escola superior de turismo
e tecnologia do mar
instituto politécnico de leiria

Internship Report and Project Report

Master in Aquaculture

Routines in a European aquaculture research facility

and

Analysis of biometrics, the *asteriscus* and *lapillus* otolith relationships in adult Atlantic bluefin tuna, *Thunnus thynnus*.

Ana Catarina Lourenço Caria

Peniche, *2018*

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Internship report submitted to the Superior School of Turism and Maritime Tecnhnology, Polytechnic Institute of Leiria, as part of the requirements to obtain the Master Degree in Aquaculture. Internship held under the supervision of Doctor Simeon Deguara (AquaBioTech Group, Malta) and Professor Marco Lemos (School of Tourism and Maritime Technology, Polytechnic Institute of Leiria).

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ACKNOWLEDGEMENTS

"*A man's friendships are one of the best measures of his worth.*"

Charles Darwin

I wish to take this opportunity to thanks several people and family and institutions for making this thesis possible.

The first one goes to AquaBioTech Group to let me do this internship and special to ICAAT for giving me the data to make my trial possible. A big thanks goes to Dr. Simeon Deguara for let me work with "big fish", for his advice and guidance to supervised my internship and recognized my work in AquaBioTech. To Professor Marco Lemos for all the knowledge given in class and for supervised my internship experience to João Franco for his advice. Also, a thanks to two big women's Tania Teixeira and Professor Susana Mendes who provide me good advices and encouraged me to go further.

I wish to say thanks to all the people who made me feel at home in Malta, the Portuguese crew in Malta, all Tuna teams, to all the friends I made there. A big fat thanks goes to a friend who been with me for some years Inês Pimparel without her I would never have survived the hard years of study, and special thanks goes for Emanuel Almada like he says for the patience and humour.

A heartfelt gratitude and thanks to my Father and all my beloved family who I value the most and in particular to my Mom for sometimes being "annoying" but almost always right and for being there for me all the time.

Thanks to all the friends I've done before and during college who have remained to this day, and have made me realize that there is more family than the one that is given since birth.

In short for all the people who have been directly or indirectly involved in this journey a sincere Thank You!

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ABSTRACT

An internship was carried out at AquaBioTech Group (ABT Group) in Mosta (Malta) to complete the Master in Aquaculture of the School of Tourism and Maritime Technology of the Polytechnic Institute of Leiria. AquaBioTech Group, is a European company involved with aquaculture and aquatic technology. The company is involved in research, fisheries and marine biotechnology, development in the aquaculture sector, together with environmental sustainability. This company operates in a sustainable way using Recirculation Aquaculture Systems (RAS) to maintain aquaculture species. In collaboration with several other companies and institutions, the ABT Group is involved and supports the development of important international research projects.

This internship lasted 6 months and encompassed two important parts: (i) learning of basic and advanced procedures at the aquaculture facility (ii) participation in the Tuna Project in partnership with The International Commission for the Conservation of Atlantic Tunas (ICCAT). The participation on this project, firstly on board of factory ships and later at the laboratory enabled to perform the study “Analysis of biometric and *asteriscus* and *lapillus* otolith relationships in adult Atlantic bluefin tuna, *Thunnus thynnus*”.

This internship allowed to expand the theoretical knowledge gained during the academic years and to develop personal and professional skills. The results of the study "Analysis of biometric and *asteriscus* and *lapillus* otolith relationships in adult Atlantic bluefin tuna, *Thunnus thynnus*" shown that: (i) it is needed to update the Biometric equations used by the Standing Committee on Research and Statistics (SCRS), (ii) the difference of weight and length, between males and females can result from the feeding and/ or its process and (iii) the sex identification of *T. thynnus* is not possible through otoliths analysis.

Keywords: AquaBioTech Group, ICCAT, Aquaculture, RAS, *Thunnus thynnus*, otolith.

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RESUMO

Este estágio foi realizado no Grupo AquaBioTech (*ABT Group*) em Malta para completar o Mestrado em Aquacultura da Escola de Turismo e Tecnologias do Mar, (ESTM) do Instituto Politécnico de Leiria (IPL). O Grupo AquaBioTech, é uma empresa que está envolvida em estudos relacionados com biotecnologia marinha desenvolvidos no setor de aquacultura e pescas focando-se na sustentabilidade ambiental. Esta empresa opera de uma forma sustentável utilizando nos seus estudos Aquacultura em Sistema de Recirculação (RAS), fazendo também colaborações com várias empresas e instituições, apoiando o desenvolvimento de importantes projetos internacionais.

O estágio teve uma duração de seis meses e foi dividido em duas vertentes diferentes: (i) a primeira ocorreu nas instalações da empresa, onde procedimentos básicos e avançados de aquacultura foram executados; (ii) participação no Projecto Atum em parceria com o *The International Commission for the Conservation of Atlantic Tunas* (ICCAT). A participação neste projecto, inicialmente a bordo de navios-fábrica e posteriormente no laboratório, possibilitou o estudo "Análise das relações de biométricas e dos otólitos *asteriscus* e *lapillus* em atum rabilho do Atlântico, *Thunnus thynnus*".

Este estágio permitiu aprofundar os conhecimentos teóricos adquiridos durante o percurso académico e desenvolver capacidades pessoais e profissionais. O estudo "Análise das relações de biométricase dos otólitos *asteriscus* e *lapillus* em atum rabilho do atlântico, *Thunnus thynnus*", demonstrou que (i) é necessária uma atualização das equações biométricas utilizadas pela Standing Committee on Research and Statistics (SCRS), (ii) a diferenças de tamanhos e pesos entre machos e fêmeas é significativa, pode resultar da alimentação e/ou do seu processamento e (iii) a identificação do sexo de *T. thynnus* não é possivel ser verificada através da análise dos otólitos.

Palavras-chave: AquaBioTech Group, ICCAT, Aquacultura, RAS, *Thunnus thynnus,* otólitos.

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LIST OF ACRONYMS

♀	Female
♂	Male
ABFT	Atlantic Bluefin Tuna
ABT	AquaBioTech
ABT Group	AquaBioTech Group
AL	*Asteriscus* Length
AM	Anterior Margin,
AW	*Asteriscus* Width
CFL	Curved Fork Length
CPUE	Catch Per Unit Effort
DM	Dorsal Margin
ESTM	School of Tourism and Maritime Technology
F&F	Fish & Fish farm
FAO	Food and Agriculture Organization of the United Nations
GBYP	Grand Bluefin Tuna Year Programme
GCP	Good Clinical Practice
GMP	Good Manufacturing Practice
ICCAT	International Commission for the Conservation of Atlantic Tunas
IPL	Institute Polytechnic of Leiria
K	Fulton's Condition Factor
LL	*Lapillus* Length
LW	*Lapillus* Width
MA	Malta sea
MAR	Malta Aquaculture Research Centre
MARIBE	Marine Investment for the Blue Economy

MB	Mare Blu Tuna Farm
MFF	Malta Fish Farm
PM	Posterior Margin,
RAS	Recirculating Aquaculture System
RWT	Round Weight
SCRS	Standing Committee on Research and Statistics
SD	Standard-Deviation
SFL	Straight Fork Length
SOP	Standard Operating Procedures
TAC	Total Admissible Catch
TU	Gulf of Gabes
TY	Tyranean sea
VICH	International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products
VIE	Visible implant elastomer
VM	Ventral Margin
VMD	Veterinary Medicines Directorate
VMS	Vessel monitoring systems
VRD	Veterinary Regulation Department

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1 INTRODUCTION

Aquaculture is one of the fastest growing food production industries in the world. This activity has evolved relatively fast in order to become an important available source of food for humanity. Marine based farming in particular, is currently growing fast (Froehlich, *et al.*, 2016). Capture fisheries and aquaculture are important sources of food, nutrition income, and livelihoods for millions of people around the world. In the beginning, fishing was the principal activity to obtain aquatic products. However, aquaculture began to grow, and started as a small scale industrial with non-commercial purpose. With the continuous growth of global population, it was necessary the increase of food production and the need for more aquatic based food, which drove the need for aquaculture growth (Godfray, *et al.*, 2010; Froehlich, *et al.*, 2016). A milestone was reached in 2014 when the aquaculture sector's contribution to the supply of fish for human consumption overtook wild-caught fish for the first time. (FAO, 2014).

Aquaculture practices are used world-wide in 3 different types of environment: freshwater, brackish water, and marine:

- Freshwater aquaculture is typically carried out either in fish ponds, fish pens, fish cages or, on a limited scale, in rice paddies;
- Brackish water aquaculture is practiced mainly in fish ponds located in coastal areas;
- Marine culture employs either fish cages or the use of substrates for molluscs and seaweeds such as stakes, ropes, and rafts (FAO, 2017).

In Europe, the aquaculture industry development is one of the fastest grown in the last three decades due to increasing of diversification, intensification and technological advances. Modern finfish aquaculture is increasing and will depended on good management and information technology for monitoring, control and optimisation. In the last five years there has been significant development of software systems to support improved operational efficiency (Janssen, *et al.*, 2016; FAO, 2017).

In the same way that aquaculture is divide in 3 types of environments, it also can be divided according to the different groups of aquatic species. The aquaculture food production in the European Union is divided into three main sectors: Marine, shellfish and freshwater.

Almost 50% of the total production resulted from shellfish aquaculture, namely from mussel, followed by oysters and clams farming (Mente and Smaal, 2016). On the other hand, Europe also produces six main finfish species, namely Rainbow Trout (*Oncorhynchus mykiss*), Atlantic Salmon (*Salmo salar*), European Seabass (*Dicentrarchus labrax*), Gilthead Seabream (*Sparus aurata*), Turbot (*Scophthalmus maximus*), Common Carp (*Cyprinus carpio*) and Bluefin tuna (*Thunnus tynnus*).

The aquaculture industry is viewed as an important financial and food contributor to the European economy as well as a mean of new employments (Janssen, *et al.*, 2016; FAO, 2017). The aquaculture sector expansion contributes to the economy by employing unskilled and skilled labours, by exporting goods and increasing the gross domestic product. For example, in Malta more recent data demonstrated that the aquaculture sector, created a total of 964 full-time jobs, with 197 of this job corresponding to the aquaculture sector itself directly. An additional 767 jobs generated by way of indirect activities, namely, transport and communication, financial intermediation, and manufacturing sector (Sacchi, 2011).

In Malta, aquaculture is marine-based. It consists of the capture based aquaculture of the Atlantic bluefin tuna (*Thunnus thynnus*), as well as the culture of European sea bass (*Dicentrarchus labrax*) and Gilthead sea bream (*Sparus aurata*) with a small production of Meagre (*Argyrosomus regius*) and amberjack (*Seriola dumerili*). The aquaculture of seabass, seabream, and meagre takes place in floating cages, within 1 kilometre off the shore. Bluefin tuna is cultured in specific designated aquaculture zones between 1km offshore and 6 km offshore (3 farms utilize the south-eastern coast to fatten up the captured tunas).

