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ESCUELA INTERNACIONAL DE DOCTORADO

TESIS DOCTORAL

Traceability markers in Atlantic bluefin tuna (*Thunnus thynnus*)

Búsqueda de marcadores de trazabilidad en ejemplares de atún rojo del Atlántico (*Thunnus thynnus*)

Dª. Inmaculada Concepción Salvat Leal

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Autora: Dª. Inmaculada Concepción Salvat Leal

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Declaración de autoría y originalidad

UNIVERSIDAD DE MURCIA

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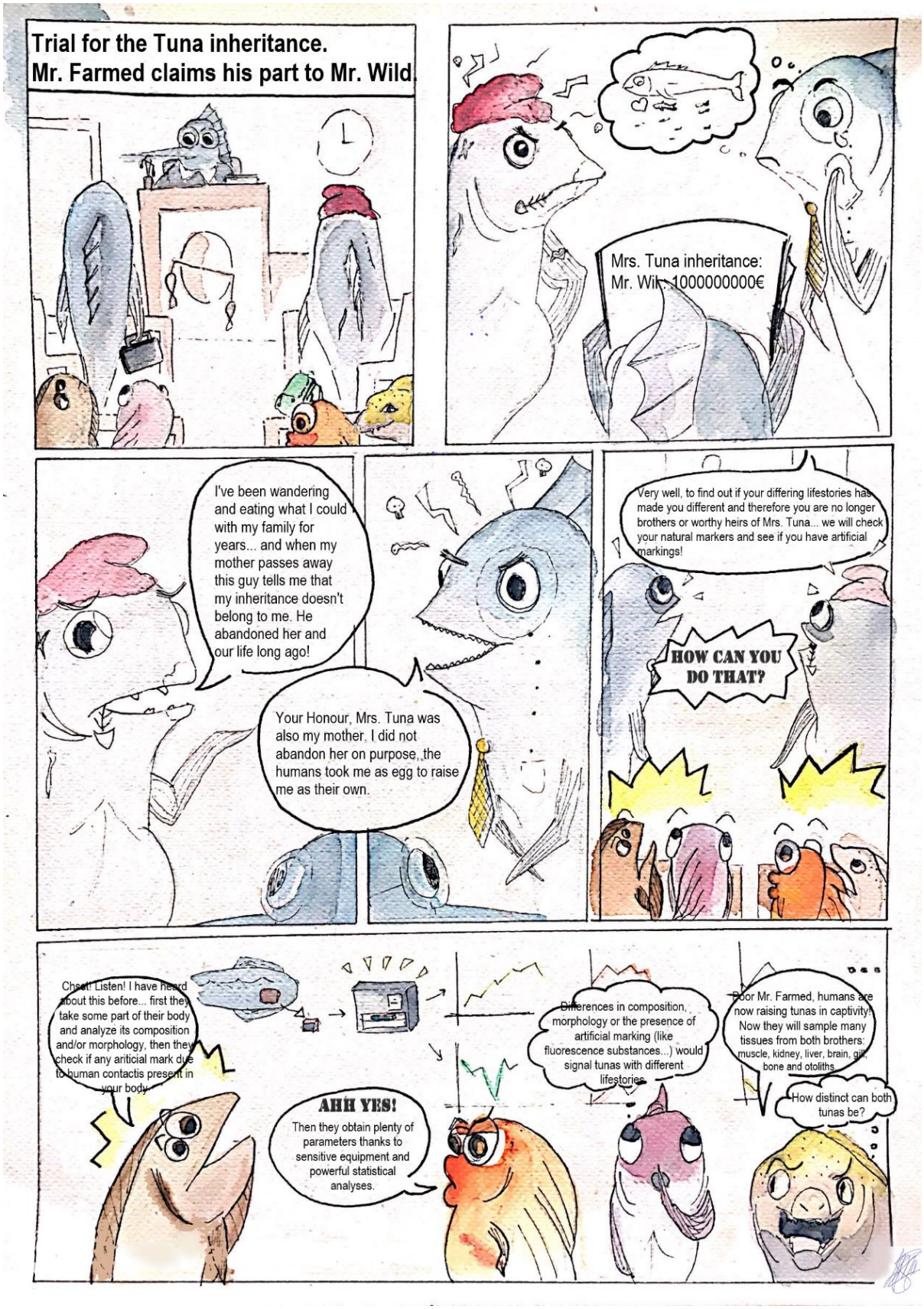

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Qué inapropiado llamar a este planeta Tierra *cuando es claramente un océano*

.

Arthur C. Clarke

Resumen en español

Estructura de la tesis

Esta Tesis presenta las principales características del atún rojo del Atlántico (ABFT, *Thunnus thynnus*), su cría en cautividad, y posibles marcadores de su trazabilidad. Nuestro objetivo general fue discriminar entre lotes de ABFT utilizando técnicas de identificación y marcaje no invasivos. Con este propósito, el **Cuerpo Principal** de la tesis se ha estructurado en tres Secciones con ocho Capítulos estructurados como publicaciones científicas. En las dos primeras Secciones consideramos el uso de trazadores naturales (de composición elemental y morfología), mientras que en la Tercera Sección probamos dos métodos de marcaje químico. Algunos de estos estudios nunca se han llevado a cabo en ABFT, como la composición química de branquias, huesos y otolitos (**Capítulos II y III**), la morfometría y/o asimetría de los otolitos **(Capítulos IV y V**), o la presencia de vaterita (**Capítulo VI**); todos con la finalidad de discriminar lotes. Además, es la primera vez que se prueba el marcaje artificial en masa en esta especie (**Capítulos VII y VIII),** siendo el más similar el estudio de Wexler et al. (2003), en el que examinaron los efectos de la inyección de oxitetraciclina en atún de aleta amarilla (*Thunnus albacares*).

Material y métodos general

Para el desarrollo de esta Tesis, en primer lugar, se capturaron ABFT con un peso inferior a 1500 g en el año 2018. Los ABFT fueron de tres lotes diferentes en los **Capítulos I y II**: i) tanques terrestres (llamados de cultivo), ii) jaulas marinas y ii) silvestres; en los **Capítulos III-VI** fueron sólo de dos lotes diferentes: i) silvestre y ii) cultivo. Estos lotes crecieron en diferentes condiciones de agua y con distinta dieta.Los resultados obtenidos durante esta Tesis fueron sometidos a análisis estadístico utilizando el software SPSS, excepto el Capítulo V, en el que algunos datos fueron procesados a través de Rstudio. A lo largo de la Tesis se utilizaron algunos análisis estadísticos clásicos, sin embargo, también se utilizaron análisis multivariantes, como el Análisis de Componentes Principales (PCA, cuyo objetivo es reducir las dimensiones de datos, generando nuevas variables llamadas componentes que pueden ser útiles como variables criterio o

predictoras) y el Análisis Canónico Discriminante (DCA, su objetivo es determinar si en función de las variables disponibles los grupos quedan suficientemente discriminados). Además, en algunos Capítulos se utilizaron fórmulas específicas para el cálculo de algunos índices (como los de asimetría).

Resultados

En la **Primera Sección de la Tesis**, contemplamos la composición de diferentes tejidos como etiquetas naturales y no invasivas. En el **Capítulo I**, se tomaron muestras de 75 especímenes ABFT que pesaban menos de 1000g (24, 22 y 29 especímenes respectivamente para los lotes de tanques terrestres, jaulas marinas y silvestres). En total se tomaron 74 muestras de hígado, 37 de riñón, 73 de músculo, y 68 de cerebro y se analizó su composición mineral. Más tarde, las muestras de tejido blando tomadas (hígado, riñón, músculo y cerebro) se congelaron inmediatamente y se almacenaron a -20 ◦C hasta su análisis. Tanto en este Capítulo como en el resto de la **Sección I**, se hizo un análisis mineral de y se sometieron 0,5 a 1,0 g de las muestras a digestión ácida para la determinación de concentraciones de elementos inorgánicos utilizando un sistema óptico de plasma acoplado inductivamente. espectrofotómetro de misión (ICP-OES). El cobre fue el único elemento con diferencias estadísticamente significativas entre grupos en todos los tejidos (hígado, riñón, músculo y cerebro). Según la literatura, la alimentación es la principal fuente de Cu y por tanto estas diferencias se deberían a la diferente dieta de los lotes. En el DCA, tanto el riñón como el cerebro fueron los tejidos que mejor discriminaron entre grupos, con más del 80% de éxito. En resumen, la composición elemental esencial en los tejidos blandos en ABFT podría usarse para discriminar diferentes lotes de atún, especialmente porque DCA puede generar fórmulas para identificar el posible lote de especímenes. En relación con estos resultados, en el estudio del **Capítulo II** de la tesis se tomaron muestras de branquia y hueso de 75 y 74 ABFT respectivamente para analizar su composición mineral. Estos son tejidos de menor valor comercial. Nuevamente, el cobre fue el único elemento con diferencias estadísticamente significativas entre los grupos tanto en branquias como en huesos. En este estudio, el hueso fue el tejido con mayores diferencias estadísticamente significativas entre grupos, pero las branquias fue el que tuvo mayor éxito en la discriminación (80%). En estos tejidos, silvestres fue el lote

mejor discriminado. Tanto en el **Capítulo I como en el II**, el PCA arroja nula (**Capítulo I**) o baja (**Capítulo II**) validez, siendo sus resultados no concluyentes para diferenciar grupos. Por lo tanto, en los siguientes capítulos, si se hicieron análisis multivariantes para la discriminación de grupos, sólo se utilizó el DCA. Estas diferencias en Cu, mayores en tejidos de ejemplares silvestres tanto en el **Capítulo I** (excepto el riñón) **como en el II**, podrían deberse a su dieta más rica y variada que incluye pequeños peces pelágicos, camarones, cefalópodos y crustáceos. Pues en cefalópodos y crustáceos el Cu juega un papel esencial en las funciones biológicas. En **el último Capítulo de esta Sección** y para los dos siguientes (**Capítulos III-V**), se muestrearon 101 ejemplares de ABFT de 2018 con un peso entre 100 y 1500 g, y se analizó la composición mineral de 101 otolitos (del lado derecho). Encontramos diferencias estadísticamente significativas en los niveles de Na, Mg, P, Sr de los otolitos que fueron mayores en los atunes de cultivo y Rb, que fue mayor en los atunes silvestres. En el DCA, P y Sr por sí solos tuvieron más del 75% de éxito en la discriminación. En resumen, Sr y P son los elementos que podrían impulsar la discriminación de lotes en aguas del Mediterráneo occidental (específicamente de la Bahía de Mazarrón) mediante el perfil de composición de otolitos. Sin embargo, debemos manejar estos resultados con precaución, ya que la química de los otolitos está determinada por interacciones complejas entre la genética, la fisiología, el medio ambiente, los cambios ontogénicos e incluso el manejo postmortem de las muestras.

En **la Segunda Sección de la Tesis**, de los mismos 101 ejemplares de los que se analizó la composición química, se analizaron la morfometría y un paramétro relacionado como la asimetría (diferencias morfológicas entre los otolitos derecho e izquierdo de un individuo) por pares de otolitos: 202 otolitos (101 derechos y 101 izquierdos). En el análisis morfológico de los **Capítulos IV-VI**, los otolitos se colocaron en un campo oscuro y se observaron (aumentos x1 y x2) bajo un estereomicroscopio conectado a un ordenador. La adquisición, procesamiento y análisis de imágenes se realizaron utilizando el software Otolab, en el que se midieron hasta siete parámetros estructurales. Además, en el **Capítulo VI**, en busca de identificar las fases minerales de la $CaCO_3$ de los

otolitos, se realizó una difracción de rayos X tras la pulverización de los mismos. Para este **último Capítulo de la Sección II** se muestrearon 46 ejemplares ABFT con un peso inferior a 1200 g: i) 23 silvestres y ii) 23 provenientes de tanques terrestres. Aquí, se analizaron 90 otolitos (46 derechos y 44 izquierdos) para detectar la presencia de vaterita y la morfología dependiendo de la composición cristalina. La vaterita es un polimorfo del aragonito ($CaCO_3$ forma normal en la mayoría de los otolitos), que se ha relacionado en la literatura con morfologías anormales, incluso apariencia de asimetría y deterioro fisiológico. En el **primer Capítulo** de esta Sección, **Capítulo IV**, encontramos diferencias entre lotes en un parámetro para los otolitos derechos y en cinco para los izquierdos. Las diferencias de lado, podrían explicarse debido a patologías de los otolitos (problemas de calcificación o variaciones de deposición…). Por otro lado, las diferencias por lote podrían deberse a las condiciones de cría, dado que la morfometría de los otolitos depende del genotipo y del ambiente del pez. En el DCA se seleccionaron el peso del otolito (bilateralmente) y la excentricidad (en el otolito izquierdo) como variables morfológicas discriminantes entre lotes. En los otolitos derechos se clasificó con éxito el 63,4% de los túnidos, mientras que en los otolitos izquierdos esto ocurrió con el 57,4%. Finalmente, las fórmulas obtenidas en este análisis se pueden aplicar a túnidos no identificados, dado que, al introducir los valores de los rasgos en las fórmulas obtenidas, el resultado puede indicarnos el probable lote del atún. En el DCA, el peso del otolito fue seleccionado como rasgo discriminante en otolitos derecho e izquierdo, siendo un rasgo confiable para la discriminación grupal en ambos lados. Sin embargo, en los otolitos izquierdos, también se seleccionó la excentricidad. El **Capítulo V**, fue el primer estudio que analizó la presencia de asimetría en otolitos ABFT. Concretamente, se encontraron dos tipos de asimetría en esta especie con claras diferencias entre los lotes presentados: silvestre y cultivo. El peso, la longitud, el ancho, la excentricidad y la compacidad de los otolitos fueron rasgos asimétricos en ambos lotes y, por lo tanto, comparables. Específicamente, la excentricidad tenía tanto antisimetría (AS) como asimetría direccional (DA) en los dos lotes. Tanto AS como DA han sido poco documentados en la literatura por sí solos. En general, existe un vínculo descrito entre los altos valores de asimetría y las condiciones de cría; sin embargo, las condiciones de vida silvestre

también pueden causar asimetría, pues los problemas ambientales que causan estrés (es decir, contaminación, tráfico marítimo...) son los desencadenantes de asimetría más mencionados. En la comparación de nuestro estudio, los especímenes de cultivo mostraron una mayor asimetría, lo cual es constante con los hallazgos de la literatura y los factores más probables en nuestro estudio para esto serían diferencias en la química del agua, la dieta y factores estresantes ambientales (como acumulación de sustancias, cambios de agua, oxigenación, fotoperíodo o densidades de población). En el **Capítulo VI** se encontraron morfologías anormales y vaterita en otolitos juveniles de ABFT. Se realizó un ensayo para ver la posible alteración de $CaCO_3$ debido al protocolo estándar de limpieza de otolitos con agua purificada, pero ni esta limpieza ni el depósito de vaterita se relacionaron con las morfologías anormales encontradas. En cuanto a la presencia de vaterita (26,67% en cultivo vs. 4,55% en silvestres) y la cantidad de vaterita por otolito (88,15% en cultivo frente a 12% en silvestres), fueron mayores en los atunes de cultivo. Además, se encontraron diferencias morfológicas entre otolitos con y sin vaterita en un mismo lote.

Ahora vamos a la **Tercera Sección de la Tesis**, aquí desarrollamos dos marcajes artificiales en ABFT de tanque terrestres: en el **Capítulo VII**, se inyectó oxitetraciclina en el músculo dorsal de juveniles ABFT en dos concentraciones (100 y 200 ppm), se muestrearon 15 especímenes, y se procesó un otolito de cada individuo. Finalmente, en el **Capítulo VIII**, se muestrearon 41 larvas de ABFT y se procesaron todos sus otolitos (dos por individuo). En cuento al procesamiento de los otolitos, en el **Capítulo VII**, estos se fijaron mediante adhesivo de montaje en un portaobjetos de microscopio, y luego pulidos para la visualización de las marcas artificiales. Debido a la naturaleza de los marcadores (fluorocromos), en los Capítulos **VII y VIII** se usó un microscopio de fluorescencia. En primer lugar, en el **Capítulo VII** descubrimos que todos los otolitos examinados tenían una marca verde visible, teniendo las dos concentraciones probadas un 100% de éxito de marcado. Además, no hubo diferencias estadísticamente significativas en la intensidad de la marca entre concentraciones, por lo que se recomendaría el uso de la concentración más baja de oxitetraciclina. Sin embargo, encontramos baja visibilidad de las marcas

en algunas muestras, lo que podría deberse a inyecciones ineficientes (subcutáneas en lugar de intramusculares) o a un marcado temprano (el etiquetado en etapas posteriores genera una marca más duradera). Finalmente, en el **Capítulo VIII** probamos el marcado rojo alizarina con 3h de inmersión y 50 ppm en huevos ABFT. Este marcado masivo fue exitoso, pues obtuvimos un 100% de tasa de marcado. Además, el rojo alizarina es conveniente y rentable para marcar peces en masa y, comparando estos resultados con la experiencia anterior en el marcado artificial de atún (**Capítulo VII**), tuvo una aplicación más sencilla: no fue necesario manipular directamente los atunes y la marca era más visible, siendo innecesaria el lijado de las muestras. Hasta donde sabemos, nunca se han realizado experimentos de marcaje de esta clase en ABFT, lo que hace que sea importante continuar con esta línea de investigación.

Conclusiones

1. Para los trazadores químicos naturales, el riñón fue el tejido que mejor discriminó a través de su perfil químico entre los lotes de ABFT, aunque el músculo y el otolito también fueron buenos discriminadores y estña mñas extendido su uso. Se recomienda incluir en el análisis de estos tejidos elementos con excelente discriminación: Mn, S y Sr), pero también otros elementos con buena discriminación y presentes en todas las muestras de ABFT: Cu, Fe, Mg, P y Zn.

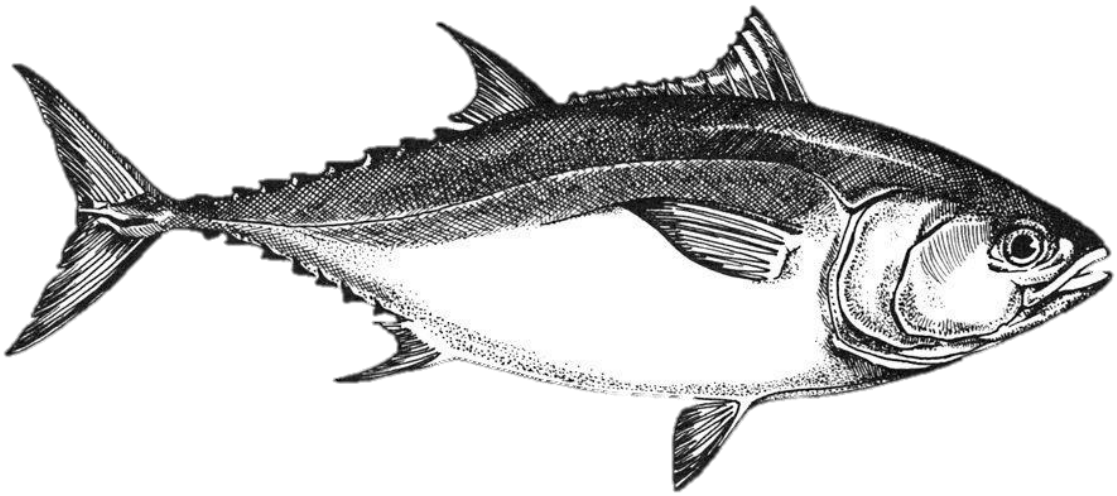
2. Para los trazadores morfométricos naturales, la morfometría de los otolitos dio los resultados discriminatorios más claros, pero combinada con asimetría y vaterita es posible obtener información del bienestar de los ejemplares.

3. En general, los otolitos se erigen como los mejores trazadores naturales, especialmente para grupos con regímenes de vida totalmente diferentes. Si es posible, fomentamos el uso tanto de la morfometría como de la química de los otolitos ya que ambos análisis se pueden realizar en las mismas muestras.

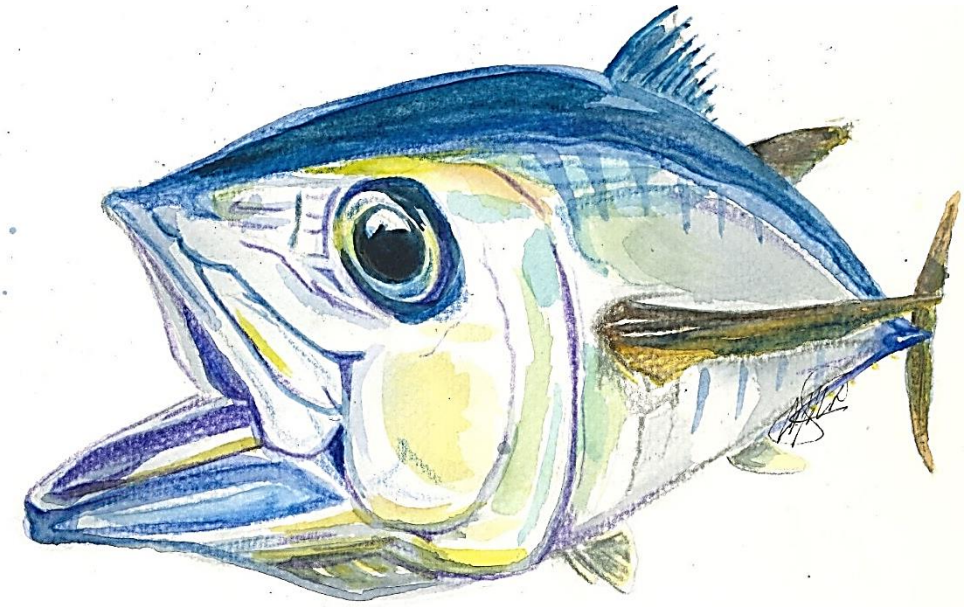
4. Entre los métodos de marcado artificial probados, el uso de la inmersión de huevos en ARS fue el más exitoso.

5. Finalmente, el otolito sería el tejido de elección para discriminar entre lotes de ABFT porque permite el marcaje artificial en estado de huevo o larva, pero

también el análisis de trazadores naturales (químicos o morfométricos) en individuos de mayor edad.



I. Table of Contents



II. Thesis Structure

The present Doctoral Thesis starts with a General Introduction which dives in the Atlantic bluefin tuna biological, commercial and production backgrounds. Then, the General Objectives of the Thesis are presented, as well as the Specific Objectives by Chapter.

The Main Scientific Body of the Thesis is composed by eight chapters, referred in the text with roman numbers, which correspond to the same scientific original studies. These Chapters are grouped in three Sections preserving the three research lines followed during the thesis experimental set up: in the two first sections we studied natural tracers (chemical and morphological, respectively), and in the last section we studied artificial markings. The Chapter I has already been published on the *Journal of Food Composition and Analysis* (Impact Factor: 4.520); the Chapter II and VIII are under review in other international scientific journals. The Chapters III, VI and VII are being prepared to be also sent to international scientific journals. Each of these chapters are divided into the common sections found in a scientific study: Introduction, Material & Methods, Results, Discussion, Conclusions and References. Some of them are redacted in the form of Short Communications (Chapters IV and VII) and therefore the sections Results and Discussion are presented as one. All the chapters and the

general sections are written in the common language for scientific publications and studies (English).

After the eight chapters, a General Discussion is presented to synthetize the findings of the studies, and present the main ideas of the Thesis, answering to the general objectives of the same. In this part we include three parts, discussing the chapters by sections, some recommendations for future studies and a comparison of the methods followed among the three Sections. Finally, the conclusions by chapter and some General Conclusions of the Thesis are presented.

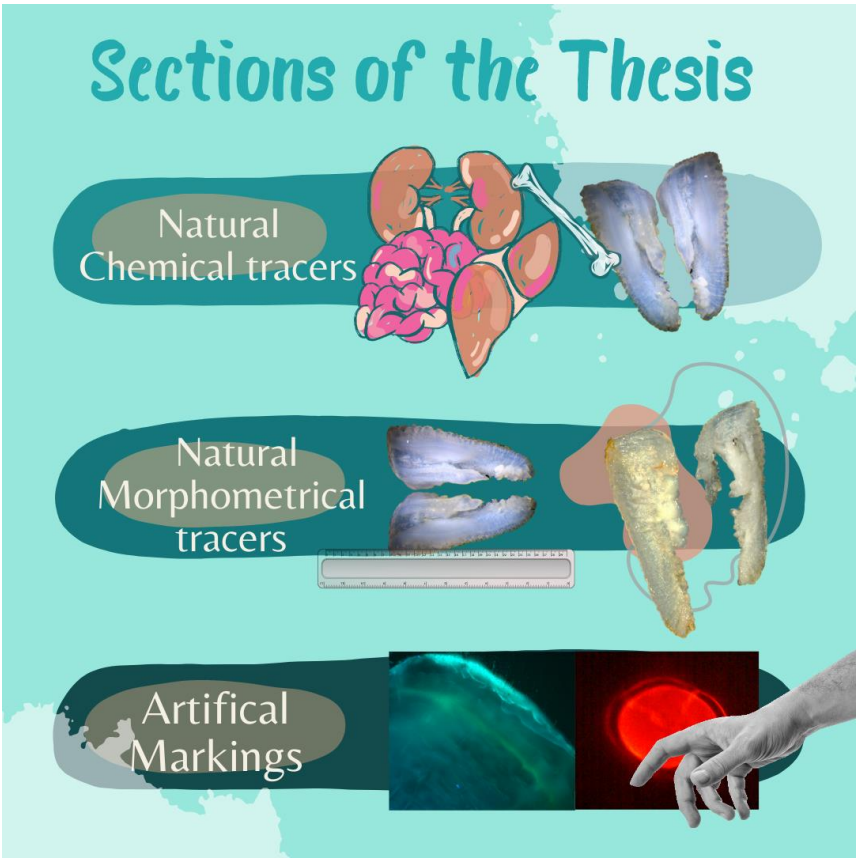


Figure II.1. Scheme of the three Sections of the Thesis, the two first study natural tracers (chemical and morphological), and the last artificial (showed as a human hand intervening) markings: I. Natural chemical tracers, II. Natural Morphometrical tracers and III. Artificial markings. The photographs are taken from the studies of the Main Scientific Body of the Thesis. Source: Inma Salvat-Leal.

III. Introduction

III.I. Atlantic bluefin tuna features

III.I.I. Phylogeny

Tuna belongs to the Scombridae family, subfamily Scombrinae, and Thunini tribe (**Figure III.1**) (Graham & Dickson, 2004). Within the Thunini tribe, we find 15 species distributed in 5 different genus. The genus *Thunnus,* made up of 7 widely distributed tunas, are mostly considered tropical and from warm waters. However, three species from this genus have adapted to colder waters, and are the largest species found in the Thunini tribe (Collette et al., 2001): Atlantic bluefin tuna (ABFT, *Thunnus thynnus*), Pacific bluefin tuna (PBFT, *Thunnus orientalis*), and southern bluefin tuna *(*SBFT, *Thunnus macoyii,*). They weigh up to 600 kg in the case of ABFT (Cort, 2007), 555 kg for the PBFT (Foreman & Ishizuka, 1990) and 260 kg for the SBFT (Nakamura, 1990). These species are unique among teleosts due to their cardio-respiratory and cardiovascular physiology, which provides blood flow through low resistance gill and systemic vascular beds at a significant pressure to ensure adequate tissue fluid exchange, in contrast to most marine species (Bushnell & Jones, 1994). This characteristic sustains their high metabolic rate and endothermic capacity, which allowed them to increase their internal temperature range by preserving more than 95% of the heat produced by the muscle (Blank et al., 2004; Graham & Dickson, 2004; Kubo et al., 2008; Block et al., 2001, 2005; Reglero et al., 2014; Cermeño et al., 2015).

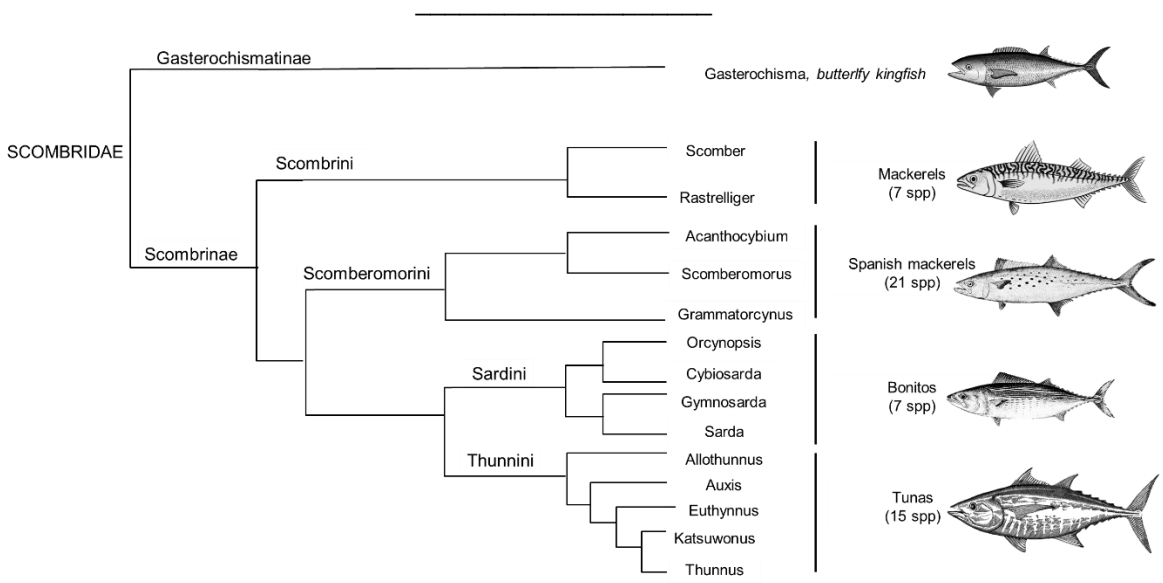


Figure III.1. Scombridae and tunas' phylogeny. Modified from: Graham & Dickson, 2000, 2004; Colette et al., 2001; Juan-Jordá et al., 2013

Adult ABFT have a high energetic consumption (more than 30% of their body mass, daily, according to Kitchell et al., 1978), derived from their high metabolic rate. This exacerbated consumption arises from the continuous swimming necessity to maintain gill ventilation (called ram ventilation) and the hydrodynamic lift that stabilizes them and maintains their position in the water column (Korsmeyer et al., 1996). All of that is sustained by their high aerobic capacity (they consume 2-5 times more oxygen than other teleosts) and makes their digestion ratios high, so that the intestinal emptying (around 12 hours) is 4-5 times faster than other piscivores of comparable size (Olson & Boggs, 1986).

III.I.II. Historical and social importance

The ABFT is the most iconic species of the Scombridae family, being the only species from the genus *Thunnus* successfully farmed in captivity in the Mediterranean (Blanco, 2018). It is a species of great ecological, recreational and commercial importance both in Atlantic and Mediterranean ecosystems, subject to rigorous fishing controls and object of interest due to its conservation status. Therefore, both from a commercial and conservational point of view, the traceability of ABFT products is something increasingly demanded and required.

This species has been exploited in the Mediterranean Sea for thousands of years (Mather et al., 1995; Doumenge, 1998), but lately ABFT fishing has become a highly profitable activity, particularly with the development of the sushi-sashimi market opportunity in Japan during the 1980s (Fromentin & Ravier, 2004; Porch, 2005). This new opportunity has increased the high-quality fish demand, attaining the ABFT very high prices in recent years (Fromentin & Powers, 2005).

This situation, led to great fishing efforts (Rodríguez-Roda, 1964; Rey, 1999; Fromentin & Powers, 2005; Morais et al., 2011) and caused tension between various fishing entities, including national and international administrations, fishermen, national scientists and NGOs (Fromentin & Ravier, 2004; Porch, 2005), and ended in the reduction of breeding populations in the Western Atlantic during the last 30 years (National Research Council, 1994; Sissenwine et al., 1998). Furthermore, there were great uncertainties around the total catches and their size composition for many Mediterranean and East Atlantic ABFT fisheries since the late 1990s, that also contributed in the reduction of the populations (ICCAT, 2005). To improve this situation, in 2007 the International Commission for the Conservation of Atlantic Tunas (ICCAT) coordinated a recovery plan for the species. The protection programs established included: allowing a limited capture period (Cort & Martínez, 2010), limiting catches through a quota system (by ICCAT in 1999, revised in 2005), and increasing the minimum age of capture by 30 kg in the Mediterranean and West Atlantic (Fromentin & Powers, 2005; Anonymous, 2007). Fortunately, the stock seems to be gradually recovering (Anonymous, 2017), mainly as a result of these protection programs. Despite this, there is a general agreement that the future of ABFT depends on both the recovery of their natural population and and the ‘domestication’ of ABFT (De la Gándara et al., 2016; FAO 2023a).

III.I.III. Distribution and migration

The ABFT is a highly migratory species with a wide geographical distribution, being able to conduct transatlantic migrations and being found from Norway to Canada to more equatorial Waters of the Atlantic Ocean and adjacent seas like

the Mediterranean, the Gulf of Mexico and the Black Sea (Fromentin & Powers, 2005; Teo & Boustany, 2016).

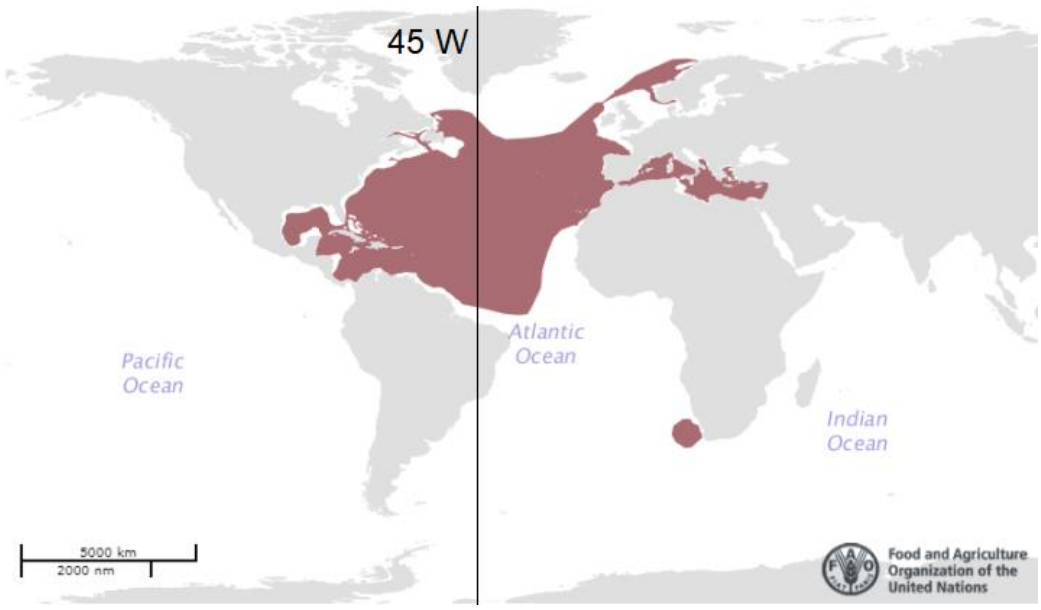


Figure III.2. ABFT distribution (in red). Source: FAO GeoInfo (2023b). The vertical line represents the stock division of ABFT's population based on 45º W meridian

For its management, ICCAT divides its population into two stocks (**Figure III.2**), one to the east and the other to the west of the 45ºW meridian (Fromentin & Powers, 2005; Carlsson et al., 2007), although many authors have documented in the last years a mixture of both stocks (Fromentin & Powers, 2005; Reglero et al., 2014). Thanks to the use of electronic satellite marking, it has been possible to map the habitats of the ABFT (Block et al., 2005), and to record its movements and oceanographic preferences (DeLong et al., 1992; Metcalfe & Arnold, 1997; Block et al., 1998a,1998b; Lutcavage et al., 1999; Kitagawa et al., 2000; Koudil et al., 2000; Le Boeuf et al., 2000; Marcinek et al., 2001; Gunn & Block, 2001), concluding that adults perform two annual migrations: (1) one at the end of spring, which goes from feeding areas to reproductive areas, being from the North Atlantic to the Gulf of Mexico for the western stock, and to the Mediterranean for the eastern stock; (2) another migration at the end of summer towards feeding areas in the north Atlantic, where both stocks overlap (Block et al., 2005; Fromentin & Powers, 2005; Aranda et al., 2013; Reglero et al., 2014).

Migration to the Mediterranean is seasonal and progressive, and for five decades it has been verified that tuna cross the Strait of Gibraltar in May and June, and

leave the area between July and August (Rodríguez-Roda, 1964). Spawning areas are persistent and present temporary differences. In the eastern Mediterranean (around Cyprus), the spawning occurs from May to June (Oray & Karakulak, 2005); and in the western zone (Balearic Islands and central Sicily, Malta and Tunisia), from June to July (Sella, 1924; Sