows the creation of detailed catalogs of available samples for future use. It is important to note that previous estimates of available samples were estimated based on the sampling conducted and the information available about whether those samples were sent to other labs or used (within AZTI) for different analyses. Although this might be a relatively good approximation, it might not necessarily be accurate since, as explained in previous reports, different circumstances faced mostly due to the late start of the yearly contracts (e.g. some samples being sent directly from the samplers to the analysts due to time constraints) affected the rigorous fulfilment of the protocols originally stablished, and the complexity of the database increased with time. Thus, the new inventory is believed to be a more accurate source of information on the samples that remain available in the sample bank.

Tables 2.5, 2.6 and 2.7 show the number of otoliths, genetic samples and spines that remain available in the sample bank, by area, subarea and size class.

Table 2.5: Number of otoliths by area, subarea and size class that remain available in the sample bank:

	AREA	larvae	YOY	Juvenile	Medium	Large	Total
Eastern Mediterranean	Levantine Sea (North)		372		102	40	514
	Aegean Sea			1			1
Central Mediterranean	Gulf of Syrta				1	25	26
	Malta		50		17	88	155
	South Sicily, Strait of Sicily		50		12		62
	Sicily (East Sicily and Ionian Sea)		120	98	37		255
	Adriatic Sea		49	154	50		253
Western Med	Tyrrhenian Sea		198		53	1	252
	Ligurian: Italian artisanal fleet		78	117	61		256
	Sardinia			9	67	20	96
	Gulf of Lion, Catalan			85	75		160
	Balearic		389	57	25		471
	Southern Spain						
Gibraltar	Gibraltar		15	16	90	42	163
East Atlantic - West African coast	Morocco					199	199
	Madeira, Canary Islands					69	69
	Mauritania						
Northeast Atlantic	Portugal				17	127	144
	Bay of Biscay			352	64		416
North Sea	Norway						
Central North Atlantic	Central and North Atlantic				8	505	513
	Azores						
North-Western Atlantic	Canada (Newfoundland-Labrador)						
	Canada (Gulf Saint Lawrence)						
	Canada (Nova Scotia)						
Gulf of Mexico & Caribean	Gulf of Mexico, Caribbean Sea						
Total			1321	889	679	1116	4005

Table 2.6: Number of genetic samples by area, subarea and size class that remain available in the sample bank:

	AREA	larvae	YOY	Juvenile	Medium	Large	Total
Eastern Mediterranean	Levantine Sea (North)		417		135	165	717
	Aegean Sea			29			29
Central Mediterranean	Gulf of Syrta				65	125	190
	Malta		26		41	179	246
	South Sicily, Strait of Sicily		50		12		62
	Sicily (East Sicily and Ionian Sea)		142	98	87		327
	Adriatic Sea		49	161	50		260
Western Med	Tyrrhenian Sea		259		96	1	356
	Ligurian: Italian artisanal fleet		79	120	70	1	270
	Sardinia			17	351	116	484
	Gulf of Lion, Catalan			86	76		162
	Balearic		385	82	48	1	516
	Southern Spain				4		4
Gibraltar	Gibraltar		15	19	114	106	254
East Atlantic - West African coast	Morocco					260	260
	Madeira, Canary Islands					73	73
	Mauritania						
Northeast Atlantic	Portugal				30	256	286
	Bay of Biscay			892	195	61	1148
North Sea	Norway					23	23
Central North Atlantic	Central and North Atlantic				80	1556	1636
	Azores						
North-Western Atlantic	Canada (Newfoundland-Labrador)					9	9
	Canada (Gulf Saint Lawrence)					23	23
	Canada (Nova Scotia)					17	17
Gulf of Mexico & Caribean	Gulf of Mexico, Caribbean Sea					181	181
Total			1422	1504	1454	3153	7533

Table 2.7: Number of spines by area, subarea and size class that remain available in the sample bank:

	AREA	larvae	YOY	Juvenile	Medium	Large	Total
Eastern Mediterranean	Levantine Sea (North)		41		96	135	272
	Aegean Sea			1			1
Central Mediterranean	Gulf of Syrta					28	28
	Malta		25				25
	South Sicily, Strait of Sicily		50		2		52
	Sicily (East Sicily and Ionian Sea)		100	94	61		255
	Adriatic Sea		49	116	50		215
Western Med	Tyrrhenian Sea		256		89	1	346
	Ligurian: Italian artisanal fleet		54	67	51		172
	Sardinia			3	205	71	279
	Gulf of Lion, Catalan			68	67		135
	Balearic		172	43	5		220
	Southern Spain				3		3
Gibraltar	Gibraltar		15	13	27	4	59
East Atlantic - West African coast	Morocco					25	25
	Madeira, Canary Islands						
	Mauritania						
Northeast Atlantic	Portugal				1	82	83
	Bay of Biscay			486	67	22	575
North Sea	Norway					2	2
Central North Atlantic	Central and North Atlantic						
	Azores					2	2
North-Western Atlantic	Canada (Newfoundland-Labrador)						
	Canada (Gulf Saint Lawrence)						
	Canada (Nova Scotia)						
Gulf of Mexico & Caribean	Gulf of Mexico, Caribbean Sea						
Total			762	891	724	372	2749

2.3 Shiny Application

A Shiny web application for R is being developed to facilitate the inspection of available samples in the biological sample bank and to aid sample selection following different criteria to help better design future experiments and analyses (see example in Figure 2.5). The Shiny application builds on the inventory of available samples conducted during Phase 5 (see previous paragraph). It allows to interactively subset the sample inventory using the predefined variables (area, year, month, size class and tissue type (namely otoliths, spines and/or genetic tissue)), and then to plot on the map the number

of available samples aggregated by position with symbols dependent on total sample size. The plotted information can be colored by Year, Month or Size class, using the legend tick box. The map can be refreshed anytime the selection criteria are changed, and the data can be downloaded in a *cvs* file. The downloaded file includes all individual fish (one row for each) contained in the final selection made by the user. For each fish, the individual ID number as well as information related to Area, Date of catch, fishing gear, length, weight and tissue availability is included.

The application is being finalized and tested. It is expected that a first consolidated version will be available only after Phase 5.. This first version can be further tested by ICCAT and any feedback can be incorporated in future improvements to the code. For the moment, it requires the user to have R and Shiny installed on their computer to run the Shiny app. It is expected to share the Shiny app in a near future without meeting these requirements, i.e., hosting the Shiny app.

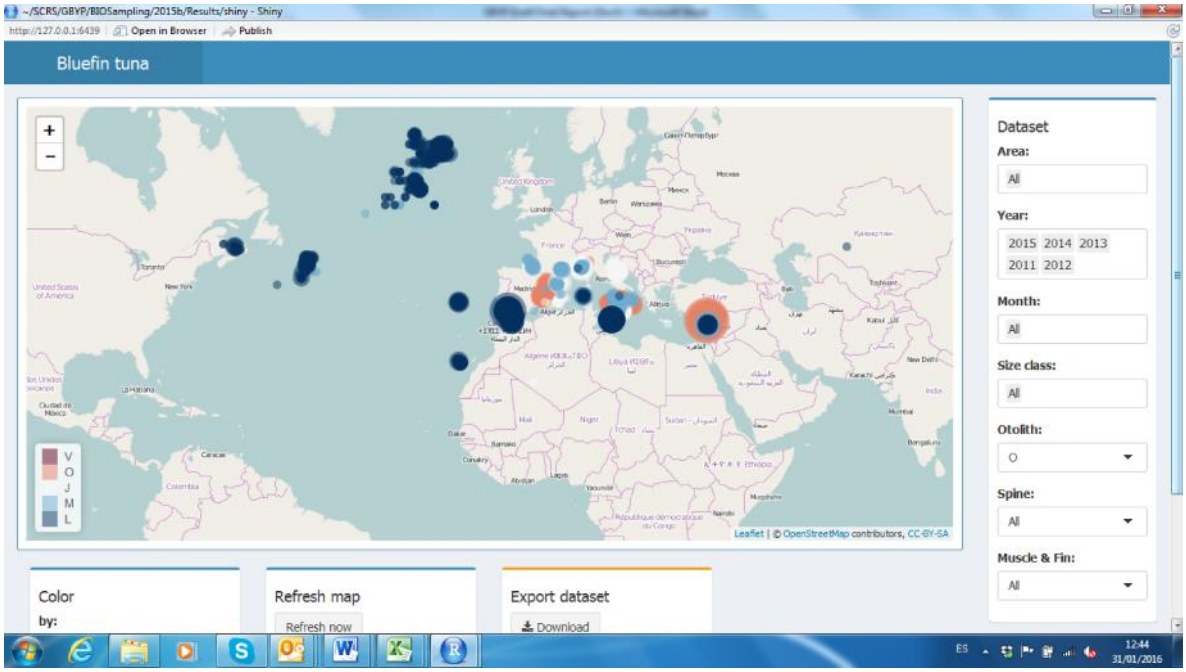


Figure 2.5: Shiny App being developed to visualize available biological samples in the sample bank held at AZTI. The dataset can be filtered using the variables in the right column (where none, one, several or all categories can be selected). The map can be refreshed using the "refresh map" button. The plotted information can be colored using different variables specified in the legend, and the information can be final subset of information can be downloaded as a cvs file.

3. ANALYSES

In the proposal, the consortium proposed to conduct 500 microchemical analyses on otoliths, 1000 individual assignments on available stable isotope data, 459 genetic analyses, 100 otolith images for otolith shape analysis, and 300 age assignments. As reflected in Deliverable 3, the late start of the contract affected the ability of some partners to conduct sampling in specific areas, send samples to AZTI, proceed with planned subcontracts, etc. Moreover, some technical difficulties (e.g. failure of the micromill) further delayed some tasks. However, most of the tasks evolved quicker during the last weeks/months. The following sections reflect the status of analyses conducted by the consortium.

The consortium is also making every effort to contribute with new stock origin data to the next stock assessment as well as the Management Strategy Evaluation (MSE) process. For the purposes of this contribution, otolith microchemistry, genetic and otolith shape data, from this and previous Phases of the GBYP program, were temporarely grouped according to the geographical strata in Lauretta et al (2015), as well as by month (or subyear) and year (the raw information remains according to the original strata and can be regrouped in any other way to fit the needs of the analyses). This information is being transferred to the appropriate modelers (e.g. Tom Carruthers in the case of the MSE) to interact as necessary and make sure it is useful in the process.

4. OTOLITH CHEMISTRY

Task Leader: Jay Rooker (TAMUG) & Igaratza Fraile (AZTI)

Participants:

AZTI: Igaratza Fraile, Haritz Arrizabalaga

TAMU: Jay Rooker

Otoliths of Atlantic bluefin tuna (*Thunnus thynnus*) have proven to be highly effective tools to study population structure and migratory pathways. Over fish's life, otoliths grow by accumulating new material in concentric layers around a central nucleus. Examining the chemical composition of different portions of otoliths inform about where fish have been at various life-stages. During GBYP Phase 5 we used otolith chemistry to answer different questions related with the ecology and stock structure of Atlantic bluefin tuna.

- We estimated mixed stock proportions of eastern and western populations throughout the North Atlantic Ocean based on stable isotopic composition (Task 1)
- We assigned the nursery origin (East vs. West) to Atlantic bluefin tuna analyzed for stable isotopic composition in previous GBYP Phases at individual level (Task 2).
- We assessed the utility of otolith trace element chemistry along the growth axis of the otolith to reconstruct the spatial movements of adult bluefin tuna along their lifetimes (Task 3).
- We used otolith trace element and stable isotope composition in young-of-the-year (YOY, age-0) bluefin tuna to distinguish different nurseries within the Mediterranean Sea (Task 4).

4.1 Determining nursery origin of bluefin tuna captured in the potential mixing zones

Introduction

The results from previous phases suggested that western origin contributions were negligible in the Mediterranean Sea, but mixing rates could be important in the open North Atlantic Ocean. In order to assess the spatial and temporal variability of mixing proportions, otoliths collected in areas with potential western contribution were analyzed for stable carbon and oxygen isotopes ($\delta^{13}C$ and $\delta^{18}O$).

Material and methods

In this section, we investigate the origin of bluefin tuna collected in the central North Atlantic Ocean (east and west of 45ºW), the East Atlantic - West African coast (Canary Islands, Moroccan Coast) and the eastern North Atlantic Ocean (Southern Portugal and Bay of Biscay) using stable $\delta^{13}C$ and $\delta^{18}O$ isotopes in otoliths. Samples utilized for this study (N=287) were collected under the GBYP. Central North Atlantic (west of 45ºW) samples were captured during August and September 2013 (43-45ºN, 47-48ºW) by observers on board of Japanese longline vessels operating in the central North Atlantic Ocean, whereas central North Atlantic (east of 45ºW) samples were collected from late October to early November of 2013 (52-59ºN, 19-22ºW). Samples from the Moroccan coast were collected in May 2014 by Moroccan traps, off the African continent (35ºN, 6ºW approximately), and during March 2014 around Canary Islands. Likewise, otoliths collected in southern Portugal were collected by Portuguese traps from May to October 2012 and samples from the Bay of Biscay, mostly juveniles and adolescents, collected from July to October 2012 by the bait boat and trolling fishery, were also included in this study (Figure 4.1).

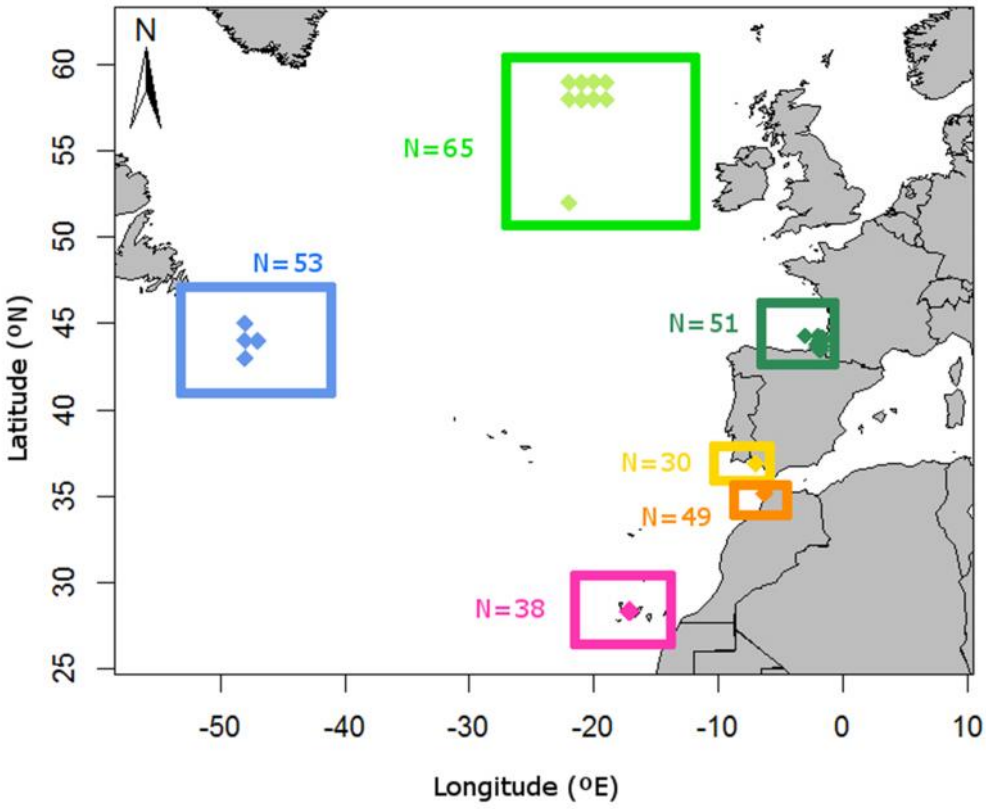


Figure 4.1: Study area in the North Atlantic Ocean. Otoliths collected in the western North Atlantic (west of 45°W, blue), central North Atlantic (east of 45°W, light green), Bay of Biscay (dark green), Moroccan traps (orange), Portuguese traps (yellow) and Canary Islands (pink).

Otolith handling followed the protocols previously described in Rooker et al. (2008). Briefly, following extraction by GBYP participants, sagittal otoliths of bluefin tuna were cleaned of excess tissue with nitric acid (1%) 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. (2008). 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.

Stable isotope signals of mixed stocks were compared with yearling samples from Mediterranean and Gulf of Mexico nurseries revised in GBYP-Phase 3 and presented in Rooker et al. (2014). HISEA software (Millar 1990) was used to generate maximum likelihood estimates (MLE) of mixed-stock proportions in each of the mixing zones.

Results

$\delta^{13}C$ and $\delta^{18}O$ were measured in the otolith cores of bluefin tuna from six locations in the Atlantic Ocean: 1) central North Atlantic Ocean (west of 45ºW), 2) central North Atlantic Ocean (east of 45ºW), 3) Bay of Biscay, 4) Atlantic coast of Morocco, 5) southern coast of Portugal and 6) Canary Islands. Mixed-stock analysis based on MLE indicated that bluefin tuna captured in the Bay of Biscay and by Portuguese traps were almost exclusively comprised by individuals from the 'eastern' or Mediterranean nursery (99.7% and 90.8%, respectively; Table 4.1 and Figure 4.2). In these two locations the confidence intervals around the estimated western proportions embrace the value of 0% and thus, using MLE, western contribution in the Bay of Biscay and southern Portugal during 2012 cannot be confirmed. Isotopic analyses revealed that mixing of eastern and western stocks occurred in the North Atlantic Ocean, both west of 45°W and east of 45°W. The presence of western migrants in the Atlantic coast of Morocco in 2014 was also notable; with the majority of bluefin samples classified to the western Atlantic population (70.3% of western origin, see also section 4.2). In contrast, catches around Canary Islands during the same year were entirely comprised by the Mediterranean population. Standard deviation around estimated percentage was ± 0%, indicating the degree of confidence in our predicted assignment was high.

Previous results suggested that movement of bluefin tuna born in the Gulf of Mexico (western origin) to the Bay of Biscay may be very limited or insignificant (Fraile et al. 2015). This trend continued, and during 2012 the predicted origin of juvenile and medium bluefin tuna from the Bay of Biscay was 99.7% 'eastern' fish. Likewise, results presented in previous phases suggested that catches by Portuguese traps in 2011 were

entirely supported by the Mediterranean population. In 2012, the percentage of western migrants detected in this region was low, indicating this region may also be comprised almost exclusively of 'eastern' bluefin tuna. In the central North Atlantic Ocean mixing of the two populations occurs at variable rates. Based on previous (Rooker *et al.*, 2014) and current results, the majority of bluefin tuna captured west of 45°W are of western origin, whereas catches east of 45ºW are primarily from the eastern population. In 2013, this fact was also mirrored by the isotopic results, but mixing rates estimated in Phase 5 in the central North Atlantic Ocean (both east of 45ºW and west of 45ºW) were found to be higher than in preceding years.

Previous analyses carried out in Phases 2 to 4 suggested a high degree of mixing in Morocco in 2011 but, the presence of western migrants was negligible in 2012 and 2013. In the present study, the estimated mixing proportions for 2014 were similar to those found in 2011, suggesting considerable interannual variability in the degree of mixing in this area. Similarly, a high mixing degree was detected in 2013 around the Canary Islands, but based on the present study, bluefin tuna captured in 2014 in this region were exclusively originated in the Mediterranean Sea, highlighting the importance of interannual variations in the spatial distribution of bluefin tuna in the North Atlantic Ocean.

Table 4.1. Maximum-likelihood predictions of the origin of large (>100 kg) bluefin tuna analyzed under the current contract. 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 (ï %).

			Predicted Origin		
Region	**Year**	**N**	**% East**	**% West**	**% SD**
Bay of Biscay	2012	52	99.7	0.03	± 1.3
Portugal	2012	30	90.8	9.2	±13.7
Central North Atlantic (west of 45°W)	2013	53	36.7	63.3	± 9.7
Central North Atlantic (east of 45°W)	2013	65	51	49	± 11.2
Morocco	2014	49	29.7	70.3	± 11.9
Canary Islands	2014	38	100	0	+ 0

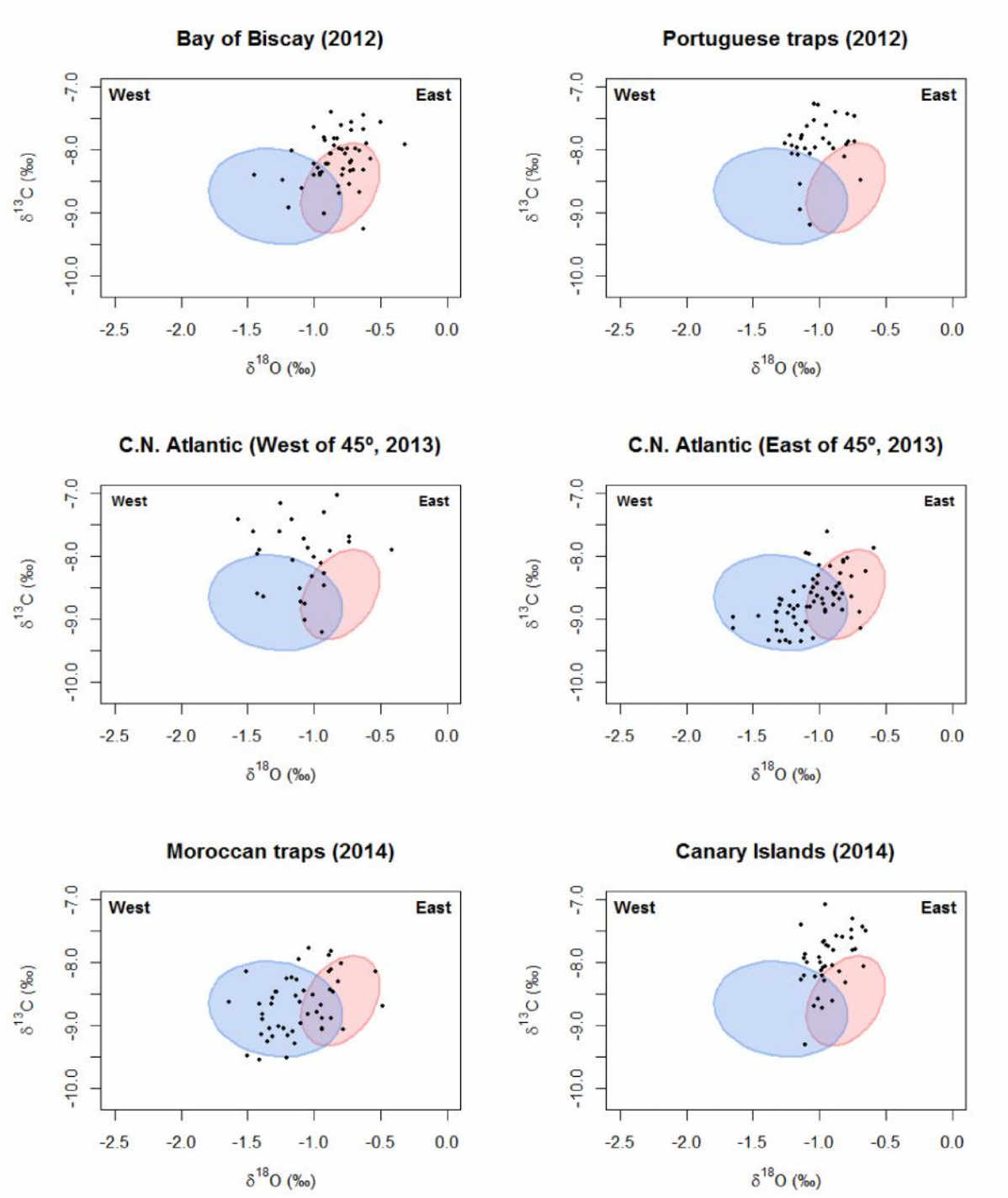


Figure 4.2: Confidence ellipses (1 SD or ca. 68% of sample) for otolith δ13C and δ18O values of yearling bluefin tuna from the east (red) and west (blue) along with the isotopic values (black dots) for otolith cores of bluefin tuna collected from the Bay of Biscay, Portuguese coast, central North Atlantic Ocean (namely west of 45ºW), central North Atlantic ocean (east of 45ºW), Atlantic Moroccan coast and Canary Islands.

4.2 Individual origin assignment

Introduction

During GBYP Phase 2 to Phase 4 otoliths from different regions of the Atlantic Ocean (Central North Atlantic, Bay of Biscay, Strait of Gibraltar and northwestern African coast) and Mediterranean Sea (Levantine Sea, Ionian Sea, Adriatic Sea, Tyrrhenian Sea, Ligurian Sea and Balearic Sea) have been analyzed for stable carbon and oxygen ($\delta^{13}C$ and $\delta^{18}O$) composition. The results from these analyses have been used to estimate eastern and western population mixing proportions in each of the areas. However, this does not allow knowing the origin of individual fish, and this information is needed for at least two main reasons: the construction of stock-age-length-keys, and the comparison/improvement of individual assignments based on different types of markers (i.e. genetic, otolith shape and stable isotopes). Moreover, it allows to table the results according to any stratification that might be used during the stock assessment or MSE process.

During Phase 5, individual classification techniques were applied to $\delta^{13}C$ and $\delta^{18}O$ values to predict the origin of bluefin tuna at individual scale.

Material and Methods

In total, 1371 individual bluefin were assigned to their natal origin (Gulf of Mexico or Mediterranean Sea), including 1084 from Phase 2 to Phase 4 and 287 presented in Task-1 of the current project. Detailed information on sample source is presented in Table 4.2.

$\delta^{13}C$ and $\delta^{18}O$ values of bluefin tuna otoliths were statistically analyzed and individuals were assigned to source populations with associated levels of probability. Classification performance of four classification methods was compared including classical standard procedures like Quadratic Discriminant Analysis (QDFA), a Bayesian estimator such as Naive Bayes (NB), and two different machine learning algorithms: Random Forest (RF) and Support Vector Machine (SVM). Each of the classification method was previously tested with the baseline dataset and attained similar classification accuracies. RF achieved the best performance, with an overall classification accuracy of 82%, followed by NB estimator (81% of accuracy) and QDFA and RF, both with 80% of correct classification. In order to increase robustness of our results, we only considered individuals with a probability >70% or <30% for the subsequent data analysis.

Table 4.2: Number of bluefin tuna sampled in several regions of the North Atlantic Ocean and Mediterranean Sea assigned individually to their natal origin using δ13C and δ18O composition of their otoliths. Regions: Adriatic Sea (AS), Balearic Sea (BA), Bay of Biscay (BB), Central Atlantic Ocean (CA), Canary Islands (CI), Strait of Gibraltar (GI), Levantine Sea (LS), Malta (MA), Atlantic Morocco (MO), south Portugal (PO), Sardinia (SA) and Tyrrhernian Sea (TY).

Region	Year-range	N samples	Size-range (FL, cm)
AS	2011	47	110-133
BA	2010-2011	42	77-263
BB	2011-2012	156	53-182
CA	2010-2013	497	121-268
CI	2013-2014	61	192-263
GI	2010-2011	100	161-269
LS	2011	48	174-282
MA	2011	82	113-262
MO	2011-2014	189	171.275
PO	2011-2012	123	170-281
SA	2011	20	123-247
TY	2010	6	149-293

Results

Overall, all classification methods lead to very similar results, indicating that individual classifications are robust (Figure 4.3). Detailed individual classifications are provided in Appendix A.

Based on QDFA and SVM methods, 226 individuals were identified as western migrants with a probability > 70%, whereas NB and RF identified 207 and 206 individuals respectively. Given the similarity of the methods, results from the QDFA were used in subsequent analyses. Most of the western migrants identified were collected in Central North Atlantic Ocean (CA) and Atlantic coast of Morocco (MO), followed by Canary Islands (Figure 4.4). This is in agreement with mixed stock proportions found in the previous GBYP Phases using maximum likelihood estimates.

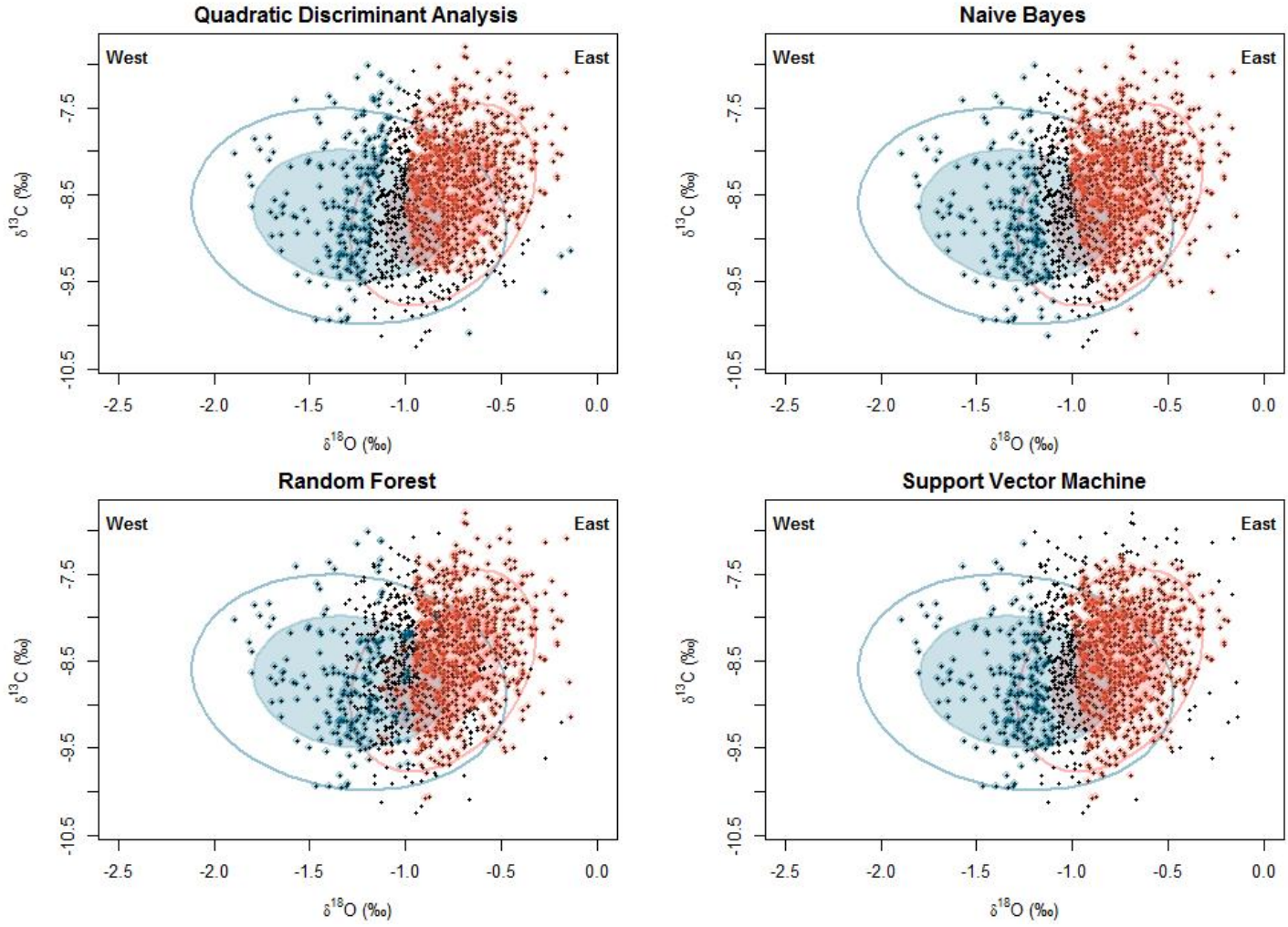


Figure 4.3: Confidence ellipses (colored ellipse: 1 SD or ca. 68% of sample, outer border: 2 SD or ca, 95% of sample) for otolith δ13C and δ18O values of baseline samples from the Mediteranean (red) and Gulf of Mexico (blue) nurseries along with the isotopic values (black dots) for otolith cores of bluefin tuna collected from the potential mixing zones. Isotopic values classified to eastern or western nurseries with a probability >70% using discriminant functions are shaded with corresponding colors.

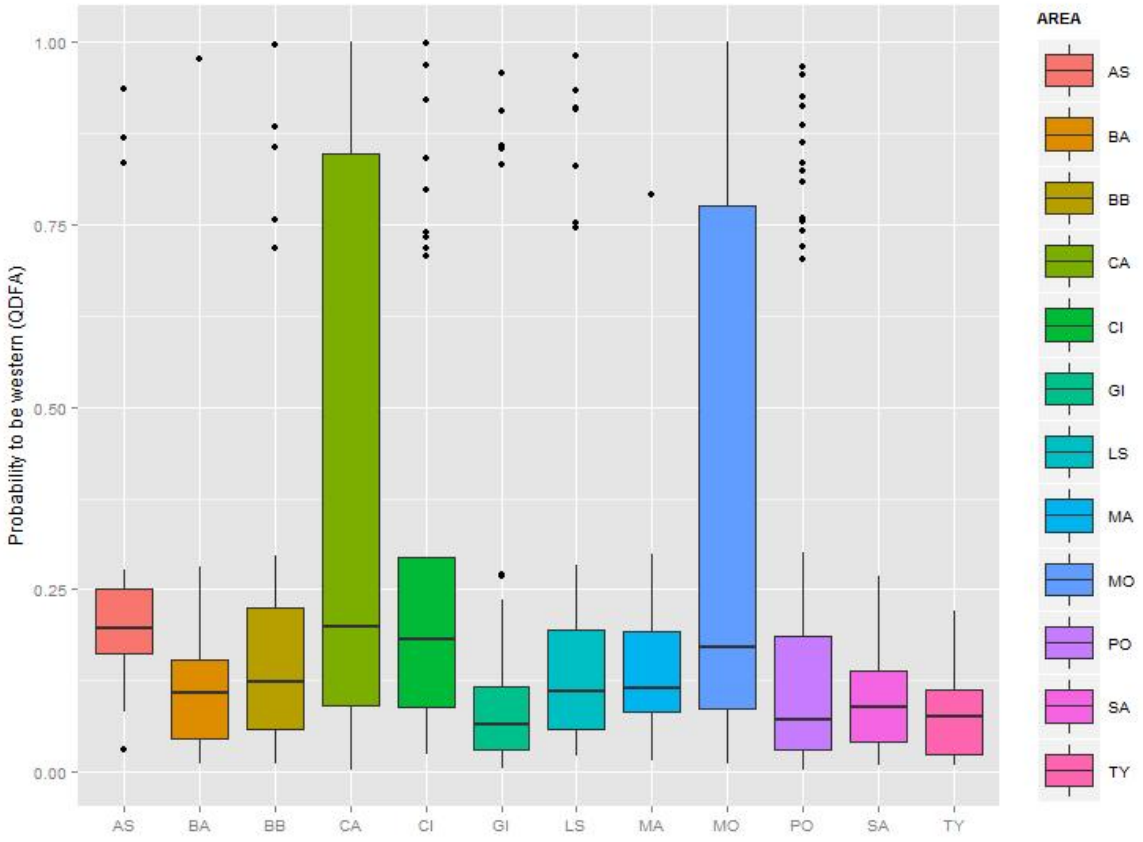


Figure 4.4: Boxplot of the probabilities of western origin estimated by QDFA (excluding probabilities between 30-70%).

In Central North Atlantic and Moroccan samples, the two main mixing zones, interannual variability was observed in the mixing proportions (Figure 4.5). In general, catches in the Central North Atlantic seem to be dominated by the eastern population, especially during 2012. Samples in 2013 reflect a similar proportion of eastern and western fish, but this is likely due to the fact that special emphasis was done this year to include a sample caught west of 45ºW. In Moroccan traps interannual variability was found to be high: catches in 2011 and 2014 were dominated by the western population, whereas 2012 and 2013 were highly dominated by Mediterranean-origin fish. Given the implications of such high mixing proportions for the stock assessment (especially for the smaller western population) and management, a year to year monitoring of the mixing is highly desirable.

It should be noted that the mixing proportions estimated in this section, through individual assignments, might not fully match the average proportions estimated by maximum likelihood in the previous section. However, considering the confidence intervals around those averages (i.e. mean+/-2*s.d), the results are generally concordant. The number of individuals assigned is slightly lower than with the

maximum likelihood approach because of the criteria followed (to assign fish only when the probability of belonging to a given population exceeds 70%), but it has the advantage that the whole dataset can be regrouped into any strata combination (e.g. areas and subyears) proposed by the different users (e.g. stock assessment or MSE experts), and that individual assignments between different methods can be compared.

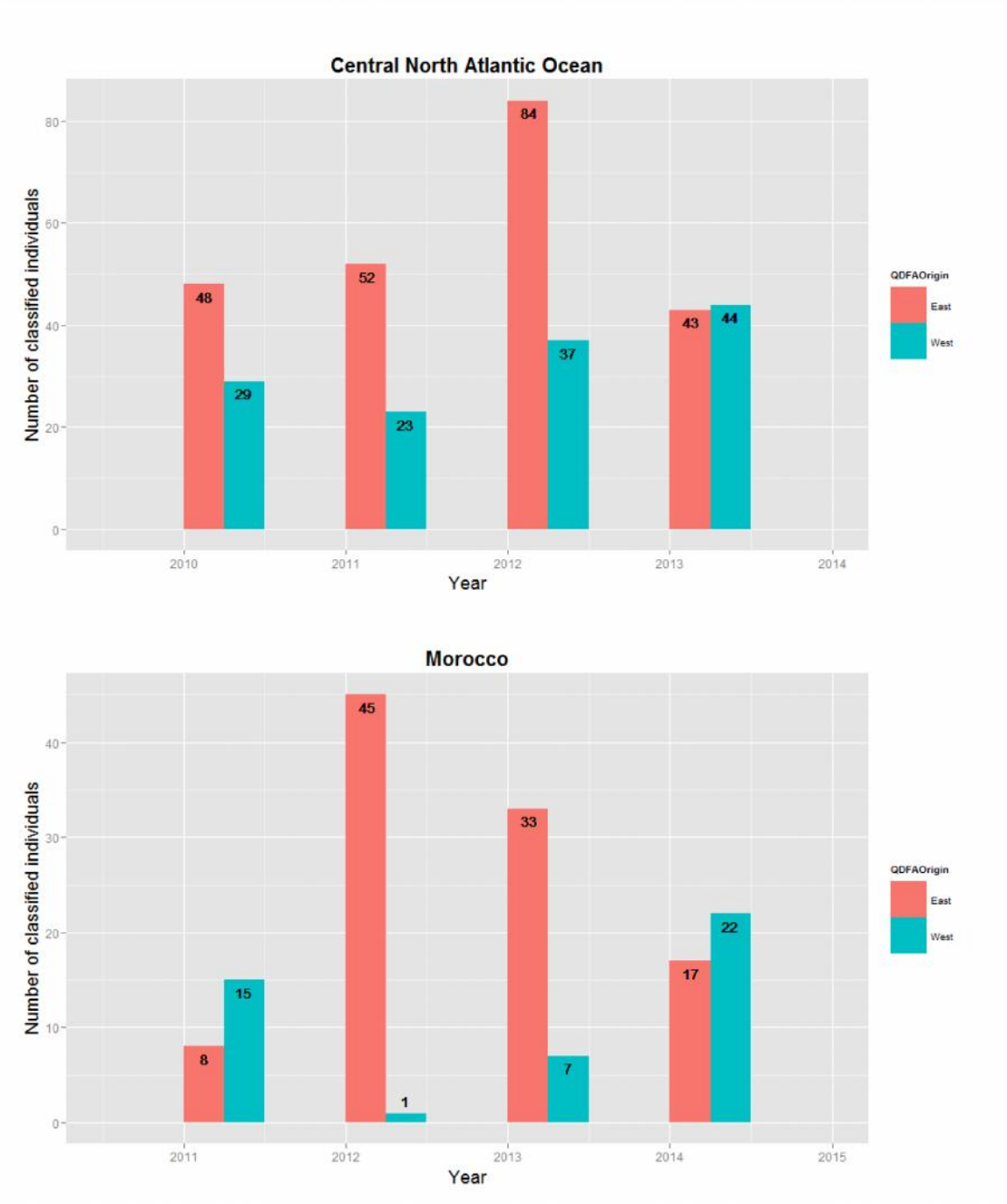


Figure 4.5: Interannual variability in the number of individuals classified to eastern (Mediterranean Sea) and western (Gulf of Mexico) nurseries using QDFA classification method (excluding individuals with probabilities between 30-70%).

4.3 Tracking habitat usage through different life stages by trace element composition

Introduction

During GBYP Phase 4 otoliths from Mediterranean Sea and open Atlantic Ocean were analyzed by Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICPMS) with the aim of developing a new marker that allows tracking bluefin tuna movement between the Mediterranean Sea and Atlantic Ocean. In the current phase we analyze additional otoliths to extend the dataset including samples from the western Atlantic Ocean.

Material and methods

In total, 127 otoliths were selected to analyze trace element concentration by LA-ICPMS. Samples included adult bluefin tuna from the eastern Mediterranean Sea (Levantine Sea, N=27), central Mediterranean Sea (Malta, N=40), Strait of Gibraltar (N=21), central-eastern North Atlantic Ocean (east of 45°W, N=13) and central-western North Atlantic Ocean (west of 45°W, N=26).

Sagittal otoliths of bluefin tuna were embedded in Epofix resin and cut with an Isomet saw to obtain transverse sections that included the core through the core. Transverse sections of approximately 0.7mm were first polished to the core with 1200 grade wet and dry sandpaper moistened with distilled water, and were further polished with a micro cloth and 0.3µm alumina powder to ensure a smooth surface. Sections were glued in a sample plate with thermoplastic glue. Magnesium (Mg), Strontium (Sr) and Barium (Ba) concentrations were measured by laser ablation inductively-coupled mass spectrometry (LA-ICP-MS) making a continuous transect from the core to the edge of the otolith, perpendicular to the growth axis. The signal corresponding to the last 40µm was then isolated to represent otolith 'edge' composition, as this portion of the otolith is supposed to reflect water mass physico-chemical properties just prior its capture (Figure 4.6). The system consisted of a laser ablation system (Nd:Yag 213 nm, New Wave Research) coupled to a mass spectrometer (X Series 2 ICP-MS, Thermo Electron Corporation). The ablation beam was of 40µm.

The calibration of the ICPMS was examined using the certified reference material, NIST 612 SRM, distributed by the National Institute of Standards and Technology. Calcium

concentration was assumed from the stoichiometry of calcium carbonate to be 400.000µg g-1.

Partial results

So far all samples were analyzed by LA-ICPMS system, but due to the late signature of the contract, results of the analyses have not been calibrated yet. The final results of this task will need to be finalized in future efforts of the project, as inter-otolith comparison cannot be completed prior to data calibration. The age will also be taken into account when interpreting the results.

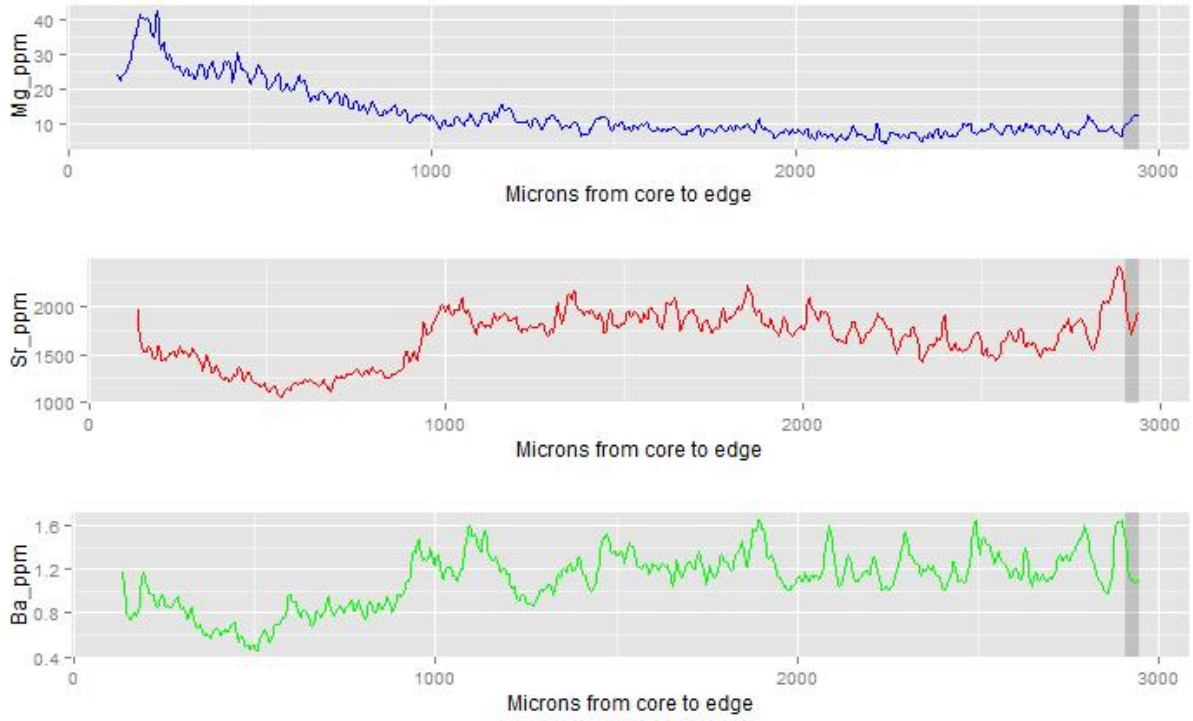


Figure 4.6: Example of trace element chemical analysis along the growth axis of an otolith of adult Atlantic bluefin tuna (Thunnus thynnus) captured in Malta. Analyses performed from the core to the edge. Last 40µm of the time series are used to represent capture location.

Preliminary results indicate differences in Mg, Sr and Ba concentrations. Generally Sr and Ba concentrations present a positive correlation, which is likely linked to high salinity values (Figure 4.7). Mg variability is often linked to differences in water temperature. These preliminary analyses suggest that discrimination among water

masses is possible if sufficient gradient in temperature and salinity exist among locations.

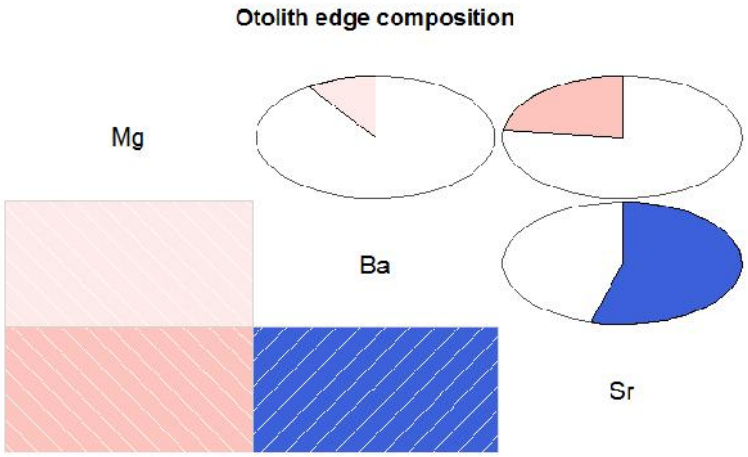


Figure 4.7: Correlation plot of trace elements measured on the edge of bluefin tuna otoliths captured in the Mediterranean Sea and North Atlantic Ocean. Blue and red colors indicate positive and negative correlations respectively, and the pie plots represent the correlation degree.

4.4 Discrimination of nursery areas within the Mediterranean Sea by trace element and stable isotope composition in young-of-the-year bluefin tuna

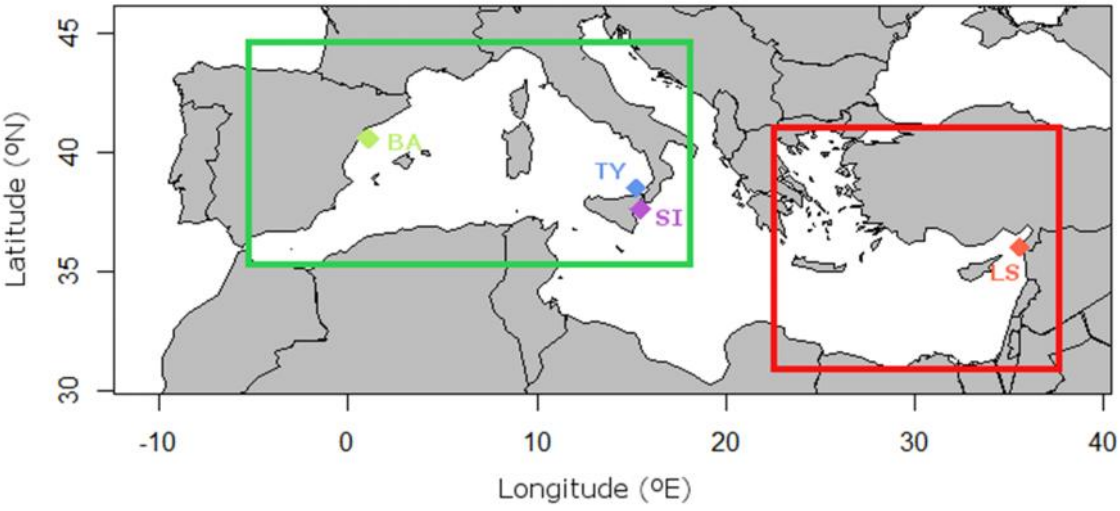
Introduction

The results from previous phases suggested that trace element composition might allow discriminating the natal origin of the Atlantic bluefin tuna from eastern, central and western Mediterranean Sea. In order to asses temporal variability and improve discrimination capacity, the study has been expanded to include samples from a previous year (2011) and combine trace element concentrations with stable isotope measurements ($\delta^{13}C$ and $\delta^{18}O$). During this phase, stable isotope and trace element

analyses have been carried out on young-of-the-year (YOY) fish captured in the Balearic Sea, southern Tyrrhenian Sea, east of Sicily and Levantine Sea. If YOY signatures are distinct among nurseries within the Mediterranean, then adult bluefin tuna that are caught in any fishery could be assigned back to their regions of origin, and each nursery's contribution to the adult population could be quantified.

Material and methods

Young-of-the-year (YOY) bluefin tuna used in this study were collected during two consecutive years (2011 and 2012) in the different nursery grounds within the Mediterranean Sea (Figure 4.8). Sagittae otoliths were extracted from each YOY fish using fine-tipped forceps, cleaned of excess tissue with nitric acid (1%) and deionized water and placed in plastic vials until further processing. One otolith per fish was used for stable isotope analysis, whereas the second pair was used for trace element



measurements.

Figure 4.8: Sample collection within the Mediterranean Sea: Balearic Sea (BA), southern Tyrrhenian Sea (TY), east Sicily (SI) and Levantine Sea (LS). Samples were grouped into western-central (BA, TY and SI) and eastern Mediterranean basin to improve discriminatory power.

Otolith preparation for stable isotopic analysis was similar to that described in Task 1: Briefly, otolith from each bluefin tuna specimen was embedded in Struers epoxy resin

(EpoFix) and sectioned using a low speed ISOMET saw to obtain 1 mm transverse sections that included the core. Following attachment to a sample plate, the portion of the otolith core corresponding to approximately the first two to three month of live 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. The pre-programmed drill path was made using a 300 μm diameter drill bit and 10 passes each at a depth of 50 μm was used to obtain core material from the otolith. Powdered core material was transferred to plastic vials 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. Overall, 153 otoliths were used for stable isotopic analysis, as detailed in Table 4.3.

In samples that were selected for trace element analyses, otolith core was identified under a dissecting microscope before embedding in Stuers epoxy resin (Epofix). Transverse sections of approximately 1 mm were cut with the low speed ISOMET ensuring that one side of the section matched exactly with the location of the core. Then, sections were polished with alumina powder of 0.3 μm using a polishing cloth to remove scratches made by the saw. After polishing, otoliths were triple rinsed Milli-Q water, dried under a laminar air flow and attached to a microscope slide using thermostatic glue (Crystalbond) prior to the laser ablation-ICP-MS analysis. Otoliths of 76 bluefin tuna were successfully processed for trace element analysis.

Otolith samples were analyzed with an IR 1030 nm femtosecond laser (Alfamet-Novalase, France) in conjunction with an Elan DRC II (Perkin Elmer) located at the University of Pau, France. A rectangle of 250 μm x 200 μm was ablated in the first inflexion point of the otolith) and results over a whole ablated surface were analyzed for trace element concentration to get the signature of the post-larval live stage. This allows avoiding possible perturbations resulting from the contamination introduced by the Crystalbond throughout micro-cracks often occurring around the core, as well as incorporation of elements due to maternal transfer. A pre-ablation step was implemented to minimize potential surface contamination (rectangle of 300 μm x 250 μm). The laser beams operated at a repetition rate of 500 Hz. Three glass reference material (NIST 610, NIST 612 and NIST 616 (National Institute of Standards and

Technology, USA)) and two fish otolith powder reference materials (FEBS-1 (National Research Council, Canada; Sturgeon 2005) and NIES No.22 (National Institute for Environmental Studies, Japan; Yoshinaga et al. 2000)) were used for laser ablation as calibration standards and quality control samples. Ten isotopes (Li^{7}, Mg^{24}, Ca^{43}, Mn^{55}, Fe^{56}, Co^{59}, Ni^{60}, Cu^{63}, Zn^{66}, Sr^{88} and Ba^{138}) were measured in each otolith by the LA-ICP-MS system. All the reference materials were measured at the beginning and the end of the session, for calibration and drift correction. ^{43}Ca was used as an internal standard for each ablation to check for variation in ablation yield. Elemental concentrations were standardized to calcium (i.e. Sr:Ca and Ba:Ca) based on the stoichiometry of calcium carbonate (380.000 µg Ca g^{-1} otolith). The data processing proceeds by identifying the background and signal windows for each measurement. Each measurement is defined here as the acquisition of data from one complete rectangle. The background signal is defined as the period during which only the carrier gas composition is measured, prior to the laser firing. The background signal was used to calculate the limit of detection (LOD) and the limit of quantification (LOQ), which were calculated as the mean background level plus 3 and 10 times standard deviation respectively. Concentrations below LOQ were not included in the statistical analysis.

Multivariate statistics were used to stable isotope and trace element data to distinguish among the different nursery grounds within the Mediterranean Sea. Multivariate Analysis of Variance (MANOVA) was performed to verify differences among the groups. Also a stepwise quadratic discriminant function analysis (QDFA) was computed to determine the combination of element that best discriminate among regions within the 2011 year-class, and to estimate classification accuracy of the resultant discriminant function (Mercier et al. 2011). A Principal Component Analysis (PCA) was applied to elemental fingerprints to reduce the dimensionality and illustrate the affinity of the elements analyzed. Interannual variability was tested using samples collected in SI over two consecutive years (2011 and 2012).

Table 4.3: Number of YOY Bluefin tuna otoliths to be analyzed in task 4, to build a baseline for discrimination of nurseries within the Mediterranean Sea: Levantine Sea (LS), southern Tyrrhenian Sea (TY), east Sicily (SI) and Balearic Sea (BA).

Method	Year	Region	N samples	Size-range (cm)
Trace elements	2011	LS	15	29-35
		TY	13	35-39
		SI	17	24-41
		BA	14	24-35
	2012	SI	17	28-40
Stable isotopes	2011	LS	20	26-55
		TY	20	35-42
		SI	18	24-41
		BA	20	22-35
	2012	LS	17	20-46
		TY	19	35-42
		SI	20	28-40
		BA	19	32-40
Trace element & Stable Isotopes	2011	LS	14	29-35
		TY	13	35-39
		SI	17	24-41
		BA	15	24-35
	2012	SI	17	28-40

Results

Stable isotopes

In total 153 otoliths of YOY bluefin tuna collected in 2011 (N=78) and 2012 (N=75) over 4 known nursery grounds were analyzed for stable $\delta^{13}C$ and $\delta^{18}O$ composition (Table 1). For both the 2011 and 2012 year-classes differences in otolith stable isotopes were mostly induced by the higher $\delta^{18}O$ and lower $\delta^{13}C$ composition of samples from the Levantine Sea compared to the other three regions, as indicated by the Tukey's HSD test. Among samples from the Balearic Sea, southern Tyrrhenian Sea and east Sicily, we found no difference in $\delta^{18}O$ composition for the 2011 cohort, whereas in 2012 differences among areas were shown by this isotopic ratio (Figure 4.9). The interannual variability

found in YOY otolith chemistry is probably a result of the complex oceanography and climate variability of the Mediterranean Sea. These results suggest that in population structure studies, it might be necessary to match adult signature to the appropriate year class when predicting the natal origin of adult bluefin tuna.

Classification success (based on QDFA) considering four potential groups baseline was 40% in 2011 and 44% in 2012 (Table 4.4, Figure 4.9). Given that differences were mostly derived from the Levantine Sea, we decided to merge samples from the Balearic Sea with those collected in southern Tyrrhenian and east of Sicily to improve classification accuracy (Figure 4.10). Results from QDFA indicated a good classification success for YOY from western-central vs. eastern Mediterranean basin (86% in 2011 and 78% in 2012). These results reflect the strength of this approach as a tool to differentiate bluefin tuna originated in the Levantine Sea with those from the central and western spawning grounds.

Table 4.4: Best element(s) and classification accuracy (estimated by QDFA) using stable isotopic composition of young-of-the-year bluefin tuna otoliths for 2011 and 2012 cohorts. Area codes correspond to Levantine Sea (LS), southern Tyrrhenian Sea (TY), east Sicily (SI) and Balearic Sea (BA).

Group division	Year	Best element(s)	Classification accuracy
BA / TY / SI / LS	2011	$\delta^{18}O + \delta^{13}C$	40%
BA / TY / SI / LS	2012	$\delta^{18}O + \delta^{13}C$	44%
East (LS) / West-Centr. (BA, TY, SI)	2011	$\delta^{18}O + \delta^{13}C$	86%
East (LS) / West-Centr. (BA, TY, SI)	2012	$\delta^{13}C$	78%

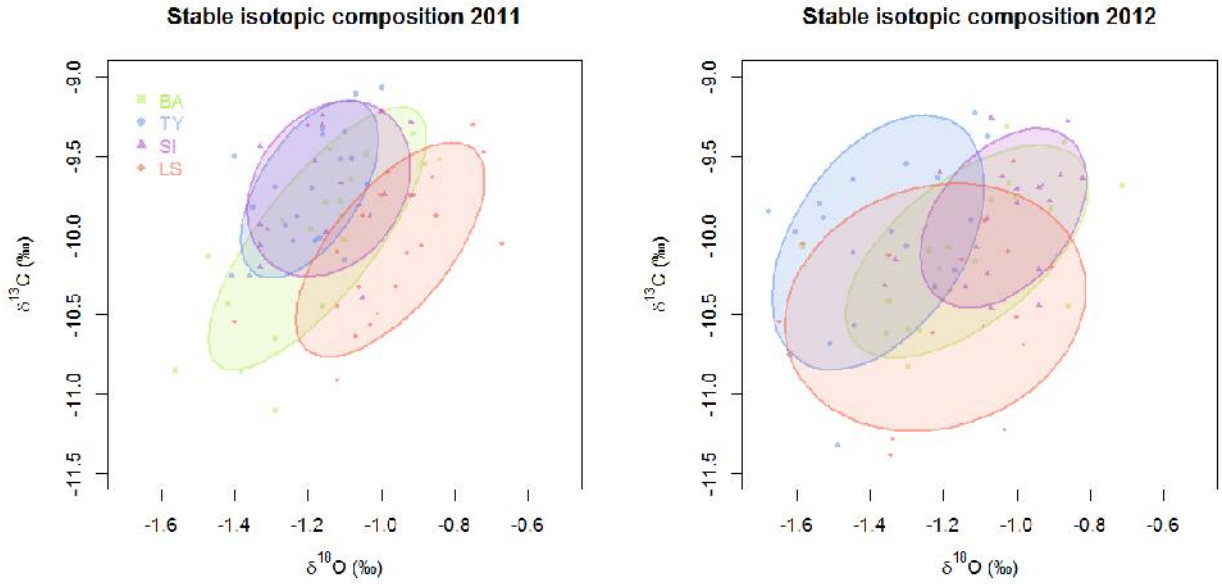


Figure 4.9: Confidence ellipses (1 SD or ca. 68% of sample) for otolith δ13C and δ18O values of young-of-the-year bluefin tuna from the Balearic Sea (green), southern Tyrrhenian Sea (blue), eastern Sicily (purple) and Levantine Sea (red) collected during 2011 and 2012.

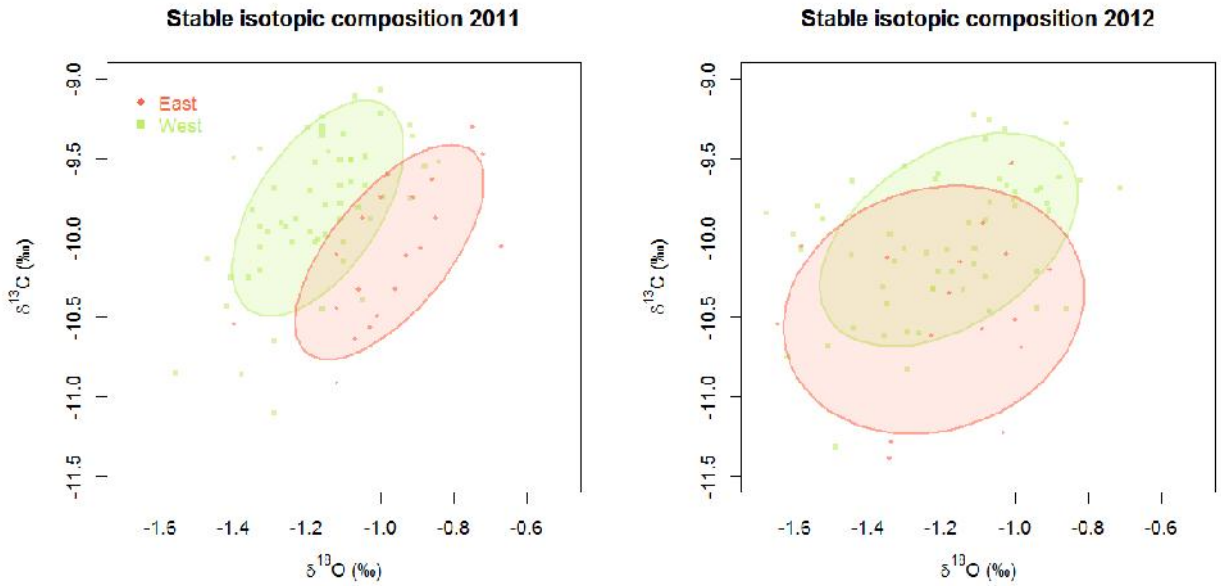


Figure 4.10: Confidence ellipses (1 SD or ca. 68% of sample) for otolith δ13C and δ18O values of young-of-the-year bluefin tuna from the eastern (Levantine Sea, in red) and western-central (Balearic Sea, southern Tyrrhenian Sea and eastern Sicily, in green) Mediterranean basins.

Trace elements

All element included in the statistical analyses were above the LOQ (Li, Mg, Mn, Fe, Cu, Zn, Sr and Ba). For the 2011 year-class we found no difference among samples from BA, TY and SI nursery grounds (MANOVA, $p > 0.05$). Thus, samples were grouped into eastern (LS) and western-central (BA, TY and SI) Mediterranean Sea to improve the discriminatory capacity. Li, Mg, Fe, Sr and Ba were found to be statistically significant between the eastern and western-central Mediterranean basins. The Tukey's HSD showed that Fe, Sr and Ba were significantly higher in the eastern Mediterranean basin, whereas Li concentration was higher in the western-central Mediterranean. Differences in Mg, Sr and Ba likely reflect east to west gradient in temperature and salinity across the Mediterranean Sea, whereas Fe and Li may also introduce meaningful information for establishing elemental signatures. However, the optimal classification accuracy (based on QDFA) was attained when using only the combination of Ba, Fe, Li and Mg. Results from QDFA indicated that YOY bluefin tuna were classified to the eastern and western-central Mediterranean basins with 98% accuracy.

A PCA was applied to these 5 elements to illustrate the affinity of the elements (Figure 4.12). The first two axis of the PCA explained the 71% of the variation in the data, and they were used to visualize the distribution of the data on a two-dimensional axis (Figure 4.13).

Interannual variability was found to be significant between 2011 and 2012, suggesting that year-class matching is necessary to use this baseline to predict the nursery origin of adult bluefin tuna within the Mediterranean Sea.

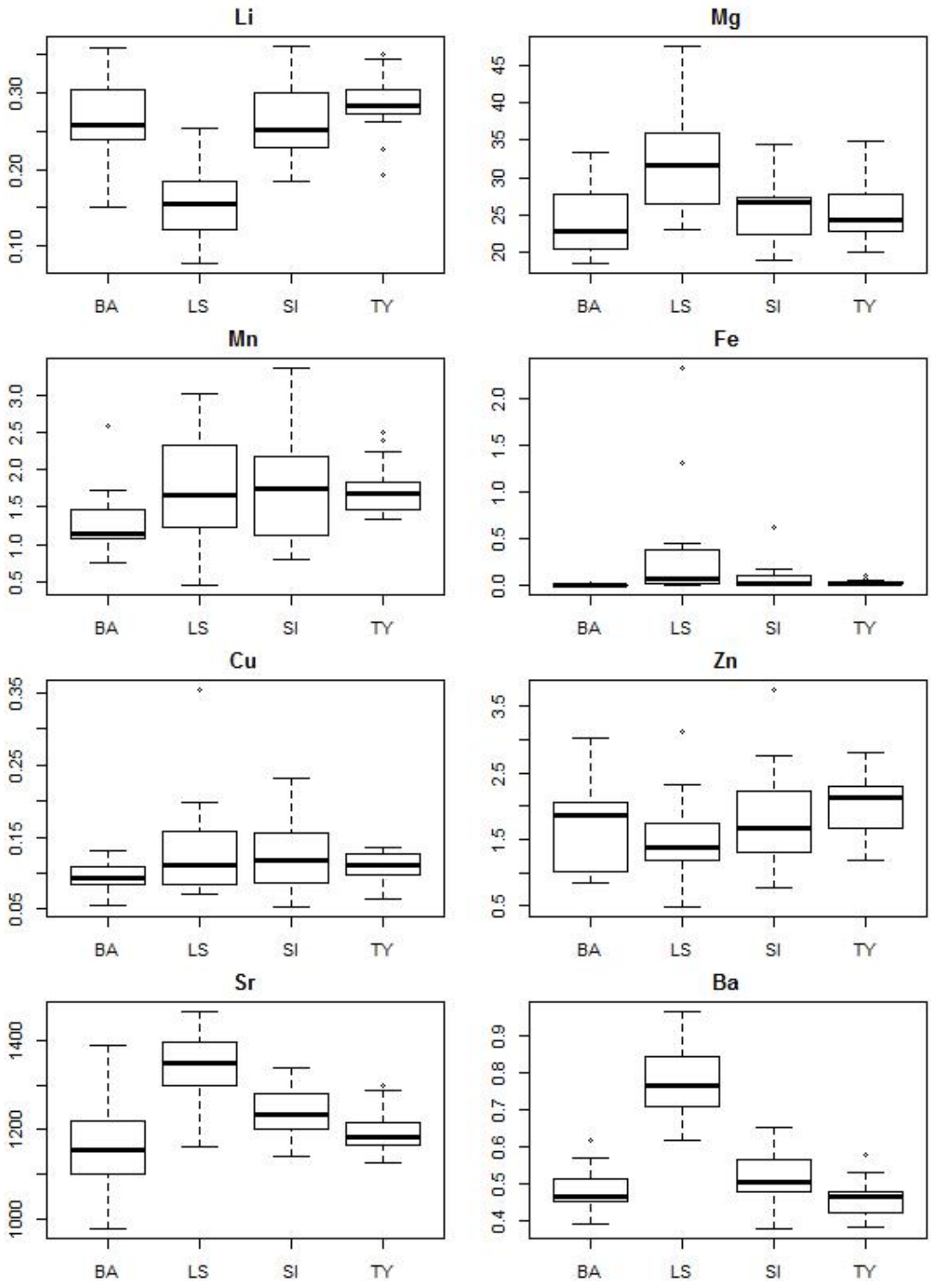


*Figure 4.11: Trace element concentration (ppm) in post-larval portion of otoliths from young-of-the-year Atlantic bluefin tuna (*Thunnus thynnus*) collected in the Balearic Sea (BA), Levantine Sea (LS), eastern Sicily (SI) and southern Tyrrhenian Sea (TY) from August to October 2011.*

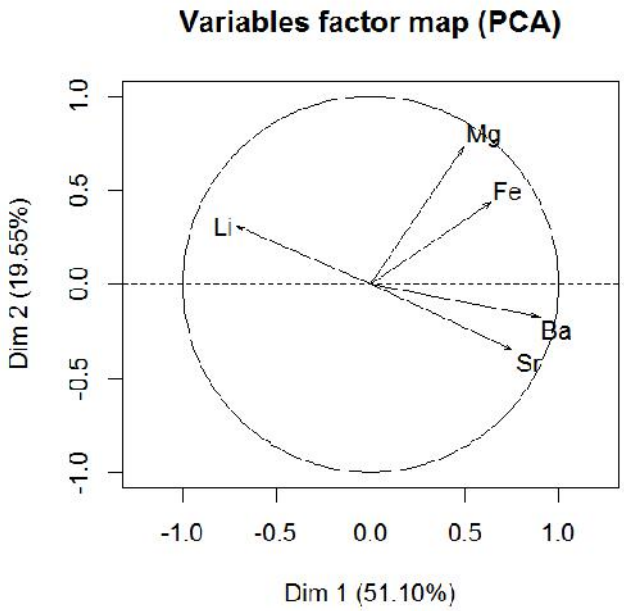


Figure 4.12: Principal Component Analysis plot of relationship among the trace element concentrations in the near-core portion of otoliths of YOY bluefin tuna (Thunnus thynnus) collected in the Mediterranean Sea.

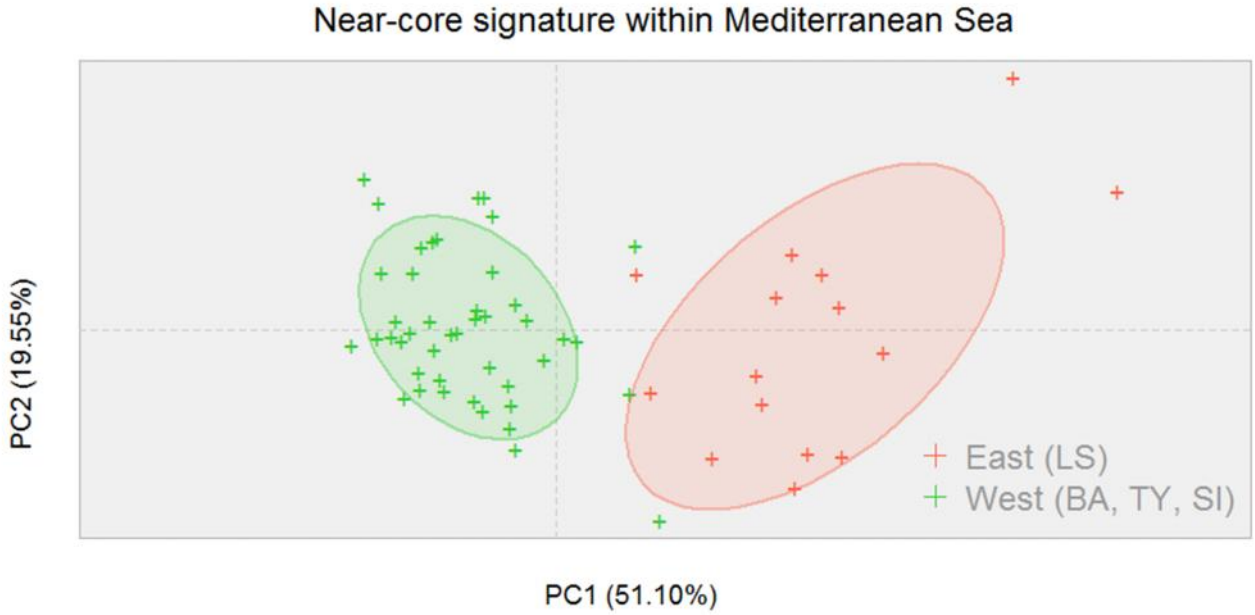


Figure 4.13: Elemental fingerprints for young-of-the-year bluefin tuna (Thunnus thynnus) otoliths from the eastern (Levantine Sea, in red) and western-central (Balearic Sea, southern Tyrrhenian Sea and eastern Sicily, in green) Mediterranean basins, based on the first two axis of the Principal Component Analysis including Li, Mg, Fe, Sr and Ba concentrations.

Combined stable isotope and trace elements

In a second step, stable Isotope values and trace element concentrations were combined to identify nurseries in the eastern and western-central Mediterranean basins. In total, 76 otoliths included both trace element and stable isotopic measurements (see table 4.3 for details). Univariate ANOVA was used to detect which elements and/or isotopes exhibited differences between the eastern and western-central regions. From all isotopic and elemental concentrations measured above the LOQ, Li, Mg, Fe, Sr, Ba, $\delta^{13}C$ and $\delta^{18}O$ were significantly different between the two regions. For 2011 year-class, a QDFA was employed to build a classification function. We identified the combination of stable isotopes and trace elements with the highest classification rate using a stepwise variable selection procedure (Mercier et al. 2011). The combination of elements identified as achieving the highest accuracy was the same as using trace element fingerprints alone (Ba, Fe, Li and Mg), with a classification success of 98% to the eastern and western-central Mediterranean basins.

A PCA was used to visualize the relationship among the trace elements and stable isotopes on a two-dimensional axis (Figure 4.14), and to illustrate differences among nursery areas in the eastern (Levantine Sea) and western-central (Balearic Sea, southern Tyrrhenian Sea and eastern Sicily) Mediterranean basins (Figure 4.15).

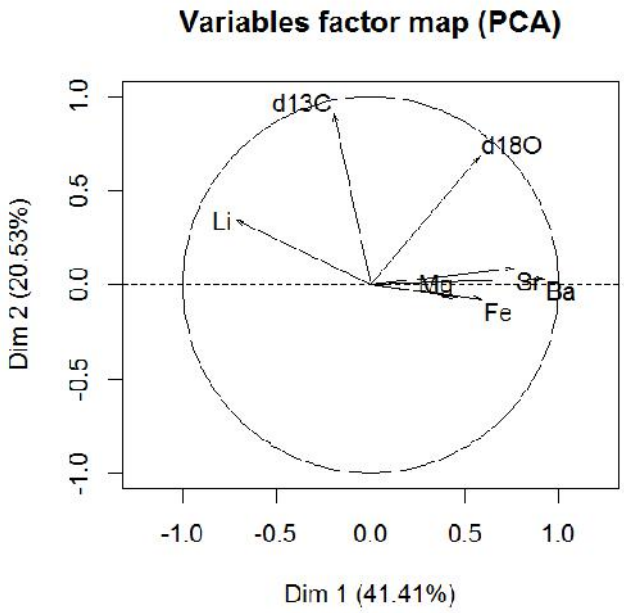


Figure 4.14: Principal Component Analysis plot of relationship among the stable isotopes and trace elements in otoliths of YOY bluefin tuna (Thunnus thynnus) collected in the Mediterranean Sea.

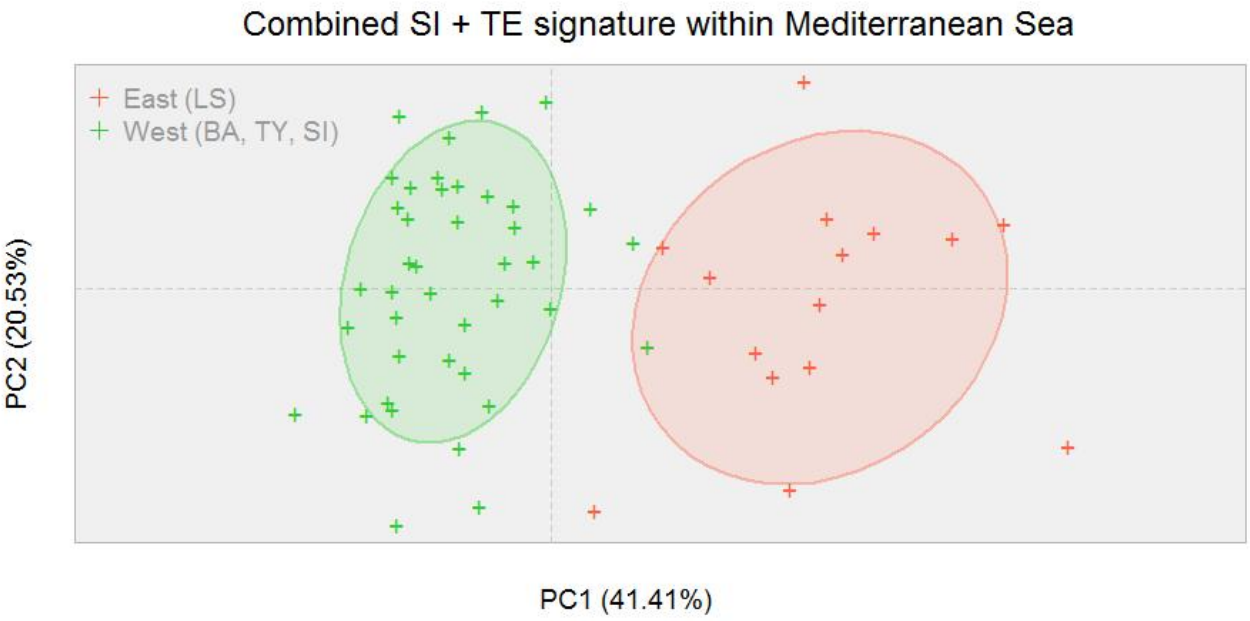


Figure 4.15: Elemental and isotopic fingerprints for young-of-the-year bluefin tuna (Thunnus thynnus) otoliths from the eastern (Levantine Sea) and western-central (Balearic Sea, southern Tyrrhenian Sea and eastern Sicily) Mediterranean basins, based on the first two axis of the Principal Component Analysis including Li, Mg, Fe, Sr, Ba concentration together with δ13C and δ18O values.

Conclussions

The results of this research show that we can distinguish fish from Levantine Sea against fish from the western and central Mediterranean Sea with a high degree of accuracy using trace element analysis of otoliths (98% discrimination accuracy). This opens up the possibility of being able to identify the source environment of adult bluefin tuna from the 2011 year-class within the Mediterranean Sea. This technique will allow determining if some spawning locations have greater contributions to the adult stock than others. Due to significant interannual variation in the chemical signatures in the Mediterranean Sea, our attempts to classify bluefin tuna from adjacent or combined year-classes will likely result in lower accuracy. Building a multiyear baseline for elemental signature is necessary when using trace element chemistry for classification of several year-classes.

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5. GENETICS

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COMBIOMA: Rita Cannas.

5.1 Introduction

The work planned for Phase 5 was divided into 4 tasks, two of which have been successfully advanced while it was not possible to complete the other two within Phase 5.. The reason for the delay on the completion of Tasks 3 and 4 has been the unexpected difficulty encountered when selecting the SNPs suitable for genotyping (Task 2), as these had to be mapped against the previously built genome assembly in order to get enough information for the design of genotyping probe and in-house computer programs had to be developed for that aim.

5.2 Material and Methods

5.2.1 Samples

For GBYPPh5-Task1, 75 reference samples (larvae and young of the year) from the Gulf of Mexico and North-West Atlantic, western Mediterranean, central Mediterranean and eastern Mediterranean were added to the 165 already used in Phase 4 to complete the RAD-seq analyses.

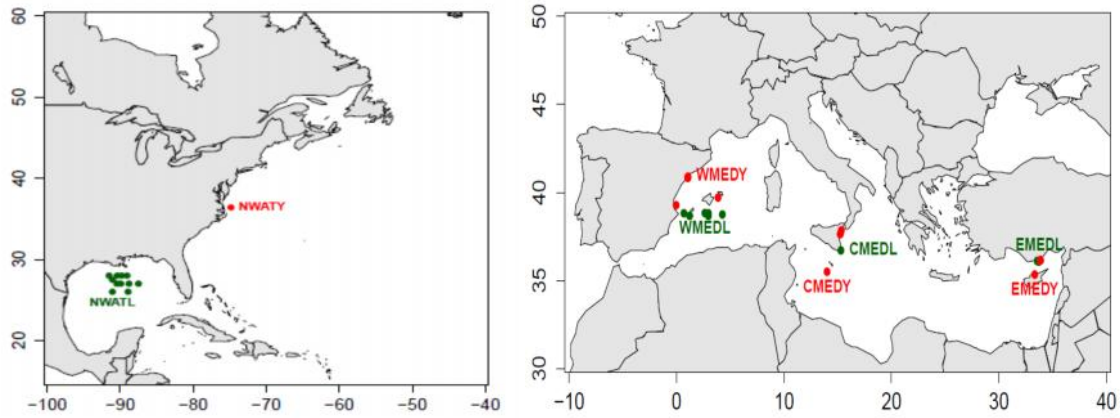

Figure 5.1: Location and code of larval (L, green) and Young of the Year (Y, red) samples. (CMED: central Mediterranean, EMED: eastern Mediterranean; WMED: western Mediterranean; NWAT: North-West Atlantic). Note that in this analysis, the NWATL and NWATLY samples are considered to be from the same population (born in the Gulf of Mexico), but the young of the year were caught in the Atlantic while the larvae were collected in the Gulf of Mexico. For this reason, and for the sole purpose of this analysis, we call this area the Northwest Atlantic (NWAT), that is compared with the 3 Mediterranean areas.

Table 5.1: Number of samples analyzed in Task 1 (CMED: central Mediterranean, EMED, eastern Mediterranean; WMED: western Mediterranean; NWATL: North-West Atlantic, YOY: Young of the year).

	Larvae	YOY	TOTAL
CMED	20	40	60
EMED	10	40	50
WMED	41	38	79
NWATL	38	13	51
TOTAL	109	131	**240**

For GBYPPh5-Task2, 188 samples (plus 4 negative controls) were selected to be genotyped with the SNPs selected from the RAD-seq analysis. Samples were selected so that they included: i) individuals used for the RAD-seq analyses (technical validation) and ii) reference samples not used for panel development. In both cases, samples already

used during GBYPPh4-Task3 (genotyped with GBS derived SNPs) where selected when possible.

Table 5.2: Samples analyzed in Task 2. For each area (CMED: central Mediterranean, EMED, eastern Mediterranean; WMED: western Mediterranean; NWATL: North-West Atlantic) and age class (YOY: Young of the year) the number of samples analysed or not with RAD-seq (RAD/NO_RAD) and/or during Phase 4, Task 3 or not (Ph4/NO_Ph4) is provided.

Area	Samples	Age	NO_RAD		RAD	
			NO_Ph4	Ph4	NO_Ph4	Ph4
WMED	47	YOY	0	20	0	4
		Larvae	0	18	0	5
NWATL	53	YOY	0	2	0	5
		Larvae	37	3	0	3
CMED	47	YOY	0	20	4	0
		Larvae	3	16	0	3
EMED	41	YOY	17	20	4	0
		Larvae	0	0	2	2

5.2.2 DNA extraction

For the new samples for which no DNA was available, DNA was extracted from newly collected samples. DNA was extracted using the Wizard® Genomic DNA Purification kit (Promega, WI, USA) following manufacturer´s instructions for "Isolating Genomic DNA from Tissue Culture Cells and Animal Tissue". The starting material was approximately 20 mg of tissue and after extraction all samples were suspended in equal volumes of Milli-Q water. DNA quantity (ng/µl) was evaluated on the Qubit® 2.0 Fluorometer (Life Technologies) and DNA integrity was assessed by electrophoresis.

5.2.3 RAD-seq library preparation, sequencing

RAD-seq libraries were constructed following the protocol from Etter et al. (2011) with some modifications. Briefly, starting DNA (ranging from 20 to 750ng, depending on integrity) was digested with the SbfI restriction enzyme and ligated to modified Illumina P1 adapters containing 5bp unique barcodes. Pools of 33 individuals were sheared using the Covaris® M220 Focused-ultrasonicator™ Instrument (Life

Technologies) and size selected to 300-500 bp by cutting agarose migrated DNA. After Illumina P2 adaptor ligation, each library was amplified using 14 PCR cycles. Each pool was paired-end sequenced (100 bp) on an Illumina HiSeq2000.

5.2.4 RAD-seq data processing

Generated RAD-tags were analyzed using Stacks v. 1.32 (Catchen et al. 2013) Quality filtering and demultiplexing was performed with the process_radtags module with default parameters and keeping only the highest quality 90 positions of the reads. Duplicates originated during the PCR were removed using the clone_filter module. Analyses with and without PCR duplicates were performed. Putative orthologous tags (stacks) per individual were assembled using ustacks with a minimum depth of coverage required to create a stack (m) of 3 or 5, and a maximum of 2 o4 4 nucleotide mismatches (M) allowed between stacks. Catalogues of loci were assembled based on all samples or only on Mediterranean samples that had more than 500,000 or 100,000 retained reads and more than 30,000 or 10,000 tags when using PCR duplicates or not respectively using cstacks; the number of mismatches allowed between sample tags when generating the catalog (n) was 3 or 6. Matches of individual RAD loci to the catalog were searched using sstacks. From each generated catalog, SNPs present in RAD loci found in at least 75% of the individuals under study were selected and exported into PLINK format using populations. Using PLINK version 1.07 (Purcell et al. 2007), SNPs with a minimum allele frequency (MAF) smaller than 0.05, a genotyping rate smaller than 0.05 and which failed the Hardy Weinberg equilibrium (HWE) test at $p < 0.05$ in at least two populations were excluded from further analyses. Each genotype dataset was exported to Structure, Bayescan and Genepop formats using PGDSpider version 2.0.5.2 (Lischer & Excoffier 2012). In total, 8 genotype subsets where created, four per each sample subset (all or only Mediterranean): not using PCR clones with M=4/n=6 using m=3 (subsets 1 and 2) or m=5 (subsets 3 and 4) and not using PCR clones with m=5 using M=2/n=3 (subsets 5 and 6) or M=4/n=6 (subsets 7 and 8).

5.2.5 Population genetic analyses

For each genotype dataset, 10 subdatasets of 5,000 randomly chosen SNPs were created and analyzed with the Bayesian clustering approach implemented in STRUCTURE (Pritchard et al. 2000). For each value of K (number of potential ancestral populations, which ranged from 1 to the number of groups of area and size class), the genetic ancestry of each individual was estimated based on the admixture model without using

sampling location as prior; estimations were obtained from 300,000 iterations that followed a burn-in period of 100,000 iterations. The 10 subdatasets obtained for each value of K were analyzed with CLUMPP (Jakobsson & Rosenberg 2007) to identify common modes, and results were plotted using DISTRUCT (Rosenberg 2004). Best K was identified according to the Evanno method (Evanno et al. 2005) as implemented in StructureHarvester (Earl & vonHoldt 2012). Principal component analyses (PCA) were performed with the R package adegenet (Jombart & Ahmed 2011) without any a priori population definition.

5.2.6 Discriminant SNP selection

For each genotype dataset, FST between Northwest Atlantic and Mediterranean (when all samples are included) and between the three Mediterranean populations were calculated per SNP following the Weir & Cockerham (1984) formulation as implemented in Genpop 4.3 (Rousset 2008). SNPs were sorted from highest to lowest FST and the 200 or the 50 first (respectively, when all or only Mediterranean samples are included) were selected. SNP flanking regions were obtained using an in-house developed script, using tag sequences corresponding to each SNP retrieved and blasted against the bluefin tuna genome assembly generated and described by the consortium during GBYP Phase 4. Selected SNPs were submitted to the Fluidigm Assay Design Website to check suitability for later genotyping.

5.3 Results

5.3.1 RAD-seq based population genetic analyses

From the 240 samples analyzed with RAD-seq, 221 remained after quality and genotyping filters (Table 3).

Table 5.3: Number of samples analyzed (left) and passing quality filters (right) per location (CMED: central Mediterranean, EMED, eastern Mediterranean; WMED: western Mediterranean; NWATL: North-West Atlantic) and age class (YOY: Young of the year).

	Larvae	YOY	TOTAL
CMED	20/18	40/40	60/58
EMED	10/10	40/39	50/49
WMED	41/34	38/38	79/72
NWATL	38/29	13/13	51/42
TOTAL	109/91	131/130	240/221

Using these samples, 8 genotype datasets were generated that contained 8527 (subset 1), 12613 (subset 2), 7588 (subset 3), 10496 (subset 4), 9871 (subset 5), 11315 (subset 6), 11246 (subset 7) and 13226 (subset 8) SNPs.

When using the dataset containing PCR clones, Structure analyses based on the eight genotype datasets show a clear structure between the Northwest Atlantic and the Mediterranean but no evidences of genetic structuring within the Mediterranean (Figure 5.2). The result is consistent whatever set of parameters is used (M=2/n=3 or M=4/n=6). Interestingly, when removing PCR clones, the differences between the Mediterranean and the North-West Atlantic are not as obvious in the structure plots (Figure 5.3), although still visible particularly for m=3.

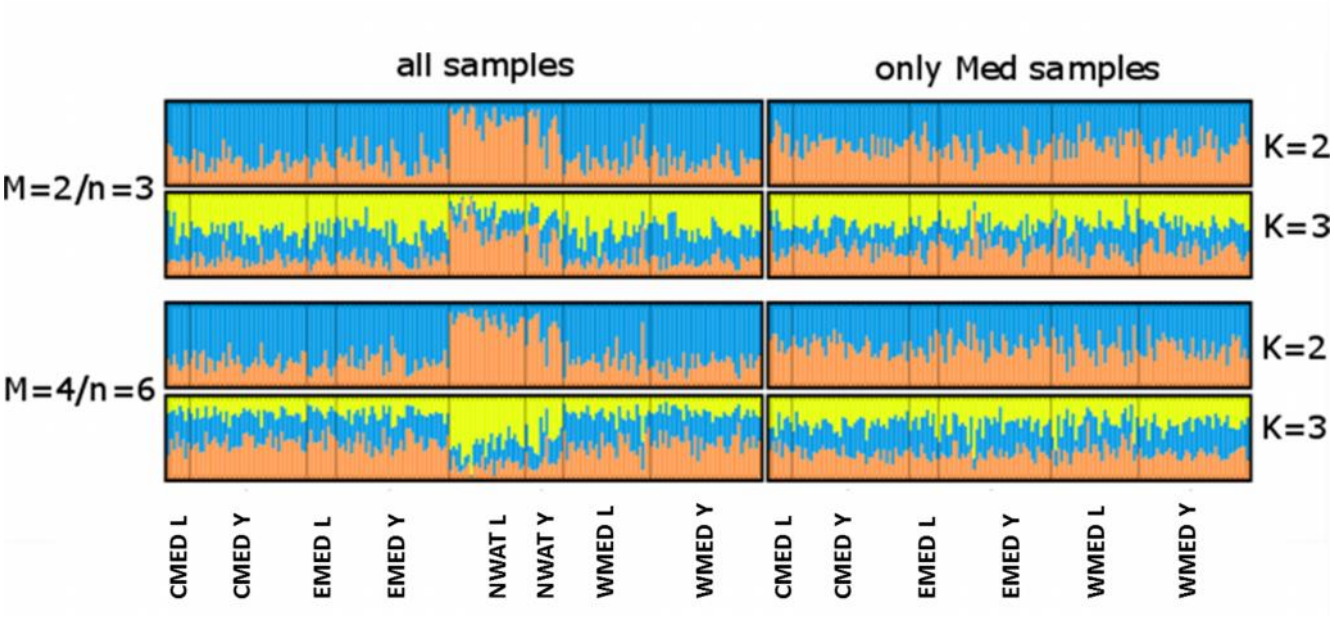

Figure 5.2: Graphical representation of individual ancestry using Structure software for the four genotype datasets including PCR clones. Each bar represents one individual and each color, its degree of belonging to each inferred group. Results of 2 or 3 (K) potential ancestral populations are shown.

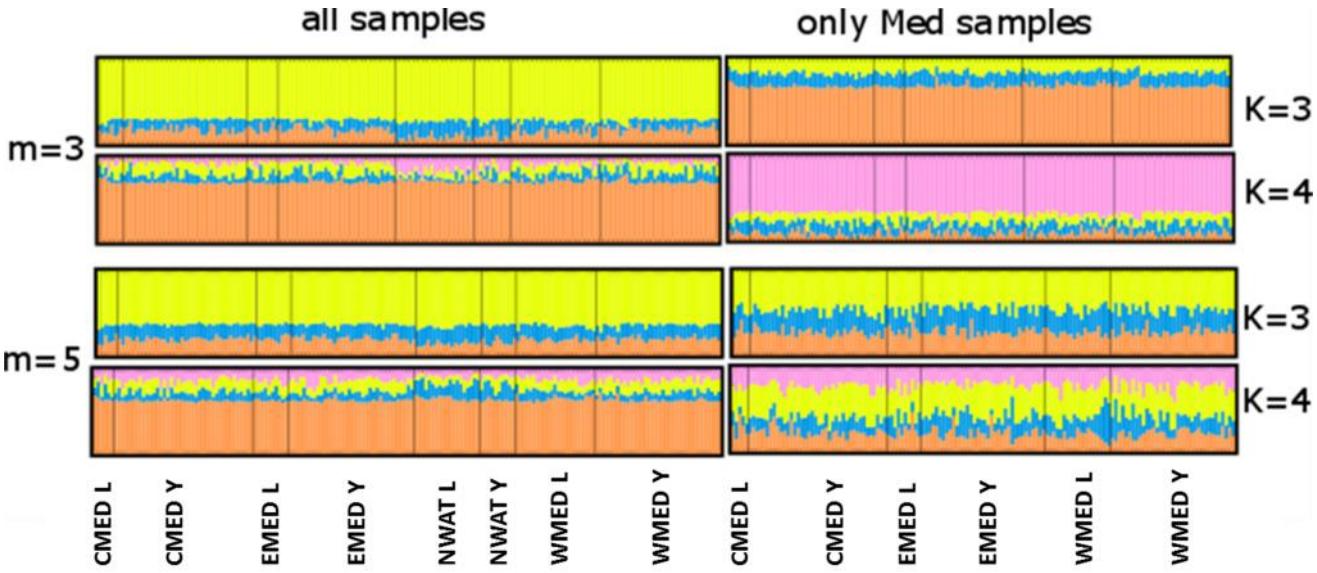

Figure 5.3: Graphical representation of individual ancestry using Structure software for the four genotype datasets not including PCR clones. Each bar represents one individual and each color, its degree of belonging to each inferred group. Results of 3 or 4 (K) potential ancestral populations are shown.

Principal Component Analyses are congruent with the Structure results and show clear differences between the Mediterranean and North-West Atlantic samples, both when PCR clones are included or not (Figures 5.4 and 5.5). Again, no differences among Mediterranean samples can be observed. In summary, our analyses support genetic differentiation between North-West Atlantic and Mediterranean samples, but do not show evidences of substructure within the Mediterranean.

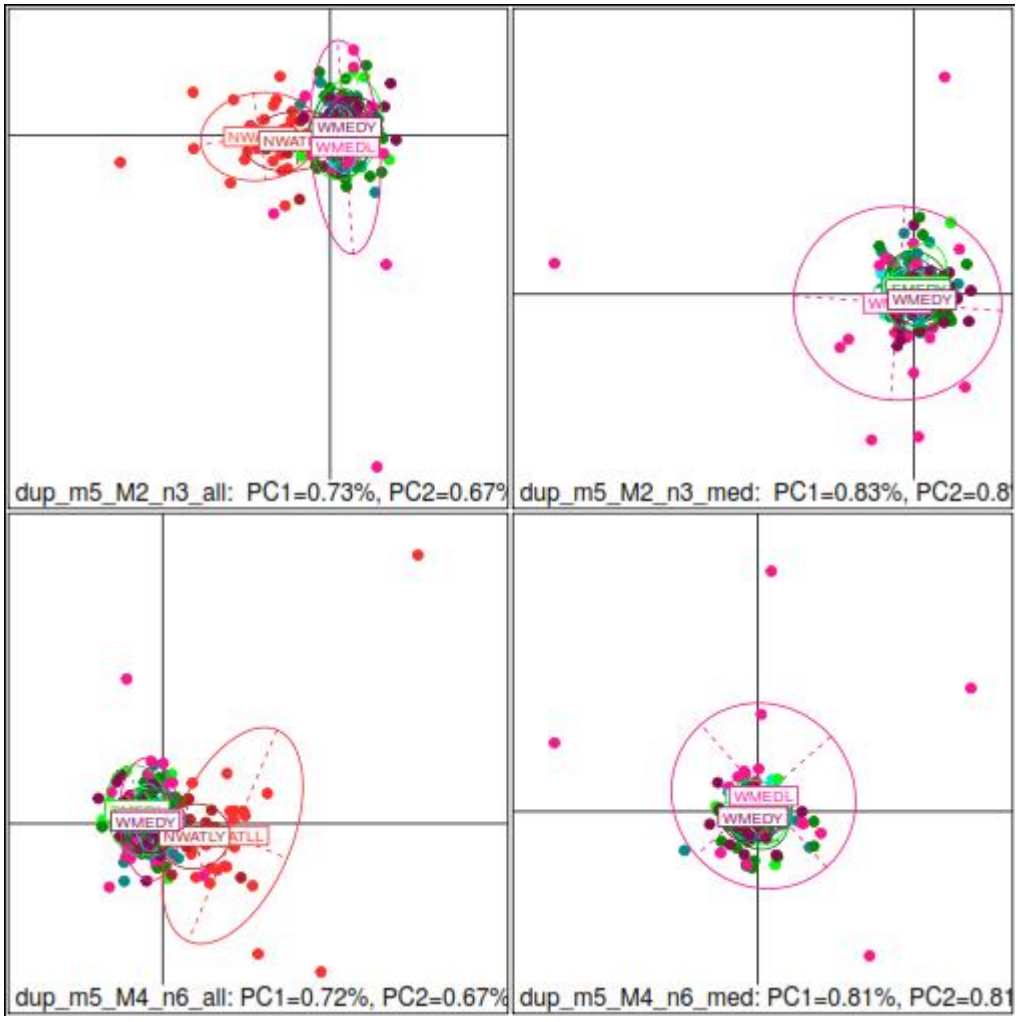

Figure 5.4: Principal Component Analysis (PCA) of allele frequencies. Each plot shows the first two principal components of the PCA obtained from the four datasets including PCR clones. Each dot represents one sample and is colored according to the area of origin. Ovals represent 95% inertia ellipses.

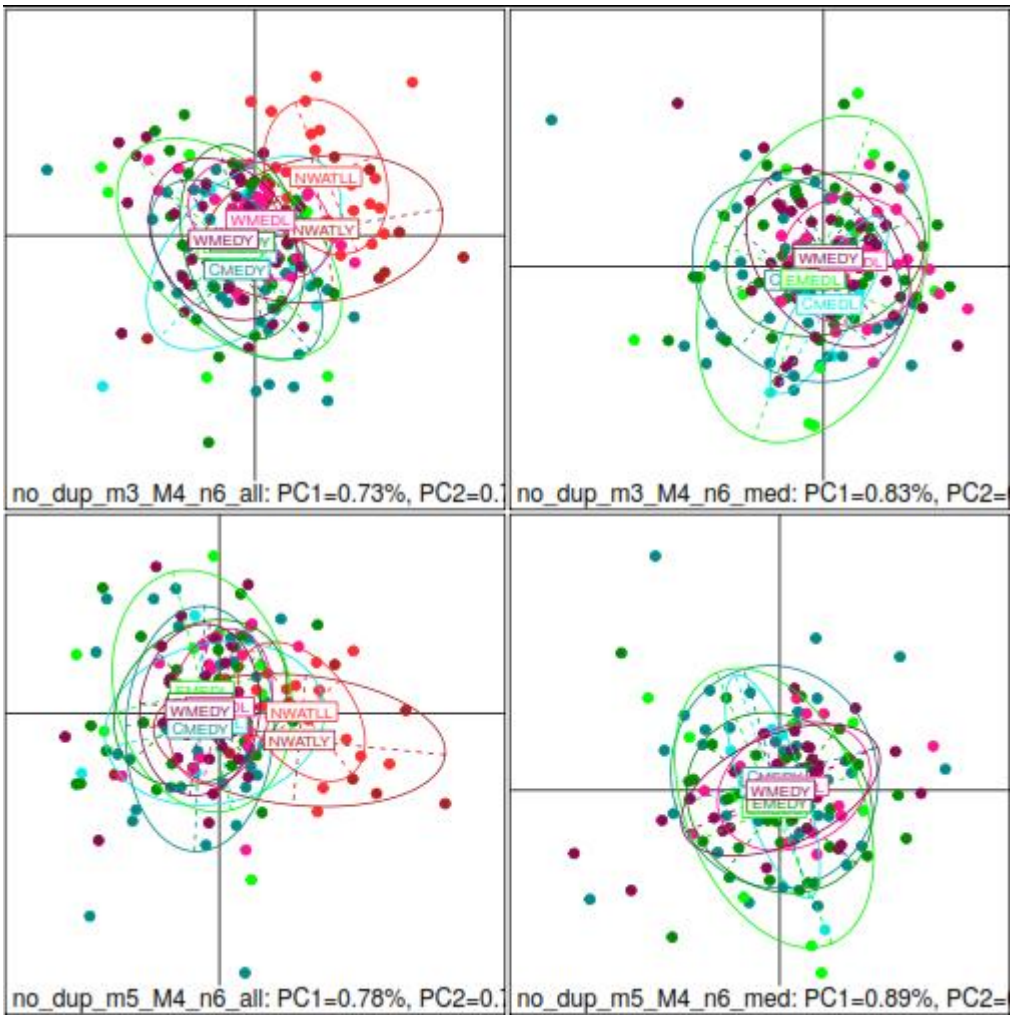

Figure 5.5: Principal Component Analysis (PCA) of allele frequencies. Each plot shows the first two principal components of the PCA obtained from the four datasets not including PCR clones. Each dot represents one sample and is colored according to the area of origin. Ovals represent 95% inertia ellipses.

5.3.2 Discriminant SNP selection

Despite the lack of genetic differentiation within the Mediterranean, SNPs discriminating the Mediterranean populations were also searched for to be validated with the Fluidigm technology. Thus, the highest FST SNPs for North-West Atlantic vs Mediterranean differentiation (284) and for the intra Mediterranean differentiation (62) were submitted to the Fluidigm Assay Design Website to check suitability for genotyping. From the suitable ones, 192 were selected (Table 4).

Table 5.4: SNPs selected per genotype dataset to be genotyped with the Fluidigm technology on the samples indicated in Table 2.

		NWATL/MED	intraMED
no clone_filter	m5M2 n3	36	14
	m5M4 n6	36	10
clone_filter	m3M4 n6	36	12
	m5M4 n6	36	12

5.4 Future analyses

Once the genotyping of 192 RAD-seq derived SNPs is completed, we will i) assess the conversion rate of the genotyping assay and the consistency of the genotypes obtained with those inferred from RAD-seq data and ii) evaluate the reliability of these markers for assignment of samples of known origin.

The best SNPs derived from the RAD-seq panel will be combined with the best SNPs derived from the GBS panel (Phase 4, Task 3) and with other SNPs obtained from the literature in order to build a final SNP panel. This panel will be validated (technical and biologically) on the samples selected for Task 3.

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6. OTOLITH SHAPE

Task leader: Deirdre Brophy (GMIT)

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Summary

- Otoliths of bluefin from the Gulf of Mexico (N=111) were used to improve the characterisation of the western stock of bluefin tuna using otolith shape.
- Otoliths were obtained from bluefin collected during the 2015 sampling season from several locations in the Mediterranean (Sardinia, Levantine Sea, Balearic Islands and Malta). The future analysis of these otoliths will improve the characterisation of the eastern stock of bluefin tuna using otolith shape.
- Bluefin from the Canadian fishery with a >80% estimated probability of originating from the Mediterranean spawning grounds based on otolith stable isotope signatures were estimated to be predominantly of western origin based on otolith shape. This indicates that otolith shape is more influenced by environmental history than natal origin.

Introduction

Otolith shape is known to vary both between and within species (Lombarte and Castellon 1991) due to the combined influence of genetic and environmental factors (Vignon 2012). Thanks to advances in image analysis, variation in otolith shape is now readily captured using geometric measurement of digitised otolith outlines (Stransky 2014). Multivariate analysis of otolith shape data can be used to characterise fish from different stocks (Paul et al. 2013) or to detect underlying structure in a mixed assemblage of unknown stock composition (Keating et al. 2014).

As part of GBYP phase 4, otolith shape descriptors were used to characterise eastern and western stocks of Atlantic bluefin tuna. Within a restricted size range (200-297cm FL) the two stocks could be distinguished with a jack-knife classification success rate of 83% (Brophy et al. 2015). The baseline samples used to characterize the western stock were obtained from the Canadian fishery and were assumed to have originated from the

Gulf of Mexico spawning population (Schloesser et al. 2010). However, the results from a subsequent analysis of oxygen stable isotopes indicate that some of the fish caught in this region may actually be of eastern origin (ICCAT 2015). In addition, Bayesian stock mixture analysis of the otolith shape data suggests that the eastern baseline (adult fish collected near Malta during the spawning season) were not fully representative of the eastern population. The objective of the otolith shape analysis task was to more definitively characterise the eastern and western stocks of bluefin tuna by including in the analysis of otolith shape, otoliths of adults collected from the Gulf of Mexico spawning grounds in the western Atlantic and from across the spawning distribution in the Mediterranean.

Materials and methods

Sample details

Sampling locations are shown in Figure 6.1. Details of the fish used in the otolith shape analysis are summarised in Table 6.1 and described below. To control for length related variation in otolith shape, and to ensure that the baseline samples were representative of spawning adults, fish <170cm FL were excluded from the analysis. Total lengths of the fish used in the analysis ranged from 174-309cm (Table 6.1).

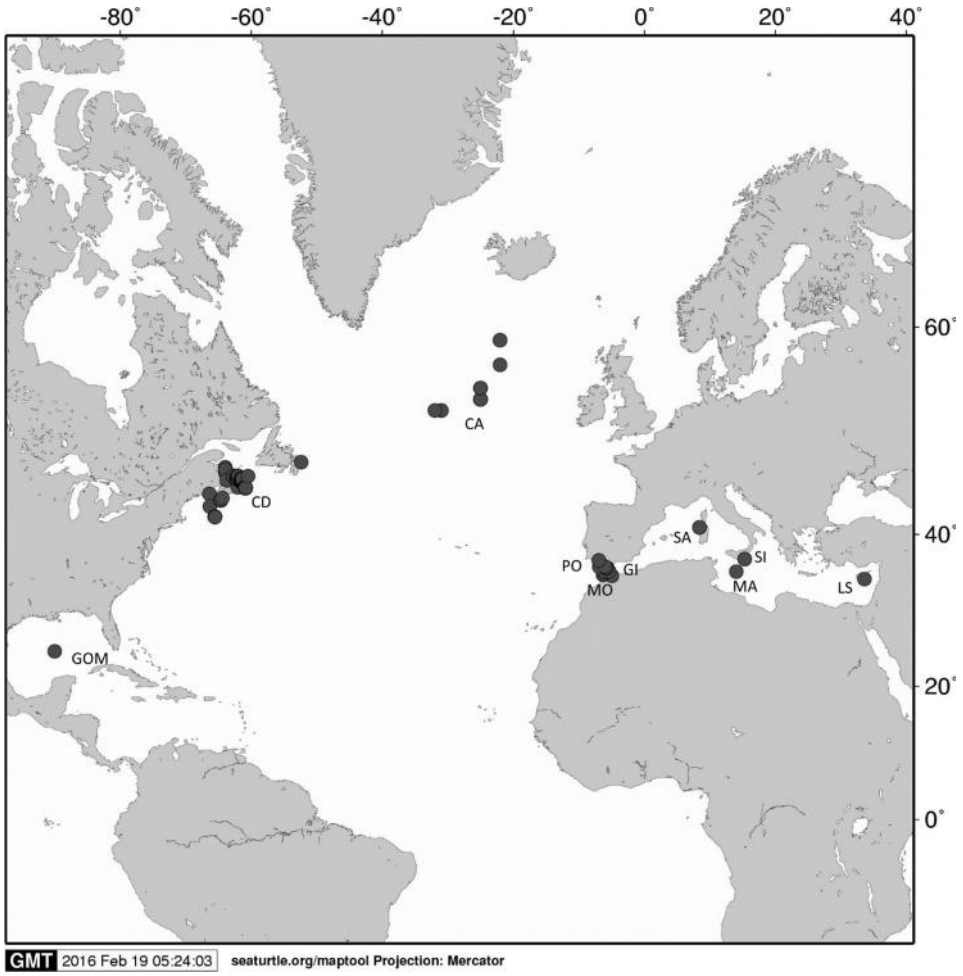

Figure 6.1. Sampling locations for Atlantic bluefin tuna (Thunnus thynnus) used in the otolith shape analysis: Gulf of Mexico (GOM) Canada (CD), central North Atlantic (CA), Portugal (PO), Straits of Gibraltar (GI), Morocco (MO), Gulf of Lion (GL), Ligurian Sea (LI), Malta (MA) and Levantine Sea (LS). The program Maptool (www.seaturtle.org) was used to produce this map.

Table 6.1: Summary of bluefin tuna (>170 fork length (cm)) used in the otolith shape analysis. The number of fish used in the analysis is provided in italic, followed by the mean length (cm) and size range. Baseline samples are shown in bold. An asterisks indicates samples that were collected outside of the GBYP sampling program.

	2009		**2010**		**2011**		**2012**		**2013**		**2014**		
Area	**Medium** (25-100 kg)	**Large** (>100kg)	**Medium** (25-100 kg)	**Large** (>100kg)	**Medium** (25-100 kg)	**Large** (>100kg)	**Medium** (25-100 kg)	**Large** (>100kg)	**Medium** (25-100 kg)	**Large** (>100kg)	**Medium** (25-100 kg)	**Large** (>100kg)	**Total N**
Gibraltar (GI)	---	---	---	---	---	*4* 219.4 (196-250)	*2* 178 (175-181)	*31* 212.8 (183-251)	---	---	---	---	*37*
Levantine (LS)	---	---	---	---	***1*** **176**	***8*** **207.3** **(178-248)**	---	---	---	---	---	---	***9***
Central North Atlantic (CA)	---	---	---	---	---	---	*2* 178.5 (174-183)	*29* 204.5 (196-222)	---	---	---	---	*31*
Morocco (MO)	---	---	---	---	---	*2* 230.5 (241-220)	---	*18* 209.9 (187-225)	---	*32* 215.6 (176-241)	---	---	*52*
Portugal (PO)	---	---	---	---	---	*16* 207 (183-235)	---	*36* 207.8 (180-281)	---	---	---	---	*52*
Malta (MA)	---	---	---	---	---	***40*** **219.2** **(181.5-261.7)**	***1*** **184**	***52*** **232** **(198-283)**	---	---	---	---	***93***
Sicily (SI)	---	---	---	---	---	---	***1*** ***176***	---	---	---	---	---	***1***
Sardinia (SA)	---	---	---	---	---	***1*** ***204***	---	---	---	---	---	---	***1***
Canada (CD)*	---	---	---	---	---	*49* 242.6 (188-309)	---	*37* 236.8 (191-294)	---	66 255.9 (203.2-300)	---	---	*152*
Gulf of Mexico (GOM)*	---	***44*** **253.7** **(205-288)**	---	***24*** **246.5** **(212-280)**	---	---	---	***11*** **247.5** **(230-273)**	---	***15*** **243.2** **(227-281)**	---	***17*** **229.2** **(181-259)**	***111***

Baseline samples

Otoliths from bluefin collected from the Gulf of Mexico between 2009 and 2014 (N=111) were provided through collaboration with Robert Allman and John Walter (NOAA). The eastern baseline samples included 88 adults collected off the coast of Malta during the spawning season which were used in the previous analysis (Brophy et al 2015), and an additional 16 fish from collections held at IEO (from Sicily, Sardinia and the Levantine Sea). Otoliths were also obtained from bluefin collected during the 2015 sampling season from several locations in the Mediterranean (Sardinia, Levantine Sea, Balearic Islands and Malta). Due to the late awarding of the contract it was not possible to process these otoliths and include them in the current analysis. The preparation of these otoliths is ongoing and their future inclusion in the analysis will greatly improve the characterisation of the eastern stock.

Mixed samples

Samples of potentially mixed origin obtained from several locations in the North-East Atlantic (Gibraltar, Portugal, Morocco), the central North Atlantic, the western Atlantic, (Canada: Gulf of St Lawrence, Newfoundland and the Scotian Shelf) and the Mediterranean Sea (Sicily, Levantine, Malta, and Sardinia) between 2011 and 2014 were included in the shape analysis. The classification functions developed from the baseline samples were used to determine the likely origin of these mixed samples. The mixed samples included 50 bluefin collected from the Canadian fishery that were used as the baseline samples in the previous analysis (Brophy et al 2015). Also included in the mixed analysis were 54 fish from the Canadian fishery which had a > 80% estimated probability of belonging to the eastern stock based on their otolith stable isotope signature and 48 fish which had a > 80% probability of belonging to the western stock (Alex Hanke, unpublished data). The otolith shape descriptors for these fish were also directly compared to each other using a series of GLM's.

Image capture and extraction of shape variables

Otolith images were captured using a stereomicroscope connected to a digital camera with a PC interface. Otoliths were photographed as a white object on a black background in a standard orientation, with the sulcus side uppermost and the rostrum pointing to left. The right otolith was used where possible. When the right otolith was unavailable the left otolith was photographed and the image was rotated. Otoliths were excluded from shape

analysis when their outline was obscured by breakage or adhering dirt/tissue (some images had been captured before the onset of this study and otoliths had since been processed for other analyses. It was therefore not possible to clean these otoliths).

Otolith images were edited to standardise their orientation and to remove visual artefacts using Paint.NET v3.5.10. Using the ImageJ software package (available from http://rsb.info.nih.gov/ij/.n), a set of morphological shape indices was obtained from physical measurements of each otolith image: circularity (4π x (area/perimeter2), aspect ratio (the ratio of the major and minor axes of the ellipse which binds the outline), roundness (4 x (π area/Major axis2). Images were converted to binary and otolith outlines were traced using edge detection and saved as a series of x-y co-ordinates.

Elliptical fourier shape descriptors were extracted from smoothed otolith outlines using the momocs package in R. The optimal number of harmonics needed to capture the variation in the outlines was determined using a combination of visual inspection and the Fourier power equation (Crampton 1995). Each harmonic is composed of 4 coefficients (an bn cn and dn). The first three coefficients of harmonic 1 (a1 b1 and c1) were used to standardise each outline for size, orientation and starting point. Thus, a total of 45 coefficients was included in the subsequent analysis.

Data analysis

The 45 elliptical Fourier coefficients and three shape indices (henceforth collectively referred to as the shape descriptors) were tested for normality and equal variances.

The shape of the otolith is under ontogenetic control and is known to change as a fish grows (Hussy, 2008). Such variation could confound the interpretation of regional differences in otolith shape. Shape descriptors that were significantly correlated with fish length and showed no heterogeneity in the size/shape relationship (length*region interaction, p>0.05) were standardised using the common within-group slope, according to the following equation:

$$Y_c = Y - b * L$$

Where Yc is the corrected variable, Y is the original variable, b is the common within group slope of the shape-size relationship (from ANCOVA), and L is the measurement of fish size (length).

Baseline analysis

A series of GLMs was used to identify which shape descriptors showed significant variation between bluefin tuna from the Gulf of Mexico and the Mediterranean. The shape descriptors that captured the majority of the variation in otolith shape were included in stepwise discriminant function analysis to distinguish between fish from the western and eastern baseline samples. Even after transformation, these 28 shape descriptors did not meet the assumptions of multivariate normality or equality of the covariance matrices. Therefore the quadratic form of the model was used (quadratic discriminant function analysis, QDFA).

Mixed analysis

The shape descriptors that were selected in the QDFA to distinguish between the two baseline groups were used in a Bayesian stock mixture analysis to estimate the proportion of fish in each mixed sample that originated from the eastern and western Atlantic stocks. The analysis was conducted using the package mixFish in R as described by Smith and Campana (2010). When using the Bayesian approach, the observations from the mixed samples classified to the base populations (the western and eastern stocks in this case) are used to update the parameter estimation of the base population (unconditional estimation). Bayesian credible intervals (95% CI) were calculated as a measure of the uncertainty associated with the estimated proportions.

Results and Discussion

Baseline analysis

In all, 27 elliptical Fourier coefficients and one shape index showed significant variation between the east and west Atlantic (GLM $P<0.05$) and were not significantly correlated with length (in some cases after standardisation).

Seven shape descriptors (B6, B10, C8, C9, D2, D3, D5, circ) were retained in the DFA by stepwise selection producing one canonical function that distinguished between otoliths from east Atlantic and west Atlantic fish ($P<0.0001$). The canonical function distinguished between fish of eastern and western origin with a mean jack-knife classification success rate of 80% (Table 6.2). The classification success was comparable but marginally lower than that achieved in the previous analysis. This may reflect the fact that the refined western baseline includes fish with more diverse environmental histories and hence more variable otolith shape than the Canadian samples that were previously used as the

baseline. The future inclusion of the Mediterranean spawners from the 2015 sampling season will allow this to be examined in more detail.

Table 6.2: Jack-knife classification matrix from the discriminant function analysis, using seven otolith shape descriptors (B6, B10, C8, C9, D2, D3, D5, circ) to discriminate between adult bluefin tuna (>170cm FL) from the Gulf of Mexico (West) and the Mediterranean (East).

	Estimated origin		
True origin	**East**	**West**	**%correct**
East	83	21	80
West	22	89	80
Total	104	88	80

Mixed analysis

The results of the Bayesian stock mixture analysis are summarised in Table 6.3. Consistent with the previous analysis (Brophy et al., 2015), samples from the central Atlantic and Gibraltar were predominantly of eastern origin. The Canadian samples which were treated as the western baseline in the previous analysis were estimated to be predominantly of western origin, justifying there use as the western baseline in the previous analysis. The Canadian samples which were estimated to have a >80% probability of being from the eastern stock (HPE) based on their otolith stable isotope signatures were classified as largely of western origin based on otolith shape. This indicates that otolith shape is more influenced by environmental history than natal origin. Nonetheless, the GLM analyses revealed small but significant differences between the HPE and HPW fish in four of the otolith shape descriptors ($P<0.05$).

The estimated % of eastern origin fish was actually higher in the HPW samples (23%) than in the HPE (9%). However, there was a large margin of error associated with these estimates, particularly for the HPW fish and the difference was not statistically significant. Overall, the performance of the classification model was relatively poor for the

HPW and HPE fish compared to the original Canadian samples (previous baseline). This may reflect the fact that the HPW and HPE samples were collected over three sampling years while the original Canadian samples were all collected in 2013. Inter-annual variability could also account for the large % error associated with the mixed samples from Morocco and Portugal. However, the baseline samples were also collected across multiple years, and the shape variables used in the classification function did not vary between years.

Table 6.3. Mean predicted percentages (±1 s.d.) and 95% Bayesian credible intervals (CI) for eastern and western origin fish in samples of Atlantic bluefin tuna collected from different locations in the central and west Atlantic based on conditional Bayesian estimation (mixFish program)

|Location	**n**	**% eastern origin**	**95% Bayesian CI**	**% western origin**	**95% Bayesian CI**	**% error (± SD)**
Central Atlantic (CA)	31	91	53.6-100	9	0.01-46.3	12.5
Canada (CD), original baseline	50	8.1%	0.01-33.5	91.9	66.6-100	9.5
Canada (CD), high probability eastern origin	54	9.1	0.01-43.2	90.9	56.8-100	12.0
Canada, high probability western origin	48	23.4	0.04-88.5	76.6	15.2-100	25.0
Gibraltar (GI)	37	91.6	55.4-100	8.4	0.01-44.6	11.9
Morocco	52	66.8	0.59-100	33.2	0.04-99.4	33.1
Portugal	52	66.8	0.59-100	33.2	0.04-99.4	33.1

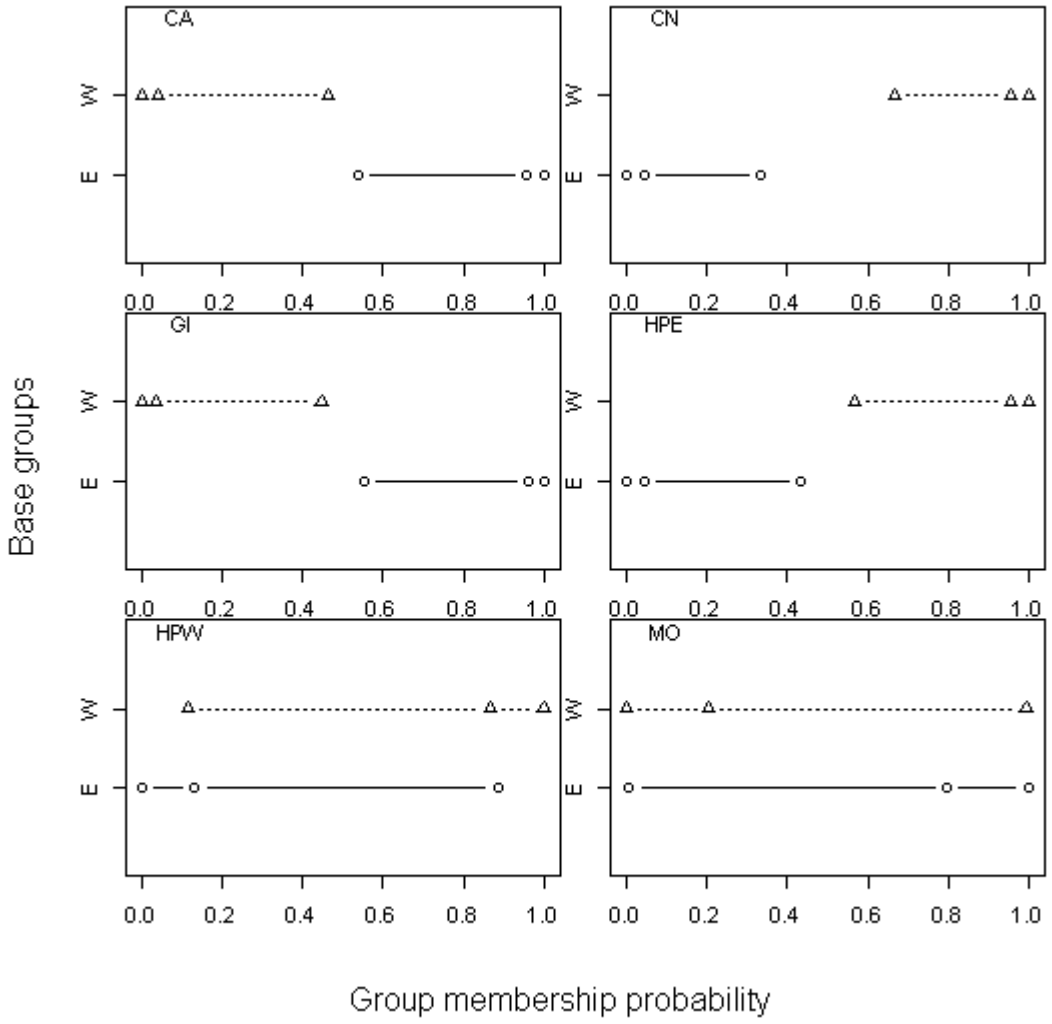

Figure 6.2. Credible intervals (95%) for the posterior estimates of the proportions of bluefin tuna in the mixed samples assigned to the East Atlantic (E) and West Atlantic (W) basegroups. Triangles (E) and circles (W) represent the position of the posterior mean and the upper and lower limits of the estimates, dotted lines (E) and solid lines (W) represent the intervals. CA, central Atlantic; CN, Canada (original baseline), HPE, Canada samples with >80% probability of being of eastern origin based on otolith stable isotope signatures, HPW, Canada samples with >80% probability of being of western origin based on otolith stable isotope signatures, GI, Straits of Gibraltar; MO, Morocco; PO, Portugal.

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7. INTEGRATED APPROACH TO STOCK DISCRIMINATION

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Summary

- An integrated stock identification database has been established and is being continually updated as material is returned from the 2015 sampling season.
- Analysis of the integrated database revealed that overall, rates of agreement between methods were reasonably good given the compounding influence of classification error associated with each method.
- Rates of agreement were lowest for fish of potential western origin (according to at least one method) collected in the Mediterranean and northeast Atlantic and fish of potential eastern origin collected in the western Atlantic (Canadian samples). This may reflect the influence of environmental history on phenotypic markers (otolith shape and chemistry).
- Otolith shape data, otolith stable isotope data and genetic tissue samples from adult bluefin from the Gulf of Mexico has been obtained through collaboration with NOAA and will facilitate the characterisation of the western stock using multiple markers.
- During the 2015 sampling season a co-ordinated approach was adopted which ensured the collection of otoliths and tissues from the same fish and representative of the Mediterranean spawning population. Future analysis of this material will facilitate the characterisation of the eastern stock using multiple markers.
- The database, together with the material and data sourced through this task will enable an integrated stock discrimination analysis of Atlantic bluefin tuna.

Introduction

Various genotypic (Carlsson *et al.*, 2007; Boustany *et al.*, 2008; Dickhut *et al.*, 2009; Albaina *et al.*, 2013)) and phenotypic (Brophy *et al.*, 2015; Rooker *et al.*, 2003; Rooker *et al.*, 2008; Dickhut *et al.*, 2009; Fraile *et al.*, 2014) population markers have been successfully used to distinguish between bluefin from the eastern and western spawning populations. However, there is a degree of uncertainty associated with each method of population assignment. For example, a classification success rate of 83% was achieved using otolith shape descriptors (Brophy *et al.*, 2015) while otolith trace elements (Rooker *et al.*, 2003) and otolith stable isotope ratios (Rooker *et al.*, 2014) have been used to distinguish between the stocks with an accuracy of 85% and 87% respectively. The potential exists to use a combination of population markers in an integrated stock mixture analysis (Smith and Campana, 2010) to improve the overall accuracy of stock assignment. Indeed, one of the recommendations arising from the recent ICCAT bluefin data preparatory meeting was to integrate information from available population markers into a single analysis in order to cross validate results obtained using different approaches and to improve the accuracy of estimated mixing rates (ICCAT 2015).

The objectives of this task are:

- To build an integrated stock identification database by compiling information from previous analyses (e.g. genetic data, otolith chemistry, otolith shape) and other basic information (e.g. size class, date, catch position, etc.). The database will contain GBYP results and, to the extent possible, will try to list other potential sources and their metadata. This database will help establish mixing proportions by 8-box or 2-box areas (ICCAT 2015).

- To compare results obtained for the same fish using different methods in order to evaluate the extent of agreement between estimates of stock origin for fish of unknown (or uncertain) spawning origin.

- To combine otolith shape, composition and genetic data for fish of known spawning origin (50-100 per region) from existing collections and as part of the

2015 sampling program and conduct an integrated analysis with a view to improving overall classification accuracy.

- To summarize the main stock structure hypotheses to be considered within an MSE framework.

Progress on this task was particularly affected by the late awarding of the contract. The delayed start date resulted in late completion of the various analyses that were to produce data for an integrated analysis (otolith shape, otolith chemistry and genetics). However, progress has been made in sourcing otolith and tissue samples and compiling the associated data into an integrated stock identification database.

7.1 Database

An integrated stock identification database was created by compiling information from analyses obtained in previous GBYP Phases (genetic data, otolith chemistry, otolith shape) and other basic information such as (sample ID, date of catch, position, length, weight, and age). First we identified the fish with individual stock origin information (namely the probability of belonging to the Eastern population). This included the following data:

- Otolith chemistry: During Phase 5, individual probabilities were calculated for all the fish analyzed in Phases 2 to Phase 5 (n=1371) , see section 4.2 above). The probabilities obtained by Quadratic discriminant function analysis (QDFA) are used for the integrated analysis.

- GBS (Phase 4): individual probabilities for adults (n=230) as well as self-assigned reference samples (n=493)

- Transcriptome analysis (Phase 2): individual probabilities for adults (n=570) as well as self-assigned reference samples (n=228)

- Otolith shape: individual probabilities of large adult fish (>170 cm, consistent with the baseline) obtained with the Random Forest methodology (n=172).

For each individual in the general ICCAT GBYP database, its probability of belonging to the eastern population, according to different analyses, was assigned (when available). Then, a subset of the database was created containing individuals for which population origin could be assigned with at least one method. This database contains 2591 individuals. 1371 individuals have otolith chemistry data, 1123 have genetic data of two different methodologies (although 722 of these are self-assigned reference samples), and 172 individuals have otolith shape data. Of these, 28 individuals had been analysed using all three methods (otolith shape, otolith chemistry, genetics) and an additional 366 had been analysed using two of the three methods. This database was used for the subsequent tasks and can be updated as new analyses become available. Also, it can be updated with analyses conducted out of the GBYP program, such as those in Fraile *et al.*, (2014), through collaborations with DFO (A. Hanke) or NOAA (R. Allman, M. Lauretta, J. Walter), and others sourced by Carruthers *et al.* (2015).

This database (Appendix 1) can be useful for the BFT WG for the upcoming assessment as well as for the Management Strategy Evaluation (MSE) process in order to inform about mixing. As mentioned in previous sections, the information contained in this database has already been transmitted by the primary analysts to Tom Carruthers (in charge of conducting the MSE work).

In table 7.1.1 we summarize the information contained in this database of individual assignments. The sample size and proportion of eastern origin is reflected for each technique. Having individual assignments allows to group the information according to different criteria (e.g. per year and/or size class) to fit the needs of the different analyses in the future. Note that only individuals with probabilities > 0.7 have been assigned to origin. This varies slightly the effective sample size, and this, as well as the use of a different methodology, makes that the proportions in this table might, to some degree, differ from those estimated using other methods (e.g. the mixed stock analyses in section 4.1, or the ones provided in other Phases).

Table 7.1.1 Summary results of proportion of eastern individuals (pE) assigned with different methods (genetics, stable isotopes, otolith shape) throughout the different Phases of the GBYP program. n=sample size (only individuals with probabilities > 0.7 have been assigned to an area of origin). Reference samples (i.e. YOY) are not included in this summary.

	Genetics		Stable isotopes		Otolith Shape	
	n	pE	n	pE	n	pE
Eastern Mediterranean	80	1,00	41	0,83		
Levantine Sea (North)	80	1,00	41	0,83		
Central Mediterranean	165	0,97	96	0,96		
Adriatic Sea	37	1,00	25	0,88		
Malta	19	1,00	71	0,99		
Sicily (East Sicily and Ionian Sea)	69	0,96				
Gulf of Syrta	40	0,95				
Western Mediterranean	205	0,97	53	0,98		
Balearic	28	1,00	32	0,97		
Gulf of Lion, Catalan	39	1,00				
Ligurian: Italian artisanal fleet	34	0,88				
Sardinia	66	0,95	15	1,00		
Tyrrhenian Sea	38	1,00	6	1,00		
Northeast Atlantic	182	0,96	509	0,84	70	0,81
Bay of Biscay	64	0,98	126	0,96		
Gibraltar	38	1,00	94	0,95	21	0,76
Madeira, Canary Islands	16	0,88	43	0,79		
Morocco	33	0,94	148	0,70	21	0,86
Portugal	31	0,90	98	0,85	28	0,82
Central North Atlantic	55	0,82	361	0,63	19	1,00
Central and North Atlantic	55	0,82	361	0,63	19	1,00
Northwest Atlantic	17	0,59				
Canada (Gulf Saint Lawrence)	17	0,59				

7.2 Degree of agreement between methods

Fish that had been analysed using two or three of the stock discrimination methods were used to compare individual assignments between methods. The analysis was based on the integrated ICCAT GBYP database described above and was complemented with individual assignments (based on otolith chemistry and otolith shape) for 152 fish from the Canadian fishery (Gulf of St Lawrence, Newfoundland and the Scotian Shelf, provided through collaboration with DFO Canada). The

individual assignments were compared first using all of the available samples and then using only samples which were assigned to a population with a probability >0.7 for each of the methods used. The levels of agreement observed between methods are displayed in table 7.2.1

Table 7.2.1 Results of a comparison of individual assignments between methods

	Disagreement (N)	Agreement (N)	Total (N)	Percent agreement (%)
Comparison	*all assignments included*			
Three way comparison	10	18	28	64
Genetics and stable isotope	30	169	199	85
Genetics and shape	8	22	30	73
Stable isotope and shape	22	58	80	73
Stable isotope and shape (Canadian samples)	68	84	152	55
	only assignments >0.7 probability included			
Three way comparison	0	7	7	100
Genetics and stable isotope	9	133	142	94
Genetics and shape	1	10	11	91
Stable isotope and shape	4	23	27	85
Stable isotope and shape (Canadian samples)	23	36	59	61

To examine in greater detail observed discrepancies between methods in the individual assignments, a set of two-way contingency tables were produced (table 7.2.2). To compare the observed levels of agreement to that expected due to chance, Cohen's Kappa statistic (K) was calculated according to the formula:

$$K = \frac{P_o - P_e}{1 - P_e}$$

Where P_o is the observed agreement and P_e is the expected agreement due to chance.

Table 7.2.2 Pairwise contingency tables showing the numbers assigned to each population by each pair of methods. Cells representing agreement are highlighted yellow.

all assignments included

	Stable isotope				Shape		
Genetics	East	West	Total	Genetics	East	West	Total
East	235	54	289	East	22	5	27
West	19	6	25	West	3	0	3
Total	254	60	314	Total	25	5	30
agreement	235	6	241	agreement	22	0	22
by chance	233.78	4.78	238.55	by chance	22.50	0.50	23.00
Kappa	0.03	*poor*		Kappa	-0.14	*less than chance*	

	Shape				Shape (Canadian samples)		
Stable isotope	East	West	Total	Stable isotope (Canadian samples)	east	west	Total
East	52	12	64	East	29	33	62
West	10	7	17	West	35	55	90
Total	62	19	81	Total	64	88	152
agreement	52	7	59	agreement	29	55	84
by chance	48.99	3.99	52.98	by chance	26.11	52.11	78.21
Kappa	0.21	*fair*		Kappa	0.08	*poor*	

only assignments >0.7 probability included

	Stable isotope				Shape		
Genetics	East	West	Total	Genetics	East	West	Total
East	171	27	198	East	10	1	11
West	7	3	10	West	0	0	
Total	178	30	208	Total	10	1	11
agreement	171	3	174	agreement	10	0	10
by chance	169.44	1.44	170.88	by chance	10.00	0.00	10.00
Kappa	0.08	*fair*		Kappa	0.00	*chance*	

	Shape				Shape (Canadian samples)		
Stable isoptope	East	West	Total	Stable isotope (Canadian samples)	east	west	Total
East	22		22	east	9	11	20
West	4	1	5	west	12	27	39
Total	26	1	27	Total	21	38	59
agreement	22	1	23	agreement	9	27	36
by chance	21.19	0.19	21.37	by chance	7.12	25.12	32.24
Kappa	0.29	*fair*			0.14	*poor*	

Evaluation of observed agreement

When evaluating the degree to which individual assignments are consistent across methods, it is important to consider the classification error rates associated with each method. Previous analyses of baseline samples demonstrate that bluefin can be assigned to their population of origin with a mean accuracy of 82%, 80% and 87%, for genetics, otolith chemistry and otolith shape respectively. Therefore if an individual fish is assigned to a population using the three methods, the probability that all three methods will assign the fish to the correct population ($P1$) can be estimated as follows:

$$P1 = 0.82*0.8*0.87= 0.57$$

Similarly, the probability that all three methods will assign a fish to the incorrect population ($P2$) can be estimated as:

$$P2 = 0.18*0.2*0.13 = 0.005$$

And the overall probability of all three methods assigning the fish to the same population is:

$$P1 + P2 = 0.58$$

Using a similar logic, the probability of two methods assigning a fish to the same population would be 0.69 for genetics and otolith chemistry, 0.74 for genetics and otolith shape and 0.72 for otolith shape and otolith chemistry.

If the rate of agreement between methods is lower than these predicted rates this could indicate a problem with one or other of the classification methods. For example such discrepancies could occur if all groups within the mixed sample are not sufficiently represented in the baseline samples.

For the GBYP samples the rates of agreement across the three methods were as good, or better than expected, given the compounding influence of the error rates for each method (table 7.2.1). However, for the Canadian samples, percentage agreement between the otolith chemistry and otolith shape methods was lower than expected. Unsurprisingly, restricting the analysis to individual assignments with a probability >0.7 increased the percentage agreement between methods but

the observed agreement for the Canadian samples was still below what was expected.

Analysis of the two-way contingency tables showed that for some components of the dataset, levels of agreement were low and as a result the Kappa statistic values were close to what would be expected by chance. In the GBYP dataset this appeared to be driven by low levels of agreement for the western. For example, 60 fish are assigned to the western population based on otolith chemistry, of these only 6 were assigned to the western population based on genetics. Similarly, for the Canadian samples percent agreement was higher for the fish that had been assigned to the western population by at least one method (55/(33+55+35) = 0.45) compared to those that were assigned to the eastern population (29/(29+35+33)=0.30).

Further examination of the database showed that the GBYP samples which had been analysed using multiple methods (N=366) were all collected in the eastern Atlantic or Mediterranean. Of these, 105 fish had been assigned to the western population by at least one of the three methods. The 152 Canadian samples, (collected from the western Atlantic) included 97 fish of eastern origin. For these fish, evidence from at least one population marker indicates a possible transatlantic migration at some point in the lifecycle. The contingency tables show that the highest levels of disagreement were associated with these potential migrants, suggesting that these fish may be more difficult to classify using the available phenotypic methods. A combined analysis of samples of known spawning origin is required to fully address this. Moreover, because otolith shape varies with age, it is important to have baseline samples of similar age at each spawning ground.

7.3 Improving classification accuracy with a combined approach

To combine the three population markers (genetics, otolith chemistry and otolith shape) into a single combined assignment method we need samples of known spawning origin that can be used to build a classification model and evaluate its accuracy. The integrated database described above (7.1) provided us with a mean to screen all of the available material for fish of known spawning origin that had been analysed using multiple methods.

The analysis of the integrated database revealed that a very limited number of samples had been analyzed using all three methods (28 fish). More fish had been analysed using two of the three methods (genetics and shape 30 fish; genetics and otolith chemistry 314 fish; shape and otolith chemistry 81 fish). Of these, 95 adults that were collected from the Mediterranean during the spawning season (May-August) had been analysed using both genetic and otolith chemistry methods and could potentially be used as baseline samples. No shape data or images were available for those fish. None of the samples from the Gulf of Mexico had been analysed using more than one method. Therefore the currently available baseline material was not sufficient to characterize the two populations using the three methods. Consequently, the main priority for this task was to obtain suitable baseline samples and associated genetic otolith chemistry and shape data as part of the 2015 sampling activities and through collaboration.

Otolith shape and stable isotope data and tissue samples for future genetic analysis were obtained for bluefin collected from the Gulf of Mexico between 2009 and 2014 (N=111) through collaboration with Robert Allman and John Walter (NOAA). From the 2015 GBYP sampling, whole otoliths and tissue are available from at least 115 adult fish (medium and large sizes) collected from the Mediterranean during the spawning season. These will provide an Eastern baseline for an integrated analysis once the genetic, chemical and shape analyses have been completed.

7.4 Main stock structure hypotheses

During the 2013 BFT WG meeting in Tenerife (ICCAT 2014), the group made schematic representations of potential population structures, given the knowledge available at that time. The main hypotheses at the Atlantic and Mediterranean level included two populations with no subpopulations, two populations with contingents, and a metapopulation (Figure 7.4.1).

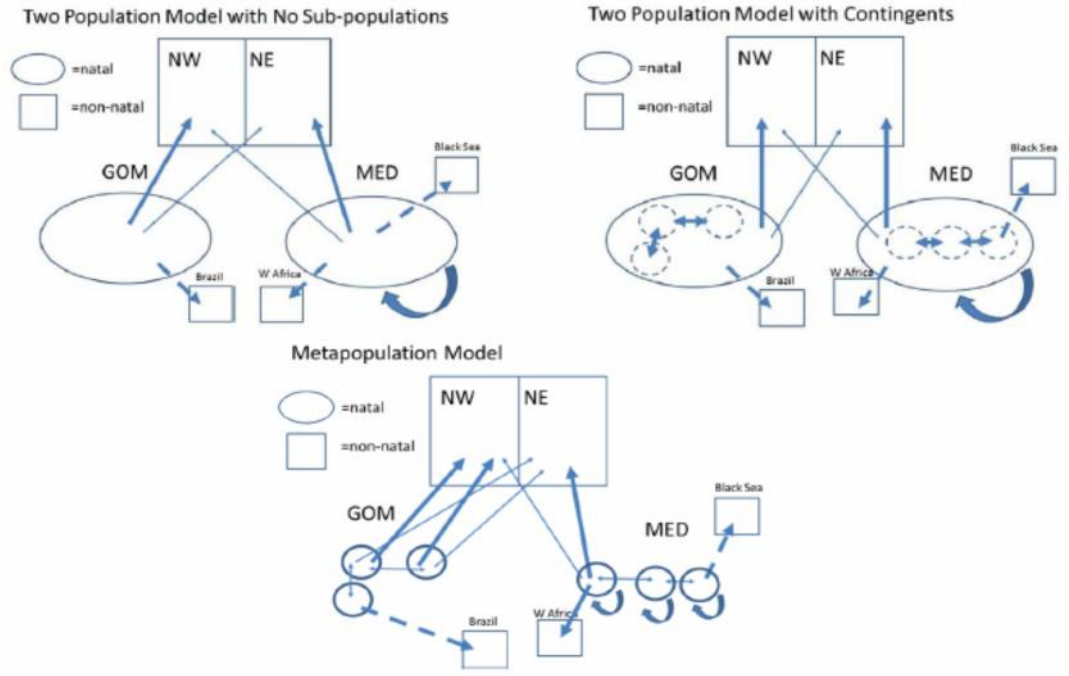

Figure 7.4.1. Main population structures considered in ICCAT (2014).

These implied three main hypotheses at the Mediterranean level (Figure 7.4.2):

- A single panmictic Mediterranean population that migrates to both the eastern Atlantic, western Atlantic and west Africa

- A single panmictic Mediterranean population with different contingents. In this case, the different contingents are genetically similar, but this is due to the genetic interchange of just a few individuals (enough to homogenize the genotypes). However, the different contingents have different life histories and migration patterns, and it is the western Mediterranean contingent

that uses the Atlantic area, while the others are residents within the Mediterranean.

- Different subpopulations within the Mediterranean. In this case, the different Mediterranean groups are genetically distinct. Again, it is the western Mediterranean subpopulation that uses the Atlantic, while the others show residency within the Mediterranean. They can spatially mix within the Mediterranean, but they show well developed natal homing behavior, maintaining genetic differences.

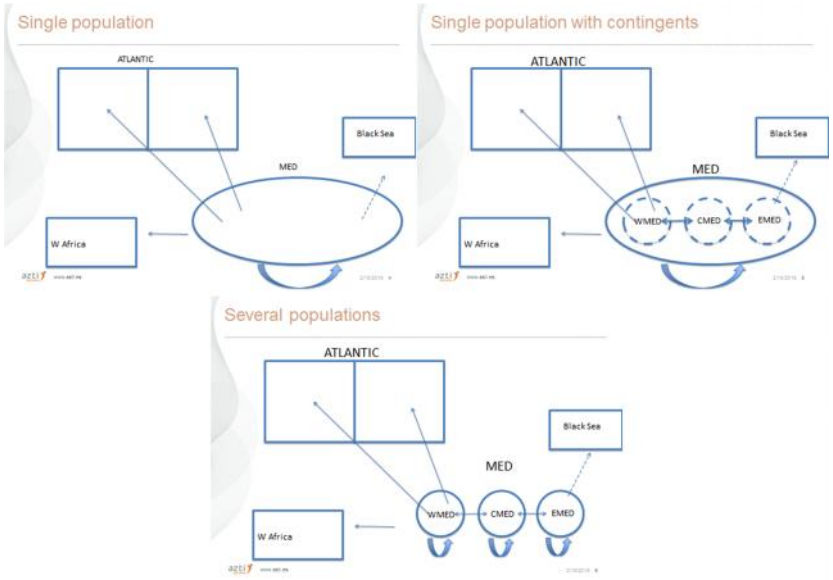

Figure 7.4.2. Main population structures for the Mediterranean population (adapted from ICCAT 2014).

Since the meeting in Tenerife, bluefin tuna research has been active and new knowledge has been generated in the field. On one hand, GBYP results have evidenced, for the first time, that Gulf of Mexico Bluefin can migrate to the western African coast and that the contribution of this population can be ver substantial and variable between years (see section 4). As for the Mediterranean population(s), Aranda *et al.*, (2013) tagged spawning individuals in Balearics and observed that all fish headed towards the Atlantic just after spawning. Likewise, Abascal *et al.*, (2016) tagged bluefin tuna during their spawning migration into the Mediterranean, and their results suggest that the tagged fish were western Mediterranean spawners, that headed back to the Atlantic just after spawning. On

the contrary, Fromentin *et al.*, (2013) and Cermeño *et al.*, (2015) found resident behavior of fish tagged within the Mediterranean occupying the western and central Mediterranean. Detailed depth and temperature profiles (e.g. from archival tags or recaptures PSATs) allow to characterize spawning activity (Aranda *et al.*, 2013, Cermeño *et al.*, 2015). This allowed identifying potential spawning events in both the western and the central Mediterranean, and certain amount of mixture between these two areas (Cermeño et al. 2015).

Quilez Badía *et al.*, (2015) suggested that both Mediterranean residents and Atlantic migrants spawn in both the western and the central Mediterranean. Subsequent electronic tagging by GBYP in Morocco confirmed that fish caught during the spawning migration would visit mostly the western Mediterranean, but also the central Mediterranean, and to a lower extent, the eastern Mediterranean (Di Natale *et al.*, 2015). Furthermore, bluefin tuna tagged in a spawning aggregation in the eastern Mediterranean revealed further evidence of migration mostly to the eastern and central Mediterranean, but also to the eastern Atlantic. Because the relatively short times at liberty, it might be that the link between the eastern Mediterranean and distant areas (such as the Atlantic) is more important than suggested by currently available e-tag studies.

The link between the western Atlantic and the three Mediterranean subareas was already shown by Walli *et al.*, (2009), where many of the fish tagged in the eastern US coast migrated into the Mediterranean, mostly to the western and central Mediterranean, but also one fish to the eastern Mediterranean.

Most of the electronic tagging research has been conducted on adults. Recent findings on the Bay of Biscay juveniles suggest a high degree of dispersal in Atlantic waters during winter, but also a high degree of fidelity to this important feeding area in the northeast Atlantic (Arregi et al, in prep).

In summary, recent research further evidences the high degree of connectivity throughout the Atlantic and Mediterranean. In order to interpret the new e-tagging information in a population structure context, it is important to either use studies that tagged spawners or that were able to characterize spawning activity. Contrary to what was represented in ICCAT (2014), it is now clear that the

Atlantic is used by all three potential contingents/subpopulations (namely the eastern, central and western Mediterranean, Figure 7.4.3.a). According to the available electronic tagging data, the Atlantic seems primarily used by western Mediterranean bluefin, and the proportion of Atlantic migrants might decrease in the Eastern Mediterranean. However, longer times at liberty in fish tagged in the eastern Mediterranean would be needed to confirm this hypothesis (Figure 7.4.3.b). Finally, no single fish has been yet observed to spawn in at least two of the three different Mediterranean subareas considered, even if available tag data should be further examined in all details. Thus, there is no clear evidence of genetic flux between these, and if natal homing is developed at the scale of these Mediterranean subareas (as is at the Atlantic scale), the existence of different subpopulations might yet be a possibility (Figure 7.4.3.c).

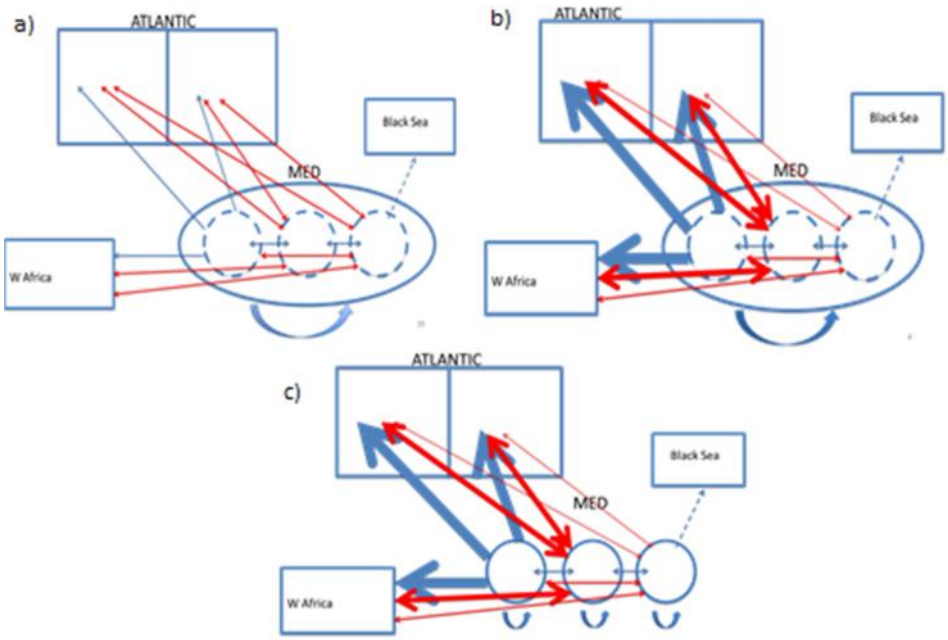

Figure 7.4.3. Main population structure hypotheses within the Mediterranean (from Arrizabalaga et al., 2016). Red arrows indicate the new links stablished after the representations in ICCAT (2014). Plot a) represents the three contingent hypothesis where all three contingents are linked with the Atlantic. Plot b) represents the same population structure, but the Atlantic is mostly used by the western Mediterranean contingent. Plot c) represents the three subpopulations

hypothesis, where the Atlantic is mostly used by the western Mediterranean subpopulation.

Some studies (e.g. Carlsson et al. 2007) indicate that genetic differences do exist within the Mediterranean, thus favoring the structure represented by Figure 7.4.3.c. However, the latest genetic results of our GBYP consortium suggest no intra-Mediterranean differentiation, which would be consistent with the population structure assumption represented by Figure 7.4.2.a. As discussed during the recent Bluefin Futures symposium in Monterey (18-20 January 2016), there is a need to reconcile the different genetic findings to agree on the genetic population structure within the Mediterranean. However, even if no genetic structure is found within the Mediterranean, this can be caused by just a few fish interchanging genetic material between areas. The importance of the contingents needs to be assessed, since Bluefin spawning in different subareas might show significantly different migration patterns and life historiesIn order to be able to assess the importance of the contingents, if genetic differences are not found, otolith chemistry techniques (as described in section 4) can be used to assign origin to Mediterranean subareas and assess whether different contingents show significantly different life histories or not. Likewise, it is important that future e-tagging protocols are adapted to take biosamples of tagged fish at tagging (biopsy); biopsy and otoliths should be taken also from those e-tagged fish when they are fished with an e-tag on,, so that the tracks can be identified with specific contingents.

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8. AGE DETERMINATION ANALYSES

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Participants

IEO: Pablo Quelle, Marta Ruiz and Elvira Ceballos.

8.1 Introduction

The biological analysis of this project includes direct ageing to obtain age composition of bluefin tuna catches and age composition of the population structure samples. In previous phases of the project, age of Atlantic bluefin tuna was estimated by reading otoliths and spiny rays of the first dorsal fin. It was decided to use a stratified sampling by size class in the selection of samples to obtain a representative age length key (ALK) of the whole size range. This selection of samples is used in other fish species with a wide size range such as monkfish (Landa et al., 2008) and has also been used to break down catch into ages for southern bluefin tuna (Anon., 2002). This method is preferred because the random sampling of main fisheries catches produce incomplete age length keys with some size ranges uncovered, which would prevent allocating certain ages to some fractions of the catch. Furthermore, in bluefin tuna fisheries, none of them capture the whole size range.

Following the recommendations that arose during SCRS and GBYP meetings, in phase 3 it was increased the number of samples analyzed for obtaining an age length key which better reflect seasonal variations in growth. To achieve this, it was conducted a size range stratified sampling of the various fisheries of this species which are also seasonal, due to the different migratory patterns of juveniles and adults of Atlantic bluefin tuna.

In previous meetings it was also highlighted the importance of the different laboratories were applying the same criteria for the age interpretation and the fact that the ageing within the "Consortium for the sampling and analysis" came from only one laboratory, the Spanish Oceanographic Institute (IEO). The IEO, as the reference laboratory for the age determination within the "Consortium", has been

working in collaboration with other laboratories in the U.S. (NOAA, GMRI, UMCES) and Canada (SABS) since 2011. From this year and as part of several national and international projects, protocols for the preparation and age interpretation of bluefin tuna calcified structures (otoliths and spiny rays) have been standardized. The standardization of the methodology of age interpretation allowed carrying out a calibration exercise in phase 4. With this calibration, the SCRS and GBYP objectives were met, verifying that independent laboratories can provide comparable interpretations from calcified structures. This calibration exchange, which was attended by 13 laboratories and 21 readers, showed that applying the standardized reading criterion a good level of precision was obtained for both experienced and non-experienced agers, although the latest readers showed some bias compared to the experienced ones. Also applying the standardized direct ageing methodology, a new age-length key for otoliths was built by rereading these calcified structures coming from phases 2 and 3 (Rodriguez-Marin et al., 2015b).

In the 2015 bluefin data preparatory meeting it was recommended to extend the age analysis by including samples from major fisheries in the Mediterranean, covering the months of higher catches and especially the purse seine fishery. Moreover, it was recalled the importance of carrying out a comprehensive analysis by specimen, with the aim of obtaining information on stock structure coupled with information on age. The objectives for phase 5 takes account of these requirements.

8.2 Material and Methods

Sampling

We have selected samples from already collected ones, with the first intention of improving the sampling coverage of summer months, the Mediterranean area and purse seiner fisheries, and secondly other factors like using samples whose natal origin had already been identified, samples from mixing zones, samples from the eastern Mediterranean (including captured by purse seiners) and samples with spines and otoliths from the same specimen.

Calcified structures preparation and age interpretation.

Otoliths were prepared and interpreted following the methodology described in Busawon et al (2015), which means that reflected light was used and opaque bands were counted. Spines were prepared and read following the methodology described in Rodriguez-Marin et al. (2012) and Luque et al. (2014), therefore, transmitted light was used and translucent bands were counted.

The preparation of otoliths included procedures in order to use the same sample for identification of nursery origin and shape analysis. Three sets of samples were used: entire otoliths from which two sections were obtained for age estimates and natal origin analysis and thin and thick sectioned otoliths which had already been used for stock identification.

Otoliths were read independently by two experienced readers. When readings differ by more than one year, a 2nd reading was conducted and if the difference continued, a consensus was reached between readers. For readings differing only one year the most experienced reader counting was used. Spines ageing were also conducted independently by two readers, but one with experience and another one without. When reading differences appear a consensus was reached.

Final age was adjusted for both structures to account for the date of harvesting and the timing of bands formation throughout the year: otoliths final age was adjusted by adding 1 year to the age when the fish was caught between January 1 and the assumed time of the opaque band formation (June 1) (ICCAT, 2015); spines final age was adjusted by substracting 1 year to the age when the fish was caught between June 1 and December 31 and the edge of the structure was translucent (Luque et al., 2014).

Precision of age estimates.

Diagnosis of paired age agreement was evaluated by precision indices through Average Percent Error (APE) and Coefficient of Variation (CV), tests of symmetry and age-bias plots (Campana et al., 1995; McBride, 2015).

Age estimates.

It was not built an age length key (ALK) for this fifth phase of the project because of the biased selection of samples. Thus, these age readings were combined with previous ones.

The annual, monthly, geographical and by gear stratification of the aged samples was explored for phase 5 and for all phases of the project. Likewise an ALK by calcified structure was built and the average size and its variation by age were examined.

8.3 Results and Discussion

Fifth phase direct ageing

In the present phase of the project, age has been interpreted from 356 calcified structures, 258 otoliths and 98 spines, of which 48 paired structures were obtained from the same specimen. Some structures, 10 otoliths and 4 spines, were discarded because of the difficulty in reading, sampling data inconsistencies or being damaged. Most samples were obtained from the years 2011 and 2012, 40% and 53% respectively. Both structures were represented in a wide size range, but spines covered better the range of 110-150 cm straight fork length (SFL) while otoliths covered the range 190-250 cm SFL (Figure 8.1A). This difference in the selection of sizes is because previous studies showed that the interpretation of the age at spines is biased relative to that of otoliths in specimens from age 10 onwards (around 170 cm SFL, Rodriguez-Marin et al., 2015a). The best sample coverage occurred in May and in the third quarter (Figure 8.1B), months that were underrepresented in relation to timing of bluefin tuna catches in the preceding age length keys. The greatest number of samples was from the Mediterranean. Atlantic samples were representative of areas with some degree of stock mixture (Figure 8.1C) (Rooker et al., 2014). In addition, the most represented fishing gears were longline followed by purse seine and trap (Figure 8.1D).

The ageing error was low for both calcified structures and between agers for the same structure, including the inexperienced reader (Table 8.1). Precision for otolith agers was: CV = 6.5%, APE = 4.6% and for spine agers was: CV = 3.1%, APE = 2.26%. These values are within the acceptable CV (10%) and APE for quality control (Secor et al. 2014). Symmetry tests and age-bias plots detected no bias (Table 8.1, Figure 8.2 and Figure 8.3).

All phases direct ageing

In the whole of the project phases, age has been interpreted from 780 otoliths and 633 spines, of which 368 were paired samples of both types of hard parts from the same bluefin tuna.

The analysis of sampling coverage by length range and time showed that 98% of the specimens, and calcified structures, come from the years 2011 and 2012 with 73 and 25% respectively (Table 8.2). This is because the most complete sampling was done in 2011 as it was decided to complete that year and minimize the use of other years to reduce the annual variability in the age length key. There was a better overall and monthly coverage for otoliths than for spines. Although we have tried to complete the sampling of every month of the year, there were still some months underrepresented and some where the entire length range was not covered, mostly as effect of the seasonality and exploitation pattern of fisheries, and the current management regulations (Table 8.2).

The monthly coverage (all years combined) for major geographic areas as the northeast Atlantic and the Mediterranean, showed an uneven monthly coverage, but with an adequate sampling by area and structure for the entire range of sizes (Table 8.3). The sampling coverage by smaller geographic areas was insufficient to cover the whole length range, since sampling reflects the fraction of the population that is targeted by the corresponding seasonal fishery in the different geographical areas.

The sampling by ocean / sea and fishing gear showed that most of the fishing gears are represented, although, according to the gear only certain sizes of bluefin tuna can be caught. The fishing gears that contributed most to the sampling and to a better coverage in sizes were traps for the northeast Atlantic and longline from the Mediterranean Sea (Table 8.4). The sampling of the Atlantic traps consists of bluefin tuna caught in Moroccan and Spanish traps in May and June which are mostly conducting reproductive migrations entering the Mediterranean and of tuna caught by Portuguese traps from July to October as a result of the opposite migration towards the Atlantic. The longline sampling in the Mediterranean Sea comes mainly from the central Mediterranean and to a lesser extent from the western sector. The Mediterranean purse seine sampling mostly corresponds to

calcified structures collected in the eastern area, either directly from the vessels or at the harvesting (Table 8.4).

There was insufficient sample size by time-area stratum (year, month, geographic area and fishing gear) to create annual or partial ALKs, although the total size range by half a year season (all years combined for January to June and July to December) and by ocean / sea are acceptably covered strata. Taking into account the compromise between incomplete ALKs and the potential blurring of cohorts, the most parsimonious option was to construct multi-year age length keys from otoliths and spines readings.

Monthly formation of edge type in otoliths, translucent or opaque, is still inconclusive and do not allow establishing an annual formation pattern, that is the reason for applying the criterion established in the 2015 Bluefin Tuna Data Preparatory Meeting (ICCAT, 2015) for adjusting age in otoliths to account for the date of harvesting and the timing of bands formation throughout the year. To see the influence of this criterion in the otoliths ALK making, two ALKs were built up: one based on estimated age from opaque bands counting and another one based on adjusted age (Table 8.5 and Table 8.6). Some differences between the two ALKs can be appreciated with a greater variability in age at length in the ALK built up with adjusted ages. The latter ALK also showed that small specimens (less than 65 cm SFL) reach unusual ages (two years old) which do not correspond to the bands counting, neither to the length of those specimens, but which age was adjusted because were caught during first five months of the year. These inconsistencies may be due to the fact that the criterion for adjusting age in otoliths does not take into account the edge type, as in the case of the spines reading criterion. However, this criterion for otoliths was adopted for tracking cohorts properly (ICCAT, 2015).

A multi-year age length key using spines readings was also built (Table 8.7). Spines ALK showed lower variability at age than the one coming from otoliths.

ALK mean length at age and variability by calcified structure using adjusted ages are described in Table 8.8. The von Bertalanffy growth model curve fitted to ALKs data showed a divergence between calcified structure growth curves with spines function growing faster than the otoliths function. Differences between functions

begin at age 7, reaching almost 10 cm SFL larger for spines in age 10 and 17 cm SFL in age 13 (Figure 8.4).

Further work will include:

1- Enlarging sample size by incorporating aged structures from national sampling programs.

2- Constructing seasonal (all months and years combined for half a year strata) and ocean/sea ALKs by calcified structure.

3- Analyzing age estimates from paired calcified structures (otoliths and spines) coming from the same specimen, by adding sampling from other national programs.

4- Analyzing the influence of methodology and reading experience in the precision and relative accuracy of aged otoliths and spines.

5- Contributing to tasks identified in the last ICCAT bluefin tuna data preparatory meeting (ICCAT, 2015) so as to compare the age assignments of the ALKs to those obtained using program AGEIT, evaluated the existing age-length and stock origin data to determine the potential to obtain age-length-stock keys (ALSKs), and other analysis.

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Table 8.1. Diagnosis of paired age agreement. Precision indices: CV = Coefficient of Variation, APE = Average Percent Error and tests of symmetry. Symmetry tests: df = degrees of freedom, chi.sq = chi-squared test, p = p-value. ERM, PQE, MRS, ECR correspond to readers' initials.

Otoliths ageing error	Precision		Symmetry tests			
	CV	APE		df	chi.sq	*p*
Both readers (n=258)	6.50	4.60	-			
ERM vs consensus (n=257)	1.96	1.38	EvansHoenig	5	9.873	7.89E-02
			Bowkers	21	26.333	1.94E-01
PQE vs consensus (n=259)	2.52	3.91	EvansHoenig	5	10.481	6.27E-02
			Bowkers	22	29.040	1.44E-01
Spines ageing error	Precision		Symmetry tests			
	CV	APE		df	chi.sq	*p*
Both readers (n=98)	3.09	2.19	-			
MRS vs consensus (n=98)	1.37	0.97	EvansHoenig	2	4.600	1.00E-01
			Bowkers	6	9.000	1.74E-01
ECR vs consensus (n=98)	2.16	1.53	EvansHoenig	2	1.000	6.07E-01
			Bowkers	6	2.333	8.87E-01

Table 8.2. Number of calcified structures aged. Annual and monthly distribution by size range. Upper table for otoliths and bottom table for spines.

Otoliths																				
Year	2010		2011							2012								2013	Total
Month	9	10	5	6	7	8	9	10	11	1	4	5	6	7	8	9	10	3	all
Size range																			
20-30						4	6												10
30-40							7	3											10
40-50								5	1										6
50-60			2		11	1			1										15
60-70			1	1	4	5													11
70-80			10	1	1	5		4											21
80-90			2	4	6	12	1							1					26
90-100			12		2	2	1												17
100-110			3	2	5	7	9			1									27
110-120			4	5	12	14	13	2		3				2					55
120-130			4	3	5		12			1							1		26
130-140			3	7	6	3	5		1						1	2	4		32
140-150			5	5	3	1	7		3						1	2	3		30
150-160			1	6	1		1		12				4			1	2		28
160-170				4					11				3		1	3	3		25
170-180				5	2			1	3			2	2	1			2		18
180-190		2	1	17		2	1	1	1			1	5	1					32
190-200			1	21		1	1	2	2		3		8				2		41
200-210			4	20	1	3	5	2	6		2	8	11			5	19	2	88
210-220	1		12	25		5	4	3	4		3	9	7			4	15	7	99
220-230			7	20	1	2	1	1	3		4	2	6		1	5	3	1	57
230-240			5	14		3	5	3	3		3	2	4	2	1	1	1	7	54
240-250			3	5	1	2		1	2		2	1	5	1		1	4	4	32
250-260							1	3	3		1		1			1	1		11
260-270			1						1				1			1	1		5
270-280							1				1								2
280-290									1			1							2
Total month	1	2	81	165	61	72	81	31	58	5	19	26	57	8	5	27	60	21	780
Total year	3		549							207								21	
								24.8											

Spines																			
Year	2010		2011							2012								2013	Total
Month			5	6	7	8	9	10	11	1	4	5	6	7	8	9	10		all
Size range																			
20-30						4	3												7
30-40							7	3											10
40-50								5	1										6
50-60			2		7				1										10
60-70			1		3	2									5				11
70-80			14	1	5	8		4											32
80-90			2		24	12	1												39
90-100			12			2	1							3	2				20
100-110			3		3	7	10			1									24
110-120			9	4	12	17	13	2		3				1	4				65
120-130			10		11	1	12							5	5	1			45
130-140			13	3		8	5		1					5	5	2	4		46
140-150			15	2	3	8	6	1	2					1	3	2	3		46
150-160			5	5	1	2			10				4	1	2	1	2		33
160-170			1	4					11				1	2	1	1			21
170-180				4				2	1				2	1					10
180-190			2	18				1					5	1					27
190-200			2	13				4					8						27
200-210			1	13				5					11				10		40
210-220				14				2	3				7				4		30
220-230				15				6	1				6						28
230-240			1	11				5	7				4		1		1		30
240-250				2				1	1				5				4		13
250-260				3				2	1				1				1		8
260-270									3				1						4
270-280																			0
280-290									1										1
Total month	0	0	93	112	69	71	58	43	44	4	0	0	55	20	28	7	29	0	633
Total year	0		490							143								0	

Table 8.3. Number of calcified structures aged. Monthly distribution by ocean/sea and by size range. Upper table for otoliths and bottom table for spines.

Otoliths

G. area	Atlantic NE									Mediterranean								
Month	1	3	5	6	7	8	9	10	Total	4	5	6	7	8	9	10	11	Total
Size range																		
20-30														4	6			10
30-40															7	3		10
40-50																5	1	6
50-60					11	1			12		2						1	3
60-70					4	5			9		1	1						2
70-80					1	3			4		10	1		2		4		17
80-90				4	6	2			12		2		1	10	1			14
90-100					2				2		12			2	1			15
100-110	1			2	3	3			9		3		2	4	9			18
110-120	3				2				5		4	5	12	14	13	2		50
120-130	1			2					3		4	1	5		13			23
130-140				5					5		3	2	6	4	7	4	1	27
140-150				2				1	3		5	3	3	2	9	2	3	27
150-160				3					3		1	7	1		2	2	12	25
160-170				5		1	2	3	11			2			1		11	14
170-180			2	7	2			3	14				1				3	4
180-190			1	18		2	1	3	25		1	4	1				1	7
190-200				27		1	1	4	33	3	1	2					2	8
200-210		2	7	27	1	3	10	20	70	2	5	4				1	6	18
210-220		7	10	23		5	9	18	72	3	11	9					4	27
220-230		1	5	17	1	3	6	4	37	4	4	9					3	20
230-240		7	3	14	2	3	6	3	38	3	4	4		1		1	3	16
240-250		4	1	7	1	2	1	1	17	2	3	3	1			4	2	15
250-260				1			2	3	6	1						1	3	5
260-270				1			1	1	3		1						1	2
270-280							1		1	1								1
280-290			1						1								1	1
Total	5	21	30	165	36	34	40	64	395	19	77	57	33	43	69	29	58	385

Spines

G. area	Atlantic NE									Mediterranean								
Month	1	3	5	6	7	8	9	10	Total	4	5	6	7	8	9	10	11	Total
Size range																		
20-30														4	3			7
30-40															7	3		10
40-50																5	1	6
50-60					7				7		2						1	3
60-70					3	7			10		1							1
70-80					5				5		14	1		8		4		27
80-90					24				24		2			12	1			15
90-100											12		3	4	1			20
100-110	1				1	4			6		3		2	3	10			18
110-120	3					3			6		9	4	13	18	13	2		59
120-130						1			1		10		16	5	13			44
130-140				2		5			7		13	1	5	8	7	4	1	39
140-150				2		7		1	10		15		4	4	8	3	2	36
150-160				2		2			4		5	7	2	2	1	2	10	29
160-170				1					1		1	4	2	1	1		11	20
170-180				4				2	6			2	1				1	4
180-190				18				1	19		2	5	1					8
190-200				18				4	22		2	3						5
200-210				22				13	35		1	2				2		5
210-220				17				6	23			4					3	7
220-230				19				6	25			2					1	3
230-240				14				5	19		1	1		1		1	7	11
240-250				6				1	7			1				4	1	6
250-260				2				2	4			2				1	1	4
260-270				1					1								3	3
270-280																		
280-290																	1	1
Total	4	0	0	128	40	29	0	41	242	0	93	39	49	70	65	31	44	391

Table 8.4. Number of calcified structures aged. Distribution by ocean / sea, fishing gear and by size range. Upper table for otoliths and bottom table for spines.

Otoliths												
G. area	Atlantic NE				Mediterranean							
F. gear	BB	LL	Trap	Total	BB	Hand	LL	PS	Trap	TROL	UNCL	Total
Size range												
20-30						4				6		10
30-40										7	3	10
40-50						1					5	6
50-60	12			12		1	2					3
60-70	9			9			2					2
70-80	4			4	2		11				4	17
80-90	12			12	10		4					14
90-100	2			2			15					15
100-110	9			9		1	15		2			18
110-120	5			5		4	43		3			50
120-130	3			3			15		8			23
130-140	3		2	5		3	10	5	9			27
140-150			3	3		2	8	9	8			27
150-160			3	3			6	14	5			25
160-170			11	11		1	1	11	1			14
170-180			14	14			1	3				4
180-190		2	23	25			4	1	2			7
190-200		1	32	33			5	2	1			8
200-210	2	7	61	70			10	7	1			18
210-220	7	5	60	72			21	5	1			27
220-230	1	6	30	37			16	3	1			20
230-240	7	4	27	38			11	5				16
240-250	4	1	12	17			8	7				15
250-260		2	4	6			1	4				5
260-270		1	2	3			1	1				2
270-280			1	1			1					1
280-290			1	1				1				1
Total	80	29	286	395	12	17	211	78	42	13	12	385

Spines												
G. area	Atlantic NE				Mediterranean							
F. gear	BB	LL	Trap	Total	BB	Hand	LL	PS	Trap	TROL	UNCL	Total
Size range												
20-30						4				3		7
30-40										7	3	10
40-50						1					5	6
50-60	7			7		1	2					3
60-70	10			10			1					1
70-80	5			5	8		15				4	27
80-90	24			24	12		3					15
90-100							20					20
100-110	5		1	6		1	15		2			18
110-120	6			6		5	45		9			59
120-130	1			1		2	29		13			44
130-140	5		2	7		6	11	5	17			39
140-150	7		3	10		2	9	9	16			36
150-160	2		2	4		1	4	12	12			29
160-170			1	1		2	4	10	4			20
170-180			6	6			2	1	1			4
180-190			19	19			1		7			8
190-200			22	22					5			5
200-210			35	35				2	3			5
210-220			23	23				3	4			7
220-230			25	25				1	2			3
230-240			19	19			1	8	2			11
240-250			7	7				5	1			6
250-260			4	4				2	2			4
260-270			1	1				3				3
270-280												
280-290								1				1
Total	72	0	170	242	20	25	162	62	100	10	12	391

Table 8.5. Multi-year otolith-based age length key for bluefin caught in the eastern Atlantic and Mediterranean stock, built up with estimated age from opaque bands counting. Numbers represent percent by number by 5 cm length class (SFL).

Otoliths	Estimated age																		
SFL (cm)	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	Total n
20-24	100																			6
25-29	100																0-20%			4
30-34	100																20-50%			6
35-39	100																50-100%			4
40-44	100																			5
45-49		100																		1
50-54		100																		1
55-59		100																		14
60-64		100																		10
65-69		100																		1
70-74		50	50																	4
75-79		47	53																	17
80-84		22	65	13																23
85-89			67	33																3
90-94			100																	2
95-99			53	33	13															15
100-104			21	50	29															14
105-109			15	38	15	31														13
110-114				9	47	25	19													32
115-119				9	30	48	13													23
120-124				9	18	55	18													11
125-129					13	60	20	7												15
130-134				10	25	25	30	10												20
135-139					17	25	33	8	17											12
140-144					18	18	18	35	12											17
145-149						31	54	15												13
150-154						21	21	29	21	7										14
155-159						7	36	43	14											14
160-164						9	18	27	36	9										11
165-169							7	50	43											14
170-174								17	33	50										6
175-179								42	33	8	17									12
180-184							6	35	41	18										17
185-189								13	27	20	33	7								15
190-194								8	42	33	17									12
195-199								10	31	24	10	14	7	3						29
200-204								18	18	23	10	10	5	13	3					39
205-209								8	24	29	20	14	2	2						49
210-214								7	20	37	13	15	2	4	2					46
215-219								8	15	25	26	17	8	2						53
220-224									11	30	22	22	5	8			3			37
225-229								10	10	10	25	25	10		5	5				20
230-234									14	14	32	19	16	5						37
235-239									12	12	24	12	6	18	12		6			17
240-244									4	13	13	26	26		9		4	4		23
245-249										22	22	11	22			11		11		9
250-254									17		17	17	17	17	17					6
255-259											20		40	20		20				5
260-264												25		25	25				25	4
265-269													100							1
270-274															100					1
275-279																	100			1
280-284															50		50			2
Total %	3.2	5.4	5.5	3.7	5.6	7.4	5.9	9.0	12.9	12.9	10.5	8.1	4.2	2.7	1.4	0.4	0.6	0.3	0.1	780

Table 8.6. Multi-year otolith-based age length key for bluefin caught in the eastern Atlantic and Mediterranean stock, built up with adjusted ages. Numbers represent percent by number by 5 cm length class (SFL).

Otoliths	Adjusted age																			
SFL (cm)	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	Total n
20-24	100																			6
25-29	100																0-20%			4
30-34	100																20-50%			6
35-39	100																50-100%			4
40-44	100																			5
45-49		100																		1
50-54			100																	1
55-59		93	7																	14
60-64		90	10																	10
65-69		100																		1
70-74		50	50																	4
75-79		24	41	35																17
80-84		22	57	22																23
85-89			67	33																3
90-94				100																2
95-99				73	27															15
100-104			7	57	36															14
105-109			15	31	23	31														13
110-114				6	47	28	19													32
115-119				4	22	61	9	4												23
120-124					27	55	9	9												11
125-129					7	53	33	7												15
130-134				10	20	25	30	15												20
135-139					17	25	33	8	17											12
140-144					12	24	12	41	12											17
145-149						15	62	23												13
150-154						21	21	29	21	7										14
155-159						7	36	43	7	7										14
160-164						9	18	27	36	9										11
165-169							7	50	43											14
170-174									33	67										6
175-179								42	33	8	17									12
180-184							6	35	41	18										17
185-189								13	27	20	20	20								15
190-194									50	33	17									12
195-199								7	31	24	14	14	7	3						29
200-204								18	15	21	13	13	3	13	5					39
205-209								6	22	24	22	20	2	2						49
210-214								7	15	35	11	20	7	4		2				46
215-219								6	13	15	32	21	6	8						53
220-224									8	22	32	22	5	8			3			37
225-229									20	5	15	30	20			10				20
230-234									8	16	24	27	11	8	5					37
235-239									12	12	24	6	12	12	6	12	6			17
240-244									4	13	9	13	39	4	9		4	4		23
245-249										22	22		22	11			11		11	9
250-254									17		17	17		33	17					6
255-259											20		40	20		20				5
260-264												25			50				25	4
265-269													100							1
270-274																100				1
275-279																	100			1
280-284															50			50		2
Total %	3.2	4.5	3.8	5.4	5.6	7.7	5.9	8.7	12.2	11.7	10.6	9.2	4.6	3.3	1.4	0.9	0.6	0.3	0.3	780

Table 8.7. Multi-year spine-based age length key for bluefin caught in the eastern Atlantic and Mediterranean stock. Numbers represent percent by number by 5 cm length class (SFL).

Spines	Age																		
SFL (cm)	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	Total n
20-24	100																			3
25-29	100																			4
30-34	100																			6
35-39	100																			4
40-44	100																			5
45-49	100																			1
50-54		100																		1
55-59	11	89																		9
60-64		89	11																	9
65-69		100																		2
70-74		33	67																	3
75-79			97	3																29
80-84			93	7																30
85-89			67	33																9
90-94			40	40	20															5
95-99				93	7															15
100-104				100																11
105-109				15	69	15														13
110-114				19	65	16														31
115-119				6	68	26														34
120-124				4	44	52														25
125-129					20	75	5													20
130-134				3	3	74	19													31
135-139					7	60	33													15
140-144						58	42													24
145-149					14	23	45	14	5											22
150-154						36	57	7												14
155-159						16	58	21	5											19
160-164						15	23	54		8										13
165-169							25	50	25											8
170-174							50	25	25											4
175-179							17		67	17										6
180-184							12	29	53	6										17
185-189								20	40	40										10
190-194								25	25	42	8									12
195-199								13	47	27	13									15
200-204								15	10	50	20	5								20
205-209									35	40	10	15								20
210-214								10		60	10	10	10							10
215-219								5	5	20	60	5		5						20
220-224								13	7	20	33	20		7						15
225-229										23	38	31		8						13
230-234									5	10	33	38	10	5						21
235-239									11	11	11	44	22							9
240-244										10	20	40	20	10						10
245-249												67		33						3
250-254											40	20	20				20			5
255-259												67	33							3
260-264													33	33		33				3
265-269													100							1
270-274																				0
275-279																				0
280-284																	100			1
Total %	3.8	3.2	10.6	7.1	11.7	16.6	9.6	6.2	7.1	8.5	7.0	5.4	1.7	1.1		0.2	0.3			633

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

Table 8.8. Mean length at age, standard deviation and number by calcified structure from multi-year age length keys.

	Multi-year otolith ALK			Multi-year spine ALK		
Age	Mean length (cm, SFL)	Stand. Deviat.	Number	Mean length (cm, SFL)	Stand. Deviat.	Number
0	31.7	7.1	25	34.5	8.2	24
1	65.6	9.8	35	60.6	5.1	20
2	80.3	11.9	30	80.3	4.6	67
3	96.9	13.7	42	101.2	10.6	45
4	114.9	11.9	44	116.8	9.3	74
5	125.4	13.7	60	132.1	12.2	105
6	138.7	16.4	46	149.7	12.9	61
7	170.7	26.5	68	177.4	21.3	39
8	195.1	25.0	95	191.1	17.6	45
9	207.7	19.4	91	206.1	15.3	54
10	216.9	16.2	83	220.7	13.6	44
11	217.5	14.7	72	230.9	13.6	34
12	230.5	17.1	36	243.1	15.4	11
13	223.1	18.0	26	234.8	15.8	7
14	240.5	24.0	11	260.0		1
15	238.2	20.8	7			
16	246.2	19.7	5	267.5	23.3	2
17	260.5	29.0	2			
18	256.1	9.7	2			

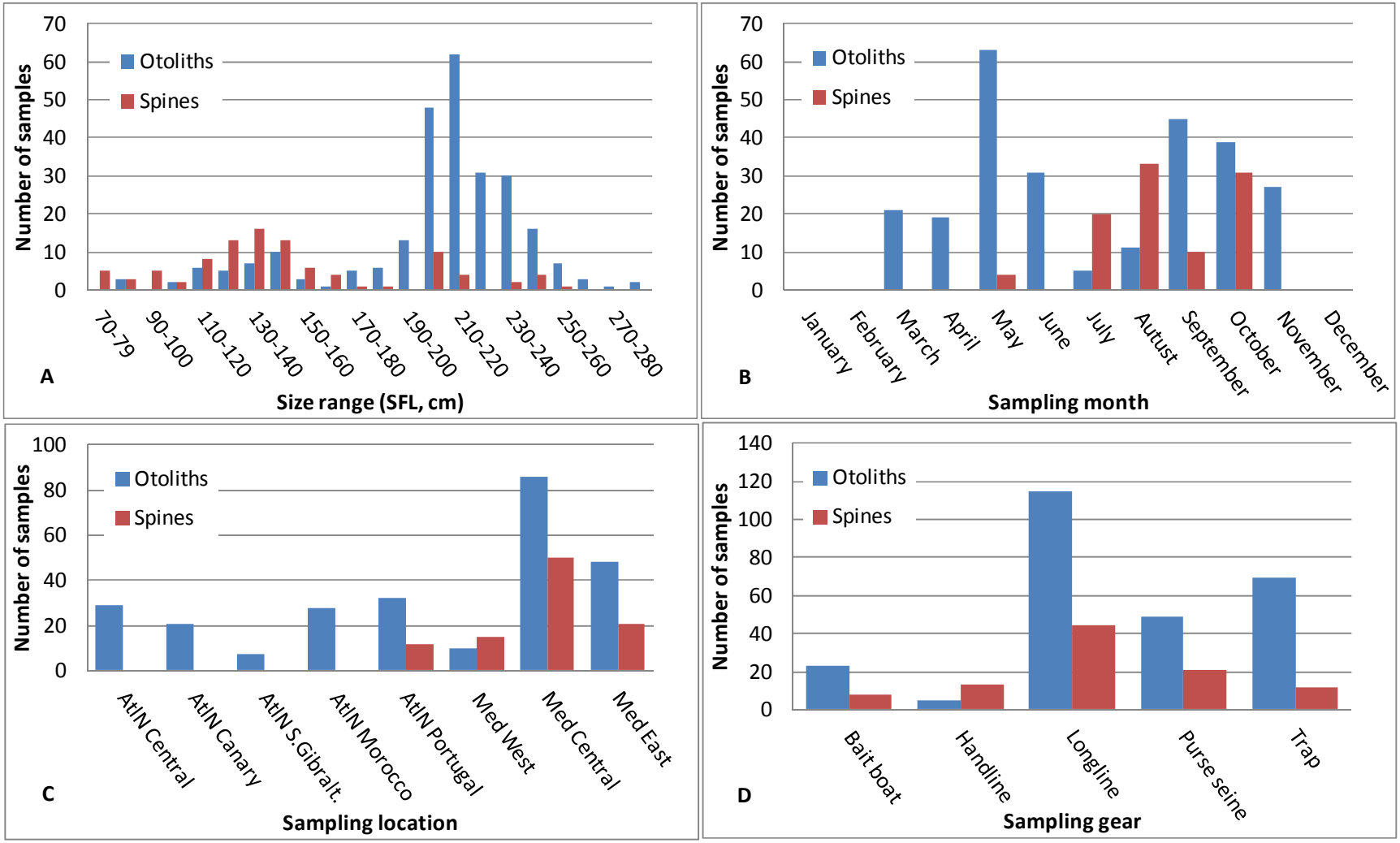

Figure 8.1. Sampling characteristics of the calcified structures used in phase 5 of the GBYP biological studies research project.

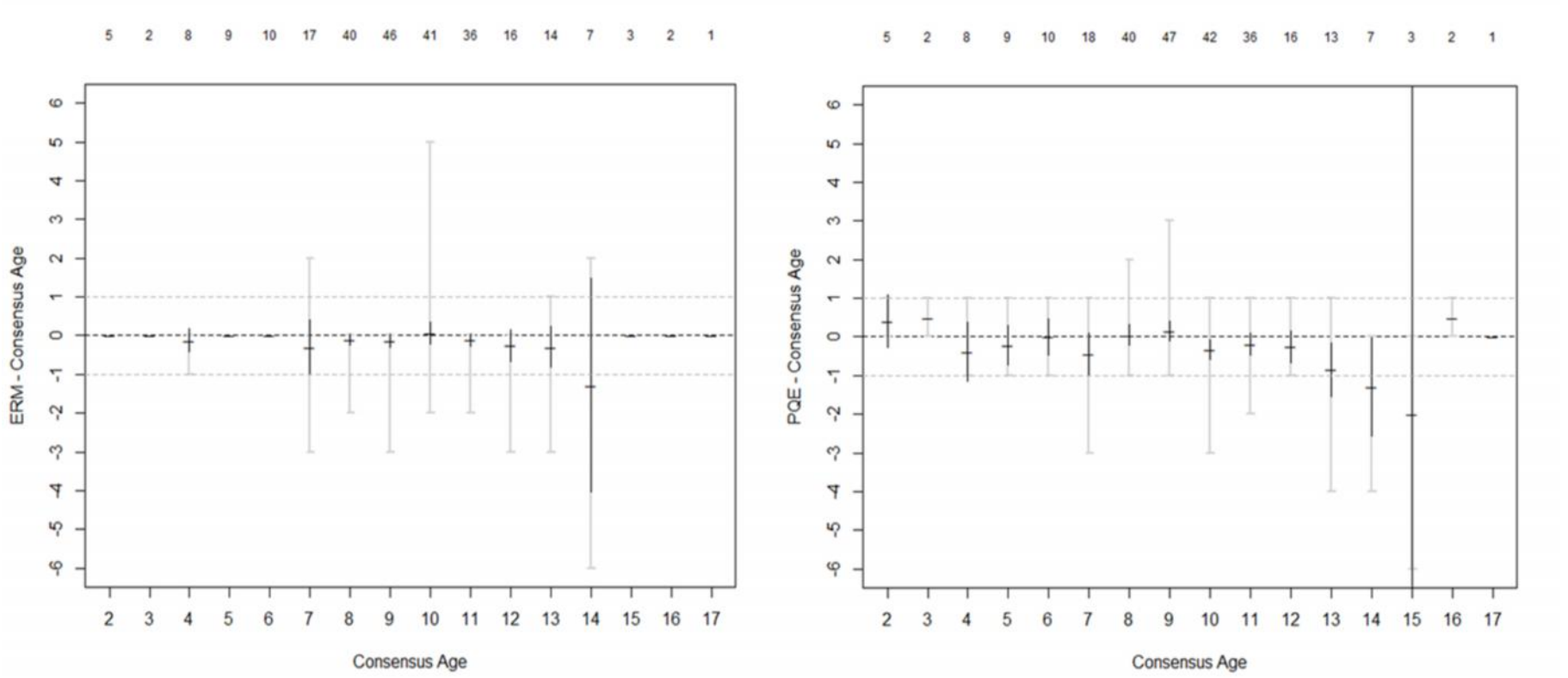

Figure 8.2. Differences in age estimates from each ager versus consensus for otoliths readings. Crosses indicate the average with black lines indicating the 95% confidence intervals and the grey lines indicating the age range. The 1:1 equivalence line (black dashed) and one year difference line (grey dashed) are also indicated. Numbers above the figure indicate number of samples by age.

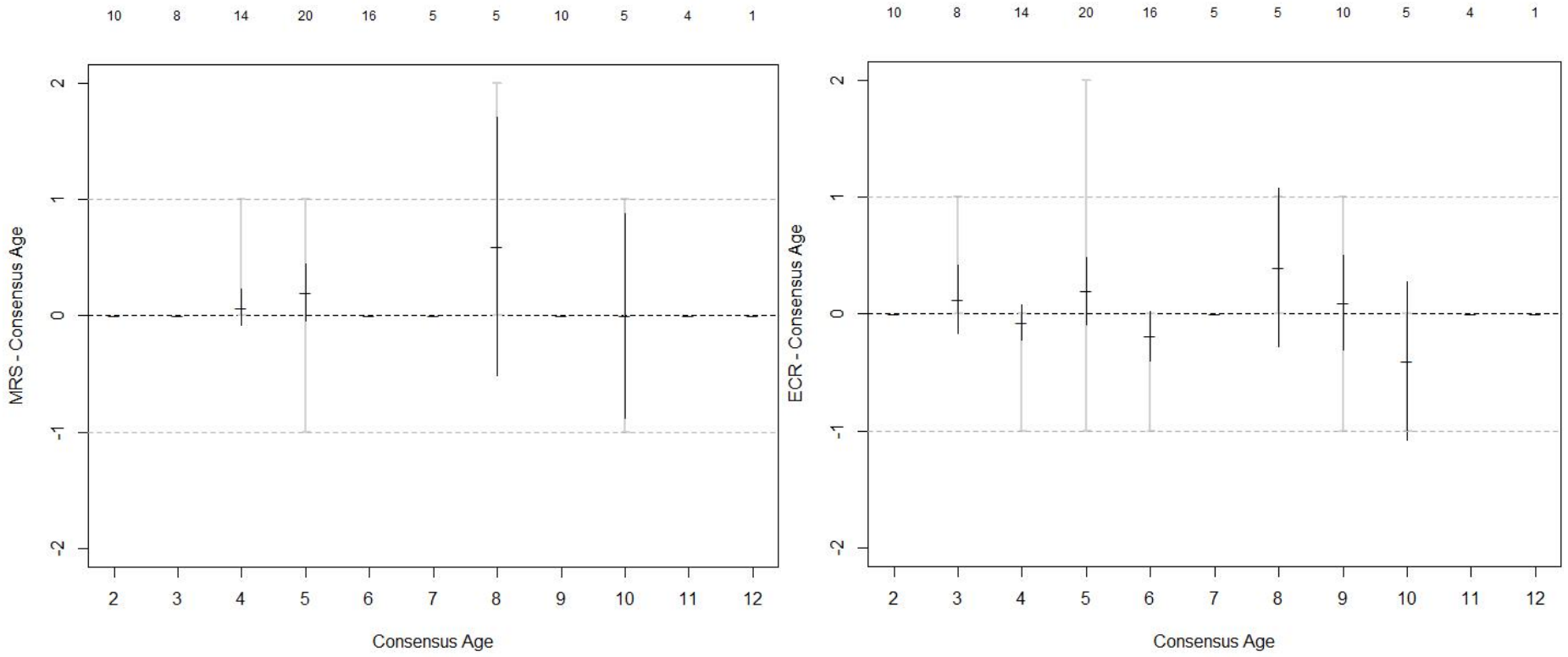

Figure 8.3. Differences in age estimates from each ager versus consensus for spines readings. Crosses indicate the average with black lines indicating the 95% confidence intervals and the grey lines indicating the age range. The 1:1 equivalence line (black dashed) and one year difference line (grey dashed) are also indicated. Numbers above the figure indicate number of samples by age.

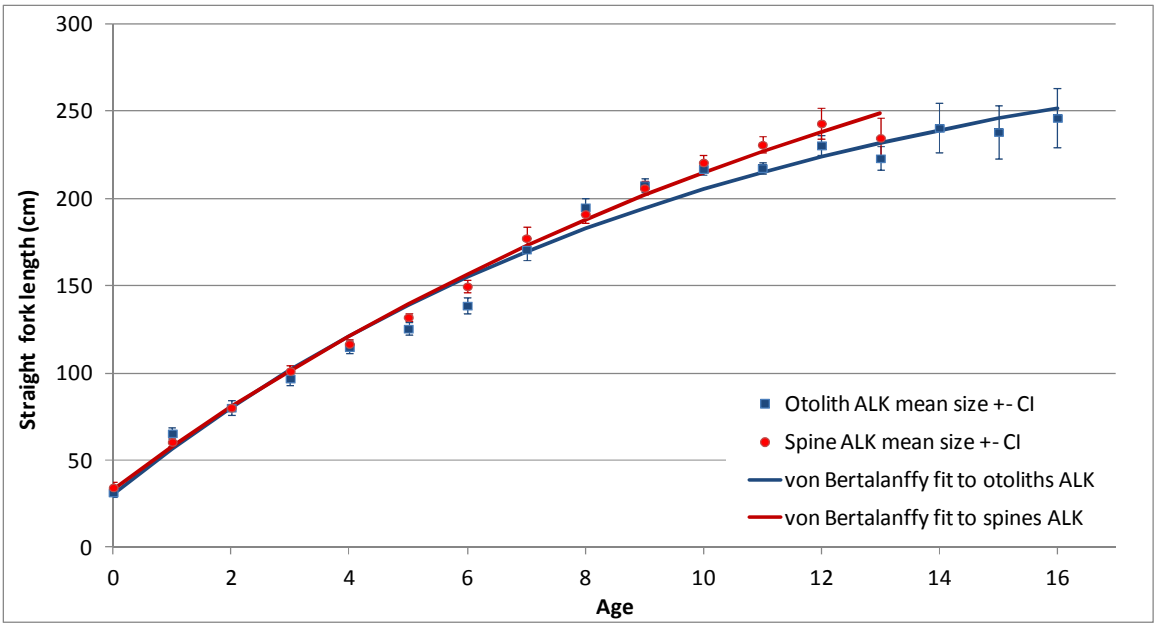

Figure 8.4. Length at age from multi-year ALKs and 95% confidence intervals for otoliths (blue dots and CI error bars), and spines (red dots and CI error bars). ALKs von Bertalanffy growth model curves fitted to observed length at age data for otoliths (blue line) and spines (red line).

9. APPENDICES

1- Appendix 1: Individual assignments to nursery areas based on otoliths chemistry, genetics (GBS and Transcriptome) and otolith shape.