There is strong competition for space and resources due to the small size of Malta, with the farms occupying various sites on the East coast of the island (figure1). Environmental issues take priority and an environmental impact assessment is required before aquaculture development is introduced in line with the Aquaculture Strategy set from (2014-2025):

- Recognise Aquaculture as an important maritime sector for Malta;
- Steering growth towards sustainability;
- Enhance clarity of relevant regulations;
- Identify appropriate locations for Aquaculture zones;

- Create new potential for growth and search new areas for potential Aquaculture zones;
- Ensure sustainability through improved environmental management;
- Ensure competitiveness through innovation. (FAO, 2005).

Figure 1- Map relative to existing aquaculture in Malta (FAO, 2005).

Untill now Malta is a successful country because in 2015, the volume of fresh fish sold amounted to 10,800 tonnes, an increase of 25.5 per cent over the preceding year. (Environment, Energy, Transport and Agriculture Statistics, 2016).

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1.1 AQUABIOTECH GROUP

1.1.1 The Company

Biotechnology and aquaculture are two areas that have advanced in parallel to contribute for the innovation and progress of aquatic fish production. AquaBioTech Group (ABT Group) was founded in 1998 with basic and small installations (figure 2). Over the years the company has been expanding its facilities as well as the services it offers. It is an international consulting company, located in Mosta, Malta. It currently occupies five floors of a building with numerous rooms for holding livestock, laboratories, and administrative offices. The company is involved in research, fisheries and marine biotechnology, development in the aquaculture sector together with environmental sustainability. ABT Innovia focuses on research into areas directly related to aquaculture productivity such as new nutritional and fish health products. ABT Aquaculture develops a wide range of aquaculture systems that indirectly contribute to creating the optimal conditions for fish growth and ABT Marine provides different services related to the study of the seabed.

Figure 2- Logo of AquaBioTech Group

1.1.2 Research areas

The profitability and potential expansion of the aquaculture industry comes with modernization, technique optimization and investment in research projects.

Space availability, water utilisation efficiency and nutrient discharge in wastewater are major challenges facing the sustainable development of aquaculture. Solving these issues are therefore of major importance. Recirculating Aquaculture System (RAS) offers a potential approach to address these issues. RAS has been developed in order to promote nutrient recycling and to minimize water consumption, minimizing it impacts on the environment. RAS is also a great solution to the increasing environmental restrictions in countries with land limitations and water access. In RAS, the water prevenient from fish tanks is recirculated after removal of the toxic nitrogenous metabolites through bioreactors. Removal of nitrogenous waste products is the core activity that ensures the successful functioning of the RAS systems. In summary, these systems work in a closed-loop facility where the tank systems are designed to retain and treat the water, allowing its partial reuse (Piedrahita, 2003; Martins, *et al.*, 2010; Nazar, *et al.*, 2013; Prabhu, *et al.*, 2017). At AquaBioTech Group, all trials are performed in RAS.

The company has been growing in different areas of aquaculture allowing partnerships with other companies. These partnerships lead to the development of projects with main focus in: health enhancement and disease prevention of fish using medicines, vaccinations and nutrition trials. ABT is currently testing feeds formulated with new ingredients, developed by an affiliated company. This feed might improve fish growth and health as well as reducing environmental impacts. By testing these new formulations, AquaBioTech Group is also providing specialized facilities that were designed specifically to perform these type of assays. ABT Group also has equipment and all the conditions to test and improve pharmacological products consequently improving the health of species. ABT Innovia perform trials that study pathogens in specific research units, develops tests to detect easily some diseases and improves the efficacy of identification tests with pathogens in aquaculture.

Presently, the ABT Group is carrying out research with European projects such as MARIBE that consists on the development of large scale activities offshore and in deep sea areas

requiring technological and non-technological challenges and the assessment of one of the most promising and sustainable business models (Available in: https://maribe.eu/2018).

BYEFOULING main goal is to provide the means for industrial, cost-effective, and robust manufacturing of antifouling coatings in Europe. BYEFOULING, is commonly defined as the undesirable accumulation of microorganisms, animals and plants on artificial surfaces immersed in seawater, that will accelerate corrosion and create biodeterioration problems. For now, the solution is to use toxic compounds that affect non-target organisms and bioaccumulate on the aquatic environment (Available in https://www.sintef.no/projectweb/byefouling/2018). The AquaBioTech Group facility provides laboratories to study and test the antifouling activity. This way, the company developed ecotoxicological protocols with s marine species (barnacle, bryozoans, mussels and algae) to study their toxic exposure.

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1.2 BLUEFIN TUNA, *Thunnus thynnus*

Cage culture involves the rearing of fish within fixed or floating net enclosures supported by frameworks set in sheltered, shallow portions of lakes, bays, rivers, and estuaries. (FAO, 2016). They overcome the low productivity of inshore aquaculture. Because the offshore aquaculture provides a better environment with good quality water and large-capacity, high-quality fish can be produced stably and consistently. Recently, the need for offshore marine aquaculture has become increasingly important in raising migratory fish species, such as, tuna and mackerel (Heungwoo, *et al.*, 2014).

Offshore marine aquaculture is a technique that has been assessed over the years. Consequently, in order to increase sustainable marine production, it is critical to first understand the potential capacity and diversity of farmed marine species, as well as the trade-offs associated with their suitability to tolerate and thrive in a range of environmental conditions (Heungwoo, *et al.*, 2014; Froehlich, *et al*., 2016).

The Atlantic bluefin tuna (*Thunnus thynnus*-Linnaeus, 1758) – ABFT – is one of the tuna species with the highest economic interest, is the biggest tuna, its official maximum weight is 726 kg, but weights up to 900 kg have been reported in various fisheries of the West Atlantic and Mediterranean Sea. Simultaneously is a large top-predator fish which inhabits the pelagic ecosystems of the North Atlantic Ocean and the Mediterranean Sea (figure 3). Like other large tunas, bluefin tuna is a migratory species, capable of tolerate big differences in temperature, with documented transoceanic and large-scale movements for feeding and reproduction (Addis, *et al.*, 2016).

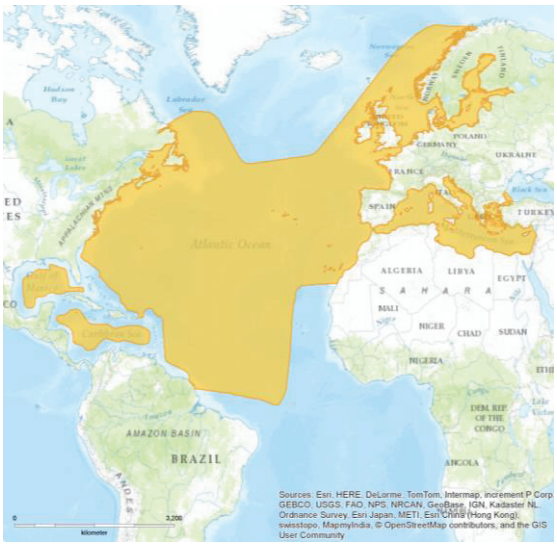

Figure 3- Distribution map of ABFT (Collette, *et al.*, 2011).

ABFT also sustains important recreational and commercial fisheries as well as the capture-based tuna aquaculture industry. The International Commission for the Conservation of Atlantic Tunas (ICCAT) research is based mainly on different separate spawning areas, one located in the Gulf of Mexico and one in the Mediterranean Sea. Later, due to differences in life history characteristics, two areas were recognized as management units: the west and east Atlantic stock and after the Mediterranean Sea. (Milatou & Megalofonou, 2014).

In the last few years, the increase in fishing pressure together with the intensity of farming activity has resulted in a dramatic biomass reduction of the Atlantic bluefin tuna stocks. The industry based on the penning and subsequent fattening of tuna has recovered somewhat as catched quotas have increased gradually from a low of 5 790 tonnes in 2011 to 11 203 tonnes in 2016 (FAO, 2017).

The global total capture fishery production in 2014 was 93.4 million tonnes, of which 81.5 million tonnes from marine waters and 11.9 million tonnes from inland waters. For marine fisheries production, China remained the major producer followed by Indonesia, the United States of America, and the Russian Federation. (Milatou & Megalofonou, 2014; FAO 2016). However, catches in 2015 of four highly valuable groups (tunas, lobsters, shrimps, and cephalopods) registered new record catches in 2014. Total catches of tuna and tuna like species were almost 7.7 million tonnes (figure 4), (FAO, 2017).

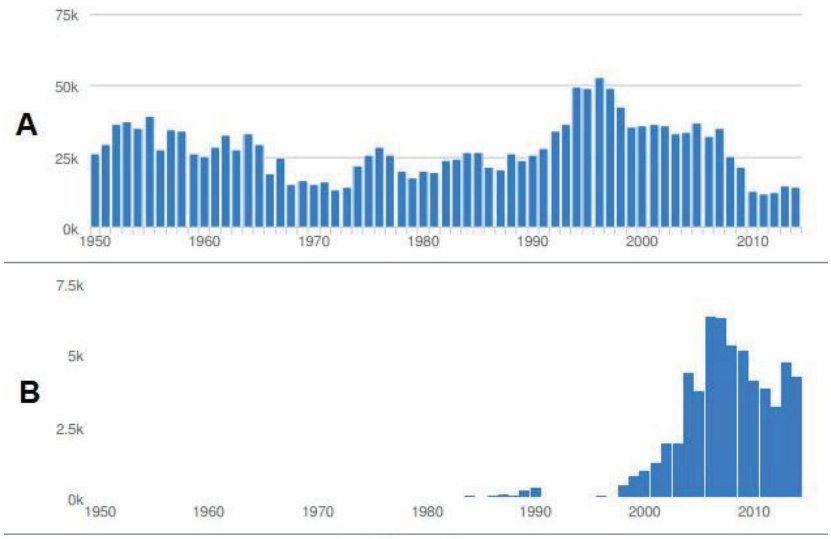

Figure 4- Graphic A- Global Capture Production and Graphic B- Global Aquaculture Production for tuna and tuna like (tonnes), Source: FAO FishStat.

Even though formulated diets are not yet in commercial use, further progress has been made in Europe with captive breeding, larval rearing and the subsequent nursery phase, but more time and funding are needed to achieve sufficiently high survival rates for commercially viable production. The hope for the development of a Mediterranean-based closed-cycle bluefin tuna aquaculture industry has been anticipated by Váradi *et al*. (2011) but so far only small steps forward have been taken (FAO, 2017).

The ABFT has not been recorded off the coast of Brazil over the last 20 to 36 years and for this reason is reported as an endangered species. So, this species as become rare according to historical levels because of massive overfishing. In addition, information available has demonstrated that catches of Bluefin Tuna from the East Atlantic and Mediterranean were seriously under-reported between the mid-1990s through 2007. The lack of compliance with Total Admissible Catch (TAC)/quotas and underreporting of the catch may have severely undermined the conservation of the stock (Collette, *et al.*, 2011).

Therefore studies at different levels on *T. thynnus* are urgently needed. Understanding the bluefin tuna migratory behaviour is crucial for management, as spatial variability governs the definition of management units, stocks, and boundaries. Since the techniques used have been improving and more hypothesis appear (Fromentin, 2006).

The use various genetic markers in bluefin tuna populations as become a hypothesis of genetic stock structure. Although the results remain somewhat controversial, most recent

and extensive studies tend to support the hypothesis of a complex population structure, with, for instance, genetic differences within the Mediterranean Sea and the central North Atlantic. To understand the bluefin tuna spatial dynamics is used an electronic tagging, however it does not provide the birth location of the migrating fish, a key information to understand the population structure. Chemical signatures in hard structures (especially otolith) have shown to be helpful to discriminate between recognized nursery grounds of West Atlantic and Mediterranean bluefin tuna (Fromentin, 2006).

1.2.1 Otoliths

Over the past 20 years, knowledge of fish calcified structures, otoliths, scales, vertebrae and fin ray spines has been applied for determining environmental histories of fish in a diverse range of aquatic environments including marine, estuarine and freshwater systems. As such, it represents one of the most powerful tools to address fundamental questions in fish ecology and fisheries science, including stock structure, site fidelity, natal origin, and migration pathway over ecological time scale. The premise of this approach is that calcified structures in fishes generally form by the periodic deposition of daily and annual increments as the fish grows, which enable scientists to determine ages and life history parameters of individual fish and fish populations. As these biogenic structures grow, trace amounts of elements (including heavy metals) are naturally incorporated into their mineral phase from the surrounding environment experienced by the fish. Coupling structure biochronology with the chemical record of fish's life (i.e. trace elements and/or isotopic composition) enables a retrospective description of individual fish environmental history including fish movements, life history traits, and ontogenetic development (Luque, *et al.*, 2016; Tzadik, *et al*., 2017).

Otoliths are small structures in the saccule or utricle of the inner ear, specifically in the vestibular labyrinth (figure 5). They are metabolically inert calcium carbonate structures that means that the newly deposited material is neither resorbed nor reworked after deposition, and hence, only ontogenetic and environmental factors should cause changes to their chemical composition. Alongside their chronological properties, otoliths (*sagittae)* represented in (figure 5A) have been the preferred bony structure to use in chemistry studies, they are the more precise way to know the age of a fish by counting the rings in them (Elsdon *et al.*, 2008; Campana & Thorrold, 2011).

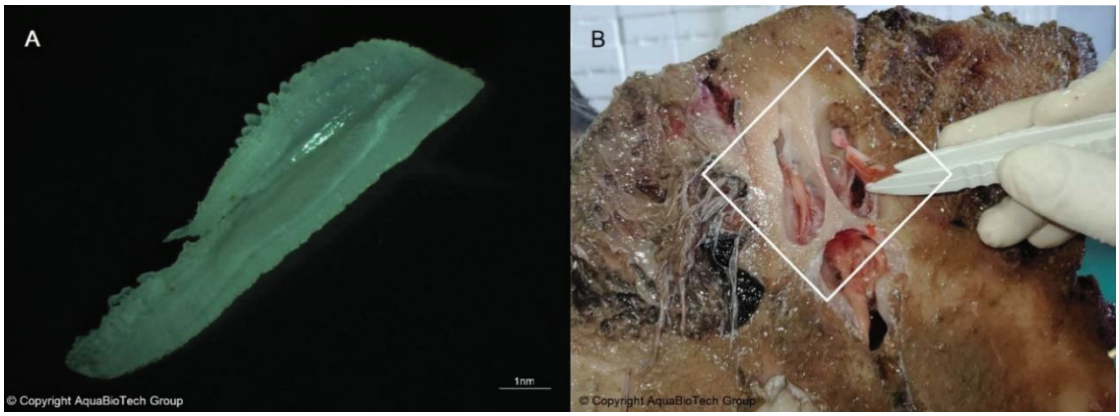

Figure 5- Otoliths, A- stereomicroscopic view of an *sagittae* otolith, B otoliths extraction.

The extraction of otoliths requires sacrificing the fish (figure 5B), which is neither allowed for rare and/or endangered species, nor practical for commercially valuable fish species such as Atlantic bluefin tuna (*Thunnus thynnus*), (Campana & Thorrold, 2011).

1.3 GOAL OF INTERNSHIP

The goals for the current internship in AquaBioTech Group were to:

- Acquire competencies related to the management and organization and understand the function and operation mode of an aquaculture company;
- Increase the practical experience and skills in the techniques applied in aquaculture;
- Maintain and take care of some of the main fish species of aquaculture and manage water quality parameters and the life support systems;
- Acquire main techniques related with fish husbandry including experimental methodology and microbiology procedures;
- Learn and take part in the research carried out by AquaBioTech Innovia at the facility and outside the facility for clients involved in aquaculture and fisheries:
    - More specifically in a program entitled Grand Bluefin Tuna Year Programme (GBYP) Biological Sampling research, for Atlantic bluefin tuna, *Thunnus thynnus*. Originating the trial carried out in this study: "Analysis of biometrics, the *asteriscus* and *lapillus* otolith relationships in adult Atlantic bluefin tuna, *Thunnus thynnus*".

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2 INTERNSHIP DESCRIPTION

2.1 COMPANY

The internship began with the reading and studying of various Standard Operating Procedures (SOP). This documentation consists of procedures carried out at the facility for repetitive techniques, in accordance with the specifications intended at obtaining a desired outcome. Then, the company gave two weeks of training, accompanied by members of the staff.

2.1.1 Biosecurity procedures

The infrastructure and organization of the facility is planned to ensure the necessary biosecurity standards.

Biosecurity is an aspect important in fish production. It helps to prevent the introduction and propagation of diseases within and between aquaculture facilities. Developing studies with pathogens requires rules that must be followed rigorously every day to maintain the quality and safety of the work and the stock. The AquaBioTech facility operates with a high level of biosecurity. This includes procedures that are designed to ensure the minimum risk for proliferation of diseases; all the staff have a rigorous training to follow the biosecurity procedures.

The aquaculture unit is divided into different rooms, named 'Bays'. Each Bay can be differentiated as:

- Clean Bays - where there is no pathogen manipulation;
- Challenge Bays - where there are trials with pathogens, that involve infectious agents.

This means that the biosecurity adopted inside of the different Bays follows different procedures, like the use of special shoes in the case of Challenge Bays and different clothes for each type of Bay. All the bays have disposable gloves available and bottles of alcohol for sterilisation of hands and equipment.

All of the experimental challenge trials are performed according to Good Clinical Practice (GCP) under the principles of the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) The AquaBioTech Group is a Good Manufacturing Practice (GMP) certified company by the United Kingdom Veterinary Medicines Directorate (VMD) and Maltese Veterinary Regulation Department (VRD). (AquaBioTech Group Company. Available in: https://www.aquabt.com/2018).

2.1.2 Daily routines

As the name implies every day, a team member is responsible for fulfil a sheet, called a checklist. The checklist is a document that will simplify the observations of each specific field (figure 6). Checklists are filled six times per day in all the Bays composed by a list of tasks that include:

Figure 6- Daily routines.

- Monitoring the whole system;
- Water quality parameters;
- Removal of feed waste and faeces of the tanks;
- Equipment maintenance;
- Feeding fish;
- Health and fish welfare;
- Remove and record the mortalities.

Beside this, the checklist is performed differently between Clean and Challenge Bays. In Challenges Bays staff must be extra careful due to the pathogens existing in the same bay. Also, due to biosecurity reasons, the checklist is always filled inside the Bay by the responsible technician. In the evening, after finishing all the observations, all the sheets are collected and the data is recorded in the computer system. This practice was developed to simplify the checking of all the systems, ensure their satisfactory operation and ensure fish safety.

2.1.3 Water quality control

The water quality is measured daily in each system. Every day an aquaculture technician has the task to analyse the following parameters:

- Ammonia, nitrites, nitrates, alkalinity, salinity, temperature, pH, Oxygen and RedOx.

First, 50 mL of water is collect from each system and the ammonia, nitrites, nitrates and alkalinity is analysed with kits tests. These tests consist in a visual colour evaluation. For each colour, there is the corresponding value. Nevertheless, when colorimetric kits are used, attention must be taken because the evaluation may be imprecise unless they are carried out carefully. However, these tests are the commonly used they are a quick and easy procedures which provides an idea of the water quality general status (Boyd & Tucker, 2012). Sensors measure the other 5 parameters permanently present inside of the tanks. These instruments provide a more accurate value. They send the data to the computer system that saves them and creates a database by systems and tanks separately. The system monitor shows if all if these values are in the optimal range, if not the tanks that normally are shown with a blue colour turns orange and if the problem isn't solved turns red and the alarm is activated.

After the measurement of all parameters of water quality, the person responsible must record the data. All data is saved over time, allowing a temporal evolution to be seen.

2.1.4 Cleaning

Cleaning is an important task as it will provide optimal hygiene conditions for a good fish maintenance; two times per week all Clean Bays pass thorough a special cleaning protocol.

In Challenge Bays the procedure is slightly different; when a trial ends disinfection takes place, including rigorous steps, and including cleaning of the system, so when it is needed again, it is ready to receive a new trial.

The responsible technician will have a sheet that will include all the cleaning task that has to be done, which includes:

- Remove faeces and food wastes by siphons or opening the purges;
- Cleaning the inside and exterior of the tanks;
- Cleaning whole system including the pipes and probes;
- Fill up the alcohol bottles and footbath;
- Check the gloves boxes;
- Remove the rubbish;
- Cleaning of all the room.

2.1.5 Feeding

Fish nutrition is one of the most important aspects in aquaculture; currently research being carried out includes studies on feed consumption and the physiological mechanisms involved in its regulation, nutrient requirements and interactions, metabolic pathways and nutrient utilization, fish growth, reproduction and early development. The investigation of nutritional influences on the ability of fish to resist environmental stressors and mount an immune response under challenge from pathogens also forms a part of fish nutrition research (Jobling, 2015). This is one of the goals of the company and these studies occur in what they call nutrition trials and in this case the feeding process have a specific schedule. For the other fish that aren't in this regime generally, the technician or intern responsible to do the daily checklist is the one responsible to do the feeding. The process will depend of the species and consist in giving the food between three or four times a day (figure 7). Exceptionally, if the fish have been exposed to stress conditions such as transportation, cleaning or handling the food can be given at non-scheduled times.

Figure 7- Feeding process.

There are two important aspects to understand:

- How the species feeds in the water column. If they eat at the surface, near the bottom, or in water column;
- Buoyancy and the quantity of the feed. The administration of the feed varies according to the needs of the fish.

These characteristics need to be considered to ensure that all nutritional requirements are met. After the last feeding, the food containers are collected and taken to the aquaculture office. Feed remaining is weighed, recorded and the feed for the next day prepared as required. Nutrition is central to the fish's health and their feeding behaviour is an indication of health. If the fish are not feeding it could be an indication that they have some disease. This way, the first preventive method should be immediately performed to avoid the disease spreading and actions taken accordingly.

2.1.6 Techniques acquired

The AquaBioTech Group teaches several techniques that are important in aquaculture fish handling. Some examples of practical techniques are:

- **Tagging -** Tagging is a procedure with two main applications, to study:
    - The fish in their natural habitat;
    - To differentiate fish in the trials.

The one that was taught was the second one, which is normally used in trails with vaccines. The tagging procedure included the use of a special preparation with different colours which are introduced under transparent or translucent tissues. Visible implant elastomer, (VIE), is a silicone-based subcutaneous tagging system used for individual identification and is widely used to mark fish and crustaceans. The fluorescent colours are visible under ambient light or fluoresce with a special VI Light (King & Heistermann, 2015). However, this technique requires some practice to manage the instrument and the fish. First, the fish need to be anesthetized to make accurate markings visible and without causing lesions. The injector is laid in the hand in a certain position and the needle will penetrate the surface of the skin. Then, a slight pressure should be applied in order to make a visible mark. Commonly, the tag mark is performed near the eyes, on the left or on the right side, but can be done on the belly near the pectoral fins (Figure 8). However, the tag size depends of the fish size or technician requirements. Normally, the small fish are very difficult to handling and care must be taken to not damage internal organs.

Figure 8- Tagging process.

- **Vaccination -** The vaccination technique used in AquaBioTech depends on the experimental protocol.

The vaccines contain antigens that when administrated induce a protective immune response without causing considerable side effects (Gudding, *et al*., 2014). The vaccination procedure is a technique that requires fish manipulation and may cause more stress. However, to avoid excessive stress the fish are anesthetized. Then, using syringes the fish are injected immediately in the pelvic fins zone with minimum of risk of damaging the underlying organs. The time taken depends of the team's skills which normally need around 2 or 3 people. Vaccines can be delivered to fish by intraperitoneal injection, by immersion (where animals are placed in a vaccine solution) or by oral administration (Rogers & Basurco, 2009).

Summing up, the most important parameters to be monitored and controlled in an aquaculture system are related to water quality, since they directly affect animal health, feed utilization, growth rates and carrying capacities. The critical water quality parameters that are taken care in RAS are dissolved oxygen, temperature, pH, alkalinity, suspended solids, ammonia, nitrite and carbon dioxide. These parameters are interrelated in a complex series of physical, biological and chemical reactions. Monitoring and making adjustments in the system to keep the levels of these parameters within acceptable ranges is very important to maintain the viability of the total system. The components that address these parameters can vary from system to system (Nazar, *et al.*, 2013).

2.2 BLUEFIN TUNA PROJECT

As mentioned previously in the company description, the company participates in projects with other companies. This internship report was based on GBYP Biological Sampling Research, a Programme for Atlantic bluefin tuna, *Thunnus thynnus*, that has various objectives:

- Improve basic data collection through data mining (including information from traps, observers, and Vessel Monitoring Systems (VMS) developing methods to estimate sizes of fish caged, elaborating accurate Catch Per Unit Effort (CPUE) indices for Mediterranean purse seine fleets, development of fisheries-independent information surveys and implementing a large scale scientific conventional tagging programme;

- Improve understanding of key biological and ecological processes through electronic tagging experiments to determine habitat and migration routes, broad scale biological sampling of live fish and dead fish landed (e.g. gonads, liver, otoliths, spines, etc.), histological analyses to determine bluefin tuna reproductive state, biological and genetic analyses to investigate mixing and population structure; and ecological processes, including predator-prey relationships;

- Improve assessment models and provision of scientific advice on stock status through improved modelling of key biological processes (including growth and stock-recruitment), further developing stock assessment models including mixing among areas, and developing and use of biologically realistic operating models for more rigorous management option testing (Available in: http://www.iccat.int/GBYP/en/2018).

The AquaBioTech Group was involved in collecting biometric data and samples for the determination of the age and genetic structure of the Atlantic Bluefin tuna in the Mediterranean Sea.

The work was divided in two parts, first at the factory ships and then at the wet laboratory.

2.2.1 Factory ship

In this part of the internship, the work actually began at 5 a.m. Four colleagues would go to the factory boat in a small boat responsible for taking the divers that will catch he Tuna in the cages and will take the fish to the main boat (figure 9).

Figure 9- Daily routine on a tuna processing boat.

After being taken from the cages, the tuna are hoisted onto the factory boat and placed on the deck. There without interfering with the processing, 3 people were responsible to tag the head with a label that had a number and measure the fish in three different ways:

- The Round Weight (RWT);
- the Straight Fork Length (SFL);this is the straight line from the end of the upper jaw (end of the snout) to the fork of the caudal fin as shown in figure 10; this can best be measured using a caliper or alternatively with a tape measure, although it must be kept straight while measuring with the fish placed on a flat surface in a horizontal position);
- the Curved Fork Length (CFL); this is the length from the upper jaw (end of the snout) to the fork following the fish curvature (figure 10).

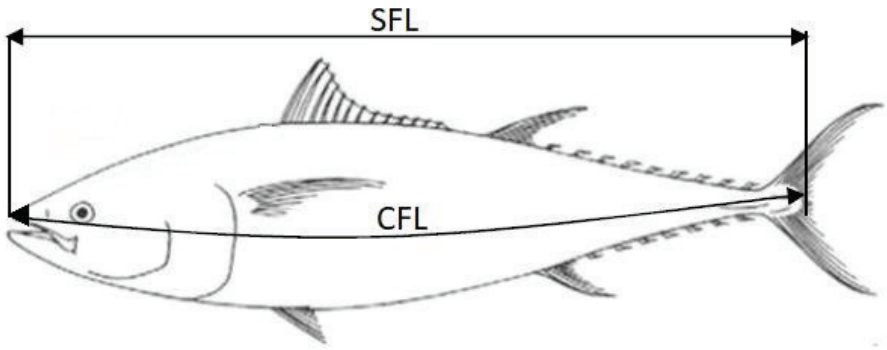

Figure 10 Representative scheme of measurements for bluefin tuna.

After weighing, the head was cuted off and placed inside a big sack which was also tagged with a number that enable us to know from which farm the heads were collected. The fourth person was responsible to follow the body of the previously labelled fish to check the sex. The sacks with the heads were then taken back by the farm boats to be frozen at 20^{o}C negative until otolith extraction as described below.

2.2.1.1 Wet lab

The heads were kept frozen until the day that 4 colleagues processed them. On that day the work started at 9 a.m. One of the workers was responsible to cut the head parallel to the nose. This needed to be done carefully to make certain that the cut did not break the otoliths. The

easiest way to cut the head was with a saw in the frontal plane above the supraorbital ridge (figure 11a).

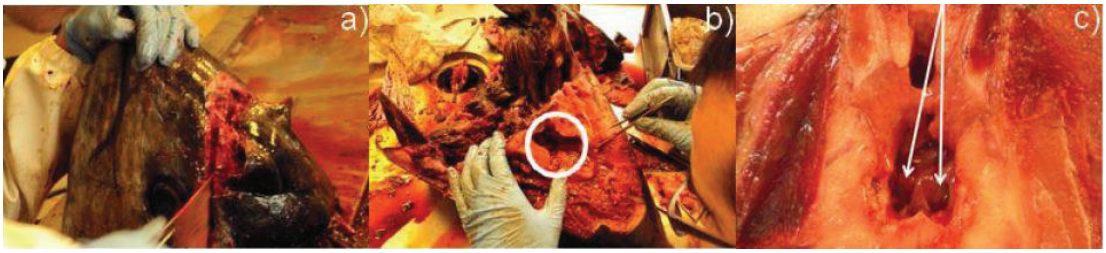

Figure 11- Otolith extraction routine, a) parallel cut to the nose, b) brain cavity c) semi-circular canals.

2.2.1.2 Otoliths extraction:

After cutting and open the head, other people were responsible to search and extract otoliths from their canals. It was very important to work carefully because the otolith can easily be damaged at this stage. The otoliths are located at the back of the brain cavity (figure 11b), inside semi-circular canals (figure 11c). The posterior end of the otolith is the most fragile. Small forceps were used to extract the otoliths from the bony capsules and gently remove the membrane surrounding the otolith immediately after extraction. After, a scalpel was used to take a muscle sample, more and less with 1 cm^2 for genetic analysis. Throughout these processes new forceps, scalpel, etc were used to prevent cross-contamination between samples.

2.2.1.3 Sampling procedure:

Two persons were in charge of labelling and processing the samples. The process consisted in taking the muscle sample from each head and placing it directly into the microtube with 96% ethanol. Since any residual ice in the sample may dilute the ethanol, it was necessary to, change the ethanol after 4-5 days, in order to ensure proper sample preservation. Otoliths were carefully cleaned as follows: The otoliths were immersed in deionized water to hydrate biological residues adhering to the otolith surface. These were removed using small forceps. Then, the otoliths were immersed into 0.1% nitric acid for 5 min. to remove surface contamination. The otoliths were then cleaned again with deionized water to remove the remaining nitric acid. Finally, the otoliths were dried for 24 hours and stored in pairs in Eppendorf with their corresponding labels.

3 ANALYSIS OF BIOMETRICS AND *ASTERISCUS* AND *LAPILLUS* OTOLITH RELATIONSHIPS IN ADULT ATLANTIC BLUEFIN TUNA, *Thunnus thynnus*.

3.1 INTRODUCTION

The Atlantic bluefin tuna (*Thunnus thynnus*)– ABFT – is one of the tuna species with the highest economic interest. The increase in fishing pressure and intensity of farming activity has resulted in a dramatic biomass reduction of stocks. Following the establishment and enforcement of a rigorous recovery plan The International Commission for the Conservation of Atlantic Tunas (ICCAT) which is responsible for managing this stock, there has been a recovery. ICCAT also puts a great deal of emphasis on obtaining scientific data to increase the knowledge about this species and make management more effective.

The source of the data used in this study originated from the GBYP Biological Sampling the scientific research project on Atlantic Bluefin tuna being carried out by ICCAT.

Between September 2016 to December 2017, *T. thynnus* were harvested from 3 different farms Mare Blue Tuna (MB); Fish & Fish (F&F) and Malta Fish Farm (MFF). Each farm provided fish from 3 different regions of the Mediterranean Sea, Gulf of Gabes (TU); Tyranean sea (TY) and Malta sea (MA), (figure 12).

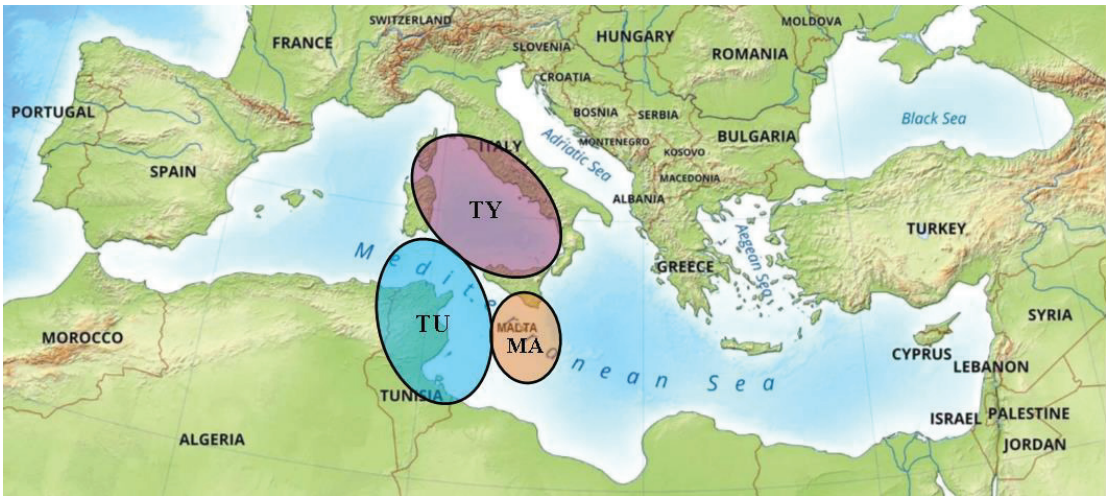

Figure 12-Mediterranean Sea map showing the 3 areas/regions studied,

3.1.1 Goal

This study was divided in three goals. First to correlate the following parameters: Straight Fork Length (SFL), Curved Fork Length (CFL); Round Weight (RWT). As well the Fulton's Condition Factor (K) for knowing the nutritional status of *Thunnus thynnus* caught in Malta. Second to study the relationship between length, weight and sex collected of ABFT and to compare the differences between farms and regions where they were catched. The third and last goal was to carry out a morphometric analysis of the otoliths: *asteriscus* and *lapillus* of Bluefin tuna to see if there exists any correlation between the sizes of the otoliths with the SFL and sex.

3.2 MATERIAL AND METHODS

3.2.1 Morphological characteristics

Between September 2016 to December 2017 *Thunnus thynnus* were harvested from three different offshore farms (MB, F&F and MFF) situated in Malta in the Mediterranean Sea. Each farm provided fish caught from three different regions/areas of the Mediterranean Sea (TU, TY and MA).

In total 1054 specimens were tagged. The tagging process consisted in putting a label with a number in the head next to jaw as you can see in figure 13B. Afterwards the specimen was measured (SFL and CFL), (figure 13A) and then weighed (RWT). After opened the abdominal area the sex was registered (figura13C).

Figure 13- Representative illustration of parameters studied; A- measurement of a tuna, B- tagged head and C- representation of the gonads in which 1- male and 2- female.

3.2.2 *Asteriscus* and *Lapillus*, otoliths observation

All the tagged heads were taken to a wet lab in Marsaxlokk, Malta.

After making a transverse cut in the ventral cranial cavity, the otoliths from left and right semi-circular canals were extracted. Then the *asteriscus* and *lapillus* were cleaned with deionised water and stored, dry in little plastic bags labelled with institute code, area code, size class, individual ID (nº) and tissue code.

The structure of 64 *Asteriscus* and 63 *Lapillus*, was studied with a dissection microscope (Optika SZP, Italy). Data on length and width were registered for each otolith through their observation with a dissection microscope with a graduated ocular lens (figure 14). Otolith sample size was calculated using an ocular and stage micrometre for calibration. First, the stage was positioned with the ocular micrometre in a way both scales were lined up completely parallel. After that the real measure was given by:

$$Real\ measure = [(x\ pitch\ of\ ocular\ micrometre\ is\ y\mu m) \times (objective\ lent)).$$

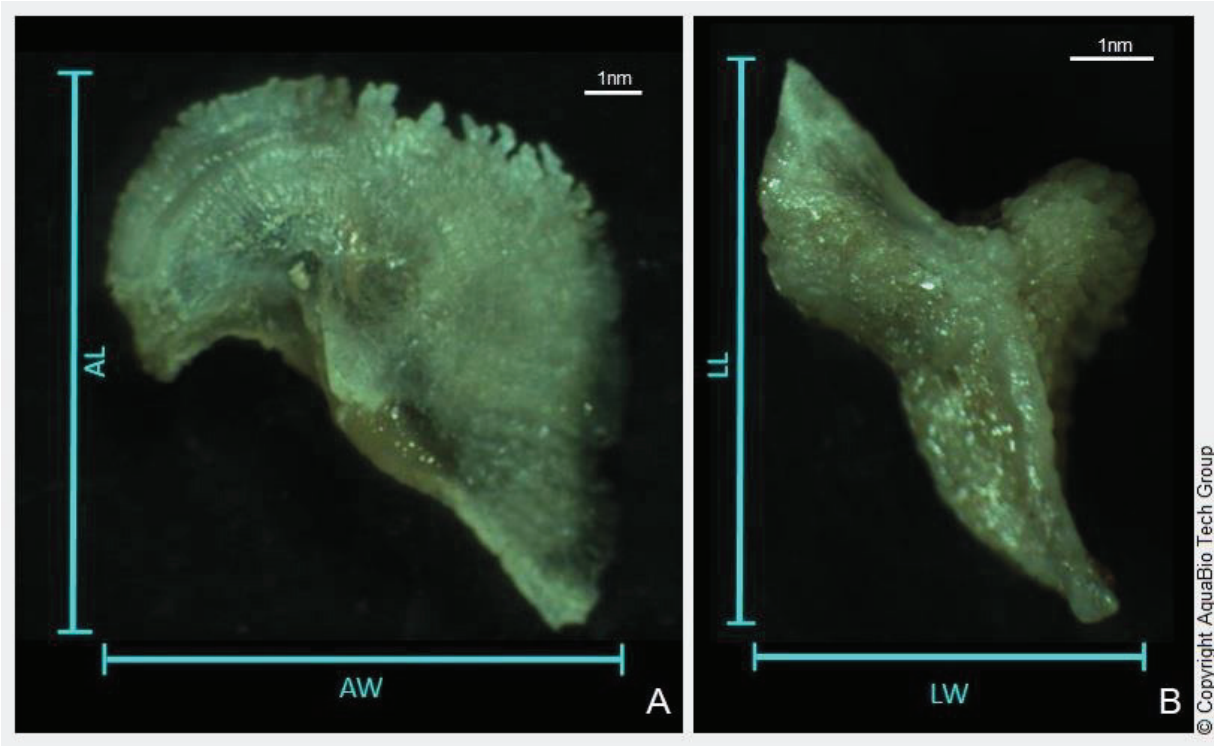

Figure 14 Representative illustration of measurements for *Asteriscus* (A) and *Lapillus* (B): *Asteriscus* Width (AW); *Asteriscus* Length (AL), *Lapillus* Width (LW) and *Lapillus* Length (LL).

After obtaining the measurement of all the samples the relationships between the lengths and widths of the otolith with SFL for all fish and then for males and females separately were studied.

3.2.3 Data analysis of morphological characteristics and otoliths observation.

The following relationships were studied: RWT vs FL, RWT vs SFL and SFL vs CFL. A similar analysis was performed for the otoliths, that is, the relationship between the

Asteriscus Width (AW) vs SFL, *Asteriscus* Length (AL) vs SFL, *Lapillus* Width (LW) vs SFL and *Lapillus* Length (LL) vs SFL.

Fulton's Condition Factor, K,(Ricker, 1975) was calculated as:

$$K = 10^5 \times {}^{RWT}/_{SFL^3}$$

Analysis of variance (ANOVA) or the Kruskal-Wallis (Zar, 2010) non-parametric test (depending on the violation or not of the normality and homogeneity of variance assumptions) were applied to compare the estimated straight fork length (cm), curved fork length (cm) and round weight (kg) between farms and between areas, separating by sex of Bluefin tuna. Games-Howell and Tukey (HSD) tests (depending on the violation or not of ANOVA assumptions) were performed as a post-hoc test when significant differences were observed (Zar, 2010).

For the morphometric analysis of the otoliths (namely, size (cm), length (cm)) for *asteriscus* and *lapillus* of Bluefin tuna, differences between sexes were determined using the Student's t-test (Zar, 2010).

Where applicable, results are presented as mean±standard-deviation (SD). Differences at p-value ≤ 0.05 level were accepted as significant. All statistical analysis was done using IBM SPSS Statistics for Windows, version 23.0 and Excel Office 2016.

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3.3 RESULTS

3.3.1 Morphological characteristics

The total number of fish studied was 1054. However, there were differences in of the numbers of fish sampled per farm and area. In the case of F&F were 147 in total, 60 females and 87 males. For MFF 217 in total, 129 females and 88 males. Finally for MB there were 690 fish in total, but in this farm fish had been caught from two different areas so the total fish for MB, MA is 168, 80 females, 88 males and for 522 TY, 146 females and 376 males (table I ,II and III).

The SFL, CFL and RWT were measured on all fish sampled. Table I represents the relationship between RWT and SFL. An exponential regression fit was used to determine the relevant relationship. The R^2 value representing the goodness of fit to the data is also given.

Table I - Refers to number of fish sampled, size range, average and RW/SFL relationship by farm, area, where they were captured, and gender.

Farm	Area	Gender	Number of fish	SFL range (cm)	$\bar{x}$ SFL (cm)	RWT/SFL relationship	R^2
F&F	MA	all	147	190-267	228	$5{,}00x10^{-6}SFL^{3{,}2775}$	0,86
		female	60	190-267	223	$3{,}00x10^{-6}SFL^{3{,}3587}$	0,84
		male	87	196-261	232	$4{,}00x10^{-5}SFL^{2{,}9048}$	0,83
MB	MA	all	168	185-265	225	$2{,}00x10^{-4}SFL^{2{,}5857}$	0,81
		female	80	185-240	219	$2{,}00x10^{-4}SFL^{2{,}5445}$	0,80
		male	88	186-265	231	$3{,}00x10^{-4}SFL^{2{,}5132}$	0,77
	TY	all	522	114-271	220	$9{,}00x10^{-5}SFL^{2{,}7374}$	0,96
		female	146	124-271	206	$7{,}00x10^{-5}SFL^{2{,}7916}$	0,97
		male	376	114-269	226	$1{,}00x10^{-4}SFL^{2{,}6868}$	0,96
MFF	TU	all	217	184-262	216	$6{,}00x10^{-4}SFL^{2{,}4059}$	0,77
		female	129	184-239	213	$5{,}00x10^{-4}SFL^{2{,}4093}$	0,78
		male	88	186-262	220	$1{,}50x10^{-3}SFL^{2{,}2233}$	0,72

Table II represents the relationship between RWT and CFL. An exponential regression fit was used to determine the relevant relationship. The R^2 value representing the goodness of fit to the data is also given.

Table II- Refers to number of fish sampled, size range and RW/CFL relationship by farm, area where they were captured and gender.

Farm	**Area**	**Gender**	**Number of fish**	**CFL range (cm)**	**$\bar{x}$ CFL (cm)**	**RW/CFL relationship**	**R^2**
F&F	MA	all	147	195-284	245	$1{,}00x10^{-5}CFL^{3{,}0743}$	0,89
		female	60	195-284	237	$9{,}00x10^{-6}CFL^{3{,}1307}$	0,91
		male	87	211-279	250	$4{,}00x10^{-5}CFL^{2{,}8666}$	0,82
MB	MA	all	168	195-275	243	$6{,}00x10^{-5}CFL^{2{,}7595}$	0,87
		female	80	199-258	236	$9{,}00x10^{-5}CFL^{2{,}6983}$	0,84
		male	88	195-275	248	$7{,}00x10^{-5}CFL^{2{,}7354}$	0,85
	TY	all	522	126-288	238	$9{,}00x10^{-5}CFL^{2{,}6915}$	0,97
		female	146	129-286	222	$9{,}00x10^{-5}CFL^{2{,}7047}$	0,97
		male	376	126-288	244	$1{,}00x10^{-4}CFL^{2{,}6627}$	0,97
MFF	TU	all	217	198-281	232	$1{,}00x10^{-4}CFL^{2{,}6696}$	0,87
		female	129	198-253	228	$2{,}00x10^{-4}CFL^{2{,}604}$	0,87
		male	88	204-281	236	$2{,}00x10^{-4}CFL^{2{,}606}$	0,86

Table III represents the relationship between SFL and CFL. A linear regression fit was used to determine the relevant relationship. The R^2 value representing the goodness of fit to the data is also given.

Table III- Refers to number of fish sampled, size range and SFL/CFL relationship by fram, area where they were captured and gender.

Farm	**Area**	**Gender**	**Number of fish**	**CFL range (cm)**	**$\bar{x}$ CFL (cm)**	**SFL/CFL relationship**	**R^2**
F&F	MA	all	147	195-284	245	$8,15x10^{-1}$CFL+28,732	0,89
		female	60	195-284	237	$8,06x10^{-1}$CFL+31,193	0,90
		male	87	211-279	250	$8,51x10^{-1}$CFL+19,365	0,86
MB	MA	all	168	195-275	243	$9,20x10^{-1}$CFL+2,2316	0,91
		female	80	199-258	236	$9,12x10^{-1}$CFL+3,8353	0,90
		male	88	195-275	248	$9,16x10^{-1}$CFL+3,3577	0,89
	TY	all	522	126-288	238	$9,03x10^{-1}$CFL+5,3499	0,98
		female	146	129-286	222	$8,93x10^{-1}$CFL+7,618	0,98
		male	376	126-288	244	$9,08x10^{-1}$CFL+4,1961	0,98
MFF	TU	all	217	198-281	232	$9,02x10^{-1}$CFL+6,84	0,87
		female	129	198-253	228	$8,90x10^{-1}$CFL+8,2112	0,87
		male	88	204-281	236	$9,15x10^{-1}$CFL+3,5004	0,85

K is generally used as an indicator of the nutritional status of the fish. The farm averages were: F&F-MA, 2,27; MB-MA, 2.10; MB-TY, 2.19 and MFF-TU, 2,33 (table IV). So the farm-area with higher average was MFF-TU and the lowest was MB-MA. When comparing the different areas where the MB fish were caught the average was found to be similar between them (figure 15).

Table IV - Refers to average of K sampled, size range and SFL/CFL relationship by farm, area where they were captured and gender.

$\bar{x}$ K	**F&F(MA)**	**MB(MA)**	**MB(TY)**	**MFF(TU)**
all	2,27	2,11	2,19	2,33
females	2,21	2,12	2,19	2,31
males	2,31	2,11	2,18	2,37

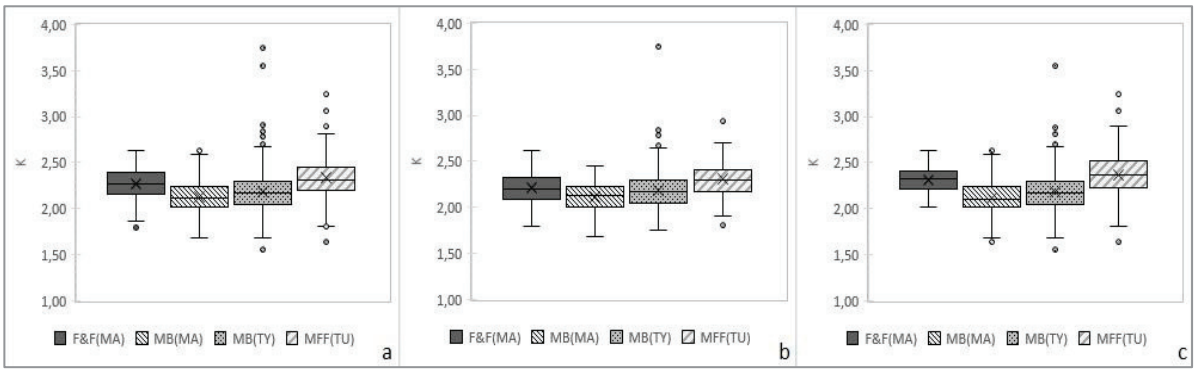

Figure 15- Box-and-Whisker plot of K of the different farms and areas; a- for all; b- only females and c- only males.

There didn't exist a big difference in K between females and males when compared overall, although in the case of the fish from F&F and MFF, K was slightly larger in males.

Observing the distribution of individuals by size classes, it is noteworthy that the most frequent SFL in females was between]210-230] cm (figure 16). Meanwhile in males, the most frequent SFL was between]230-240] cm, except for the MFF, TU figure 16d where the peak for females and males occurred between]210-220] cm. Additionally, it was possible to observe that males had their peak frequency at larger sizes than females except for MFF, TU (figure 16d).

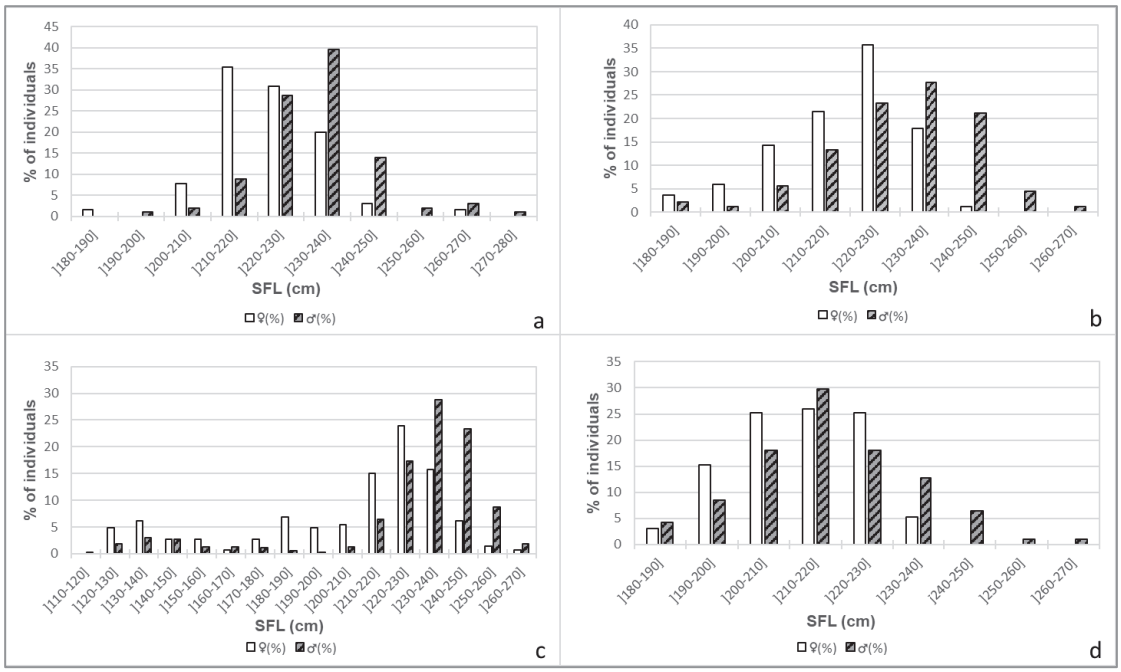

Figure 16- Percentage for females, ♀, and male, ♂ divided for class of 10 cm. For different farms and areas: a-F&F, MA; b- MB, MA; c- MB, TY and d- MFF, TU.

The results obtained by sex for the measurements studied (SFL, CFL and RW) revealed statistical significant differences when comparing catch areas (Kruskal-Wallis test, p-value ≤ 0.05; figure 17a,b,c) and farms (Kruskal-Wallis test, p-value ≤ 0.05; figure 17d,e,f). Analysing the areas under study (MA, TU and TY) showed that, for females, differences in SFL (cm) were observed between MA and TU (Games-Howell test, p-value ≤ 0.05; figure 17a), However, for the SFL (cm) of males, MA differed from TU and TY, and TU differed from TY (Games-Howell test, p-value ≤ 0.05; figure 17a). Concerning the CFL (cm) of females, TU differs from MA and TY (Games-Howell test, p-value ≤ 0.05; figure 17b, while for males MA differs from TU and TY (Games-Howell test, p-value ≤ 0.05; figure 17b. Finally, for RWT (kg) the differences were seen in males. Specifically, these were observed between MA and TU, as well as between MA and TY (Games-Howell test, p-value ≤ 0.05; figure 17c).

When looking at the farms (F&F, MFF and MB), (Kruskal-Wallis test, p-value ≤ 0.05; figure 11d,e,f) in the case of females, differences in SFL (cm) were observed between F&F and MFF and between MB and MFF (Games-Howell test, p-value ≤ 0.05; Figure 11d). Regarding SFL in males, F&F differed from MB and MFF, and MB differed from MFF (Games-Howell test, p-value ≤ 0.05; figure 11d). Similarly, the CFL (cm) of females from F&F differed from those from MFF and MB differed from MFF (Games-Howell test, p-value ≤ 0.05; figure 11e). whilst in the males, F&F, differed from MB and F&F differed from MFF also MB differed from MFF (Games-Howell test, p-value ≤ 0.05; Fig.11e). Lastly, for RWT (kg) the differences in females were between F&F and MB and between F&F and MFF, while in the males, F&F differed from MB and F&F differed from MFF (Games-Howell test, p-value ≤ 0.05; figure 11f).

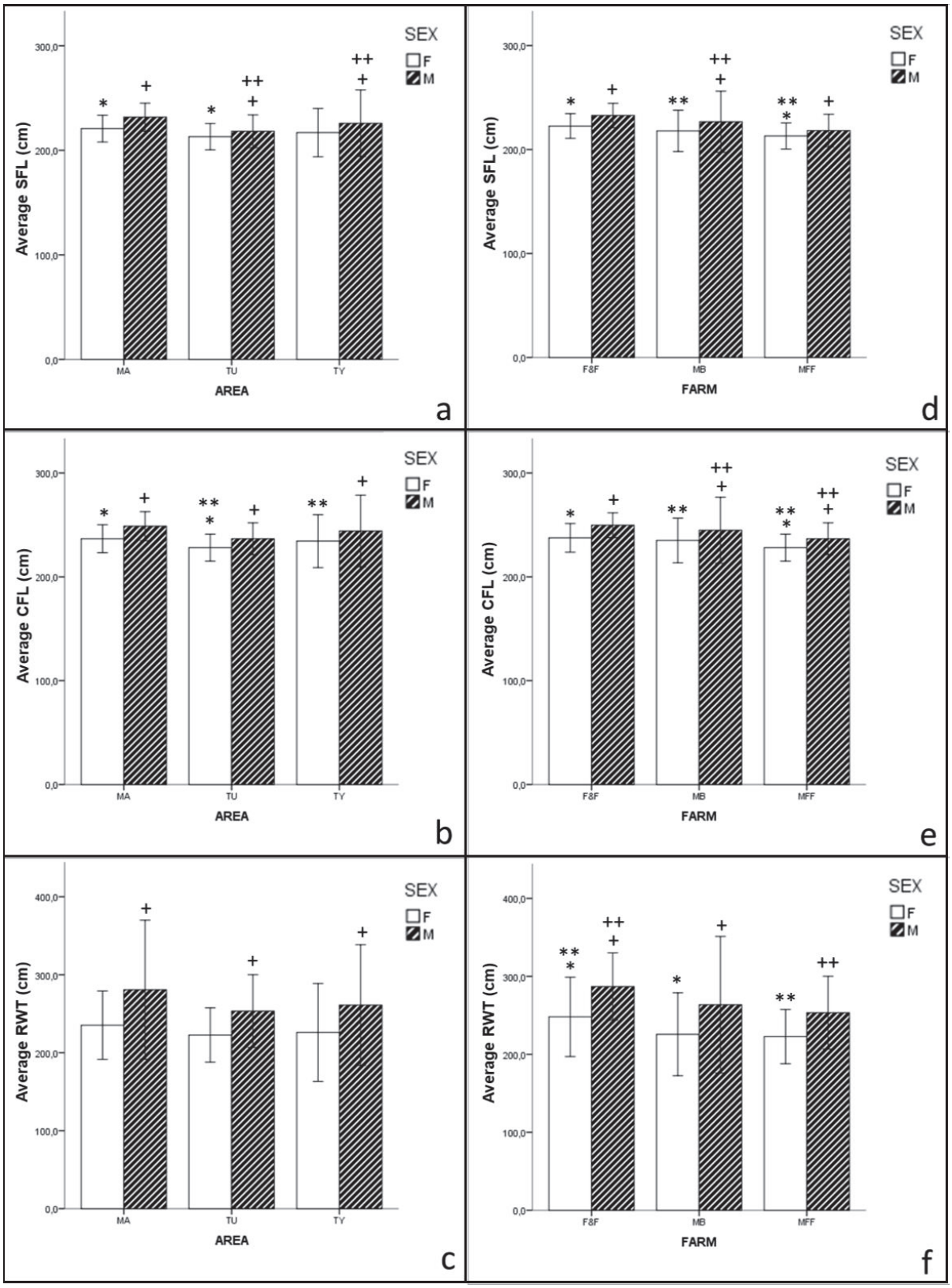

Figure 17- Distribution of measurements (cm) by area, a (SFL), b (CFL) ,c (RW);and Farms, d (SFL), e (CFL) ,f (RW) ; divided by sex. The results are presented as mean±SD.*,**, means statistically significant differences observed between females in different farms or areas. +,++ means statistically significant differences observed between males in different farms or areas.

3.3.2 Otoliths observation

3.3.2.1 *Asteriscus* description

The shape of the *asteriscus* can vary between specimens, but there were no differences between right and left otoliths, as in the *sagittae*.

A blunt projection is present in the anterior margin which divides the *asteriscus* into two areas: a dorsal area with a larger surface than the ventral area (figure 18). The anterior margin has sections that can be rectilinear from the dorsal to the ventral margins.

The posterior margin is curved and shows irregularities (figure 18). This curved section presents a groove all around the dorsal and ventral margin, which divides the otolith in two parts: the first side has a larger sized radius in the external aspect (posterior external margin) and the second has a shorter sized radius that forms the posterior internal margin.

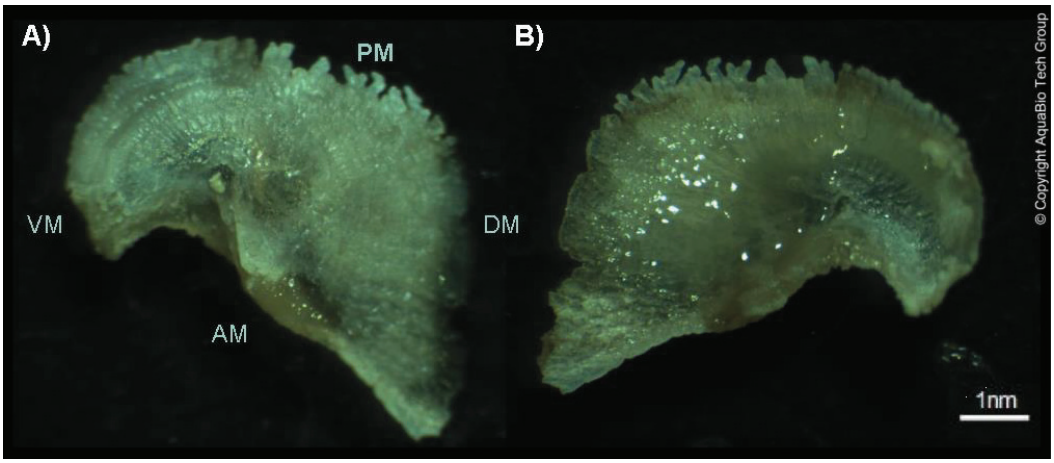

Figure 18- Representative photograph of an *Asteriscus*. A) external and B) internal aspect. AM- anterior margin, PM- posterior margin, DM- dorsal margin and VM- ventral margin.

The internal aspect of the otolith is concave and the external aspect is convex. Small indentations are present in all *asteriscus* (figure 18).

3.3.2.2 *Asteriscus* measurement

The table V shows the relationship between the AW vs SFL and AL vs SFL separated by gender and the average of the measurements, AW and AL. As can be observed the linear

regression are not similar, however the average shows that there is not a difference between gender. It should be noted that the goodness (R^2) of the equations is weak.

Table V- Refers to number of fish sampled, size range, AW/SFL and AL/SFL relationships by gender.

Gender	Number of fish	SFL range (cm)	AW/SFL relationship	R^2	$\bar{x}$ AW (cm)	AL/SFL relationship	R^2	$\bar{x}$ AL (cm)
all	64	180-267	$6{,}20x10^{-3}SFL+3{,}8536$	0,06	5,22	$7{,}40x10^{-3}SFL+2{,}2862$	0,06	3,90
female	34	185-267	$7{,}30x10^{-3}SFL+3{,}5901$	0,08	5,17	$1{,}22x10^{-2}SFL+1{,}2432$	0,18	3,88
male	30	180-262	$4{,}50x10^{-3}SFL+4{,}273$	0,04	5,28	$3{,}30x10^{-3}SFL+3{,}1825$	0,01	3,89

The results achieved for SFL (cm), AW (cm) and AL (cm) when compared between females and males revealed no significative statistical differences (Student's t-test, p-value>0.05; figure 19).

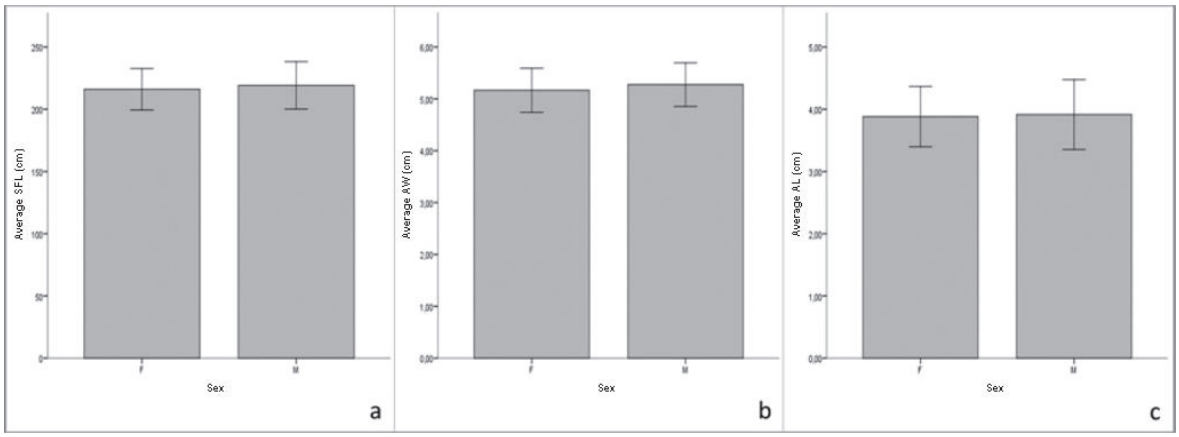

Figure 19- Distribution of measurements (cm)by sex: a (SFL), b (AW) ,c (AL). The results are presented as mean±SD

3.3.2.3 Description of the *lapillus*.

The shape of the *laplilus* can vary between specimens, but there were no differences between right and left otoliths, as in the *sagittae* and the *asteriscus*.

The *lapillus* has a shark teeth shape. The anterior margin of the *lapillus* is oriented towards the front of the fish. Dorsal and ventral margins go from the anterior margin towards the central part of the otolith making a fan-shaped structure. The ventral margin is rounded and the dorsal edge of this structure is notably larger (figure 20).

The otolith is divided in several lobes by radios. The posterior border has a small sulcus which is supposed to enters in contact with the acoustic macula, and extends along the dorsal and ventral margins. All the otolith shows indentations and other irregularities (figure 20).

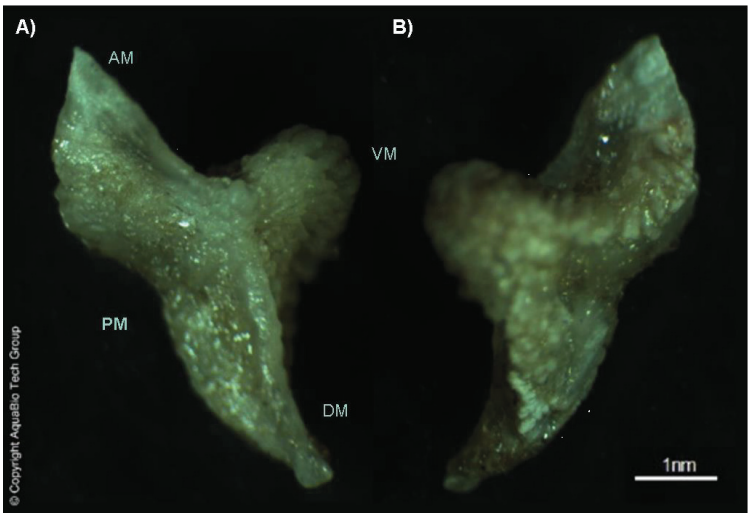

Figure 20 Representative photograph of a *Lapillus*. A) internal and B) external aspect. AM- anterior margin, PM- posterior margin, DM- dorsal margin and VM- ventral margin.

3.3.2.4 *Lapillus* measurement

The table VI shows the relationship between the LW vs SFL and LL vs SFL separated by gender and the average of the measurements, LW and LL. As can be observed the linear regressions are not similar, however the average shows that there is not a difference between gender. It should be noted that the goodness (R^2) of the equations is weak.

Table VI- Refers to number of fish sampled, size range, LW/SFL and LL/SFL relationships by gender

Gender	Number of fish	SFL range (cm)	AW/SFL relationship	R^2	$\bar{x}$ LW (cm)	AL/SFL relationship	R^2	$\bar{x}$ LL (cm)
all	63	151-271	$-1{,}50\times10^{-3}SFL+2{,}7688$	0,01	2,43	$-6{,}50\times10^{-3}SFL+5{,}6084$	0,07	4,18
female	27	151-271	$-6{,}00\times10^{-5}SFL+2{,}6292$	2×10^{-5}	2,62	$-6{,}50\times10^{-3}SFL+5{,}7195$	0,11	4,34
male	36	180-252	$-6{,}00\times10^{-4}SFL+2{,}4649$	0,00	2,34	$-4{,}00\times10^{-3}SFL+4{,}9601$	0,02	4,06

The results achieved for LL (cm) when compared by sex revealed no significative statistical differences (Student's t-test, p-value>0.05; figure 21a,c). However, for the LW (cm) the results showed significative statistical differences between males and females (Student's t-test, t(46)=2,568; p-value≤0.05; figure 21b). In addition, it was possible to observe that females presented higher values of LW when compared with males (figure 21b).

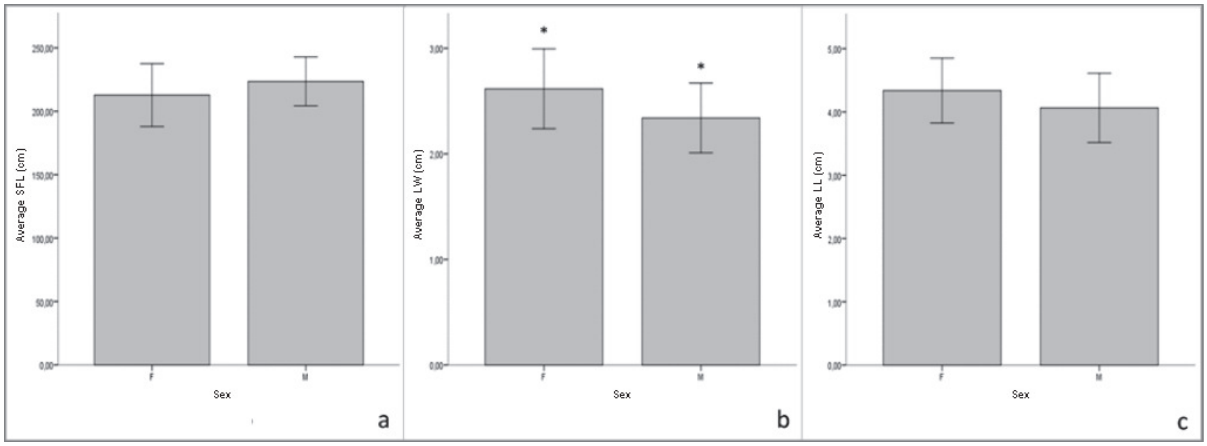

Figure 21- Distribution of measurements (cm) by sex: a (SFL), b (LW), c (LL). (*) denotes significant differences sex. The results are presented as mean±SD

3.4 DISCUSSION

3.4.1 Morphological characteristics

Biometric studies of length-weight relationships are an important tool for growth assessment and as an indicator of animals health. These morphometric relationships are required to obtain conversion factors to be used in fisheries organizations dealing with different types of data. Curiously, in spite of the interest of ICCAT in Atlantic bluefin tuna (*Thunnus thynnus*), few length-weight relationships have yet been defined for this species (Alot, *et al.*, 2011).

The observed differences were mainly due to the calculation of the condition factor of fish. The equation used currently by the Standing Committee on Research and Statistics (SCRS) is annual and does not take into consideration the seasonal variability of this biological parameter (Abid, *et al.*, 2012). Furthermore, the difference in the size range of fish could also explain the difference in the pattern of the two relationships (Abid, *et al.*, 2012).

Fulton's Condition Factor (K), is a biological parameter that probably has biological limits to the values. These limits would vary according to biological constraints related to the implication of the nutritional status of the fish. Below a certain value of K, fish cannot function physiologically and would die of starvation. K is generally used as an indicator of the nutritional status of the fish and is used in the aquaculture industry (Ricker, 1975). Regarding the differences between the values in terms of K, (figure13 and table IV), MFF is the farm that provides a diet with a higher lipidic content to their fish. This can also mean that this farm cachtes bigger fish at moment of harvesting. Younger bluefin tuna do not retain lipids in their body mass like the bigger bluefin tuna. Giménez and Garcia (2005) referred that fattened bluefin tuna under the size of 180 cm are not as overweighted as the larger specimens. The high growth and metabolical rate of smaller tuna means that they may devote most of the energy input to maintaining standard requirements. For this reason, the small ABFT fed to satiety and fattened never reach the degree of overweight observed in larger bluefin tuna. Also, tunas have been described as 'energy speculators' based on their high rates of energy turnover in a nutrient poor pelagic environment, where prey are patchily distributed and feeding success depends upon the ability to find, capture, and process food

items as rapidly as possible (Mourente, *et al.*, 2002). Consequently, when kept in cages, an environment where they have a bigger quantity of nutrients, there will be a faster increase in K than in wild life. This also explains why larger fattened bluefin tuna, whose meat lipid content is higher, are preferred by Japanese consumers (Giménez & García, 2005).

After analysing the results, minor differences were observed between females and males (figure 16). As Rooker *et al.,* (2007) reported, males grow faster than females and reach a greater size at a given age, with these differences becoming more evident at age of 10.

Tunas have relatively high rates of growth. Females can devote considerable energy into egg production (Mourente, *et al.*, 2002, and Karakulak, *et al.*, 2004). There is a lack of documentation on female ABFT feeding during their reproductive migration, from the Strait of Gibraltar to the Mediterranean spawning grounds depending exclusively of fat stores to obtain the energy required for reproduction (Mourente, *et al.*, 2002). In addition, as they age, growing fish invest increasing amount of energy into reproduction and less in growth (Api, *et al.*, 2018). But in cages they are in a completely different situation, with higher availability of food which translates in equal or superior values of K.

K is influenced by age and obviously by the availability of food. Oceanographic data of the western Mediterranean revealed higher levels of productivity indicators in this area which is also very attractive to other large pelagic predators, despite their different feeding habits. Mediterranean waters have a rather low productivity and food resources for ABFT and for this reason this species is more scattered in the Mediterranean than in the North Atlantic, making the north-western Mediterranean a unique feeding hotspot for ABFT (Bauer, *et al.*, 2017). So, the statistical significant differences (figure 15, a, b and c) between different areas can be translated by the lack of food on the Mediterranean Sea which results on a higher spending of energy to search for food. This fact might explain the migratory behaviour of ABFT and the consequent loss of energy that is redirected to their growth. In fact, ABFT are present in high-use areas throughout the year, particularly during summer-autumn, when thermal stratification is strong (Bauer, *et al.*, 2017). This observation is likely linked to the local seasonal migrations of sardines and anchovies to coastal waters during summer that ABFT appear to follow (Bauer, *et al.*, 2017). As proved by the results showed in figure 15 a, b and c, the areas can have an impact on the fattening process of the farms. To ensure the quality of these data a study would have to be made before the harvesting of the Tuna. The

statistical significant differences observed between farms (figure15 d, e, f) result from the different feeding processes applied in the cages and the different diets. These differences can be justified by the quality and quantity of food given to the fish during harvesting that might reduce the stress of farming, increasing the estimated growth from capture to the end of fattening (Api, *et al.*, 2018).

3.4.2 Otoliths measurement

Several studies have demonstrated that trace elements and stable isotopic composition of otoliths, can serve as natural markers of environmental history for individual fishes, in either freshwater or marine environments (Luque, *et al.*, 2016).

The otolith samples used in this analysis were obtained during fishery season. It was expected that the otoliths would reflect the selectivity of Malta, of the three fisheries, MB, MFF, and F&F from which they were obtained. The otoliths extraction is not an easy process. These structures are extremely fragile as most of the times they can be easily broken.

In this study were analysed the morphological characteristics of *asteriscus* and *lapillus*. The shape of the *asteriscus* and *lapillus* can change between specimens, but most of the studies show that there do not exist differences between the right and left otoliths (Hernández *et al.*, 2008 and Bar *et al.*, 2015).

Hernández *et al.* (2008) demonstrated that the size of the *sagittae* or the *asteriscus* of *Thunnus albacores* can determine either the fish is a female or a male. Differences in size between gender are not statistically significant, as observed in this study. Only *lapillus* length (cm) (figure 21) showed significative statistical differences between males and females. However, the results are not reliable, as is showed in Table V and VI, by the R^2.

One possible justification for R^2 low values is related to the fact that most of the *asteriscus* and *lapillus* were broken. These structures as referred above are very fragile. The hole where the otoliths were found was most of the times smaller than the height in case of the *sagittae.* To remove the otolith without any damage the membrane needs to be pulled out together with the structure. To clean the *sagittae* is necessary to remove the membrane very carefully to prevent damaging the *asteriscus.* The other explanation for the broken *asteriscus* and

lapillus is the way that the vestibular labyrinth was stored. These structures showed be stored in proper freezer tubes instead of a bag.

3.5 CONCLUSION AND FUTURE PERSPECTIVES

In conclusion, it is necessary to periodically update the equations used by the SCRS to take into consideration the seasonal conditions and the different captivity conditions.

It is clear that there are slightly differences of weight and length, between females and males, which can be related to the area where they were caught and to the differences between the feeding processes and the diets of different farms.

Also, differences between sexes for the otoliths measured were not statistical significant. Although some authors demonstrated that it is possible to identify the sex of the tuna by the size of the *sagittae* or the *asteriscus*, it was not the case in this study, possibly due to the difficulty in extracting and processing of the samples.

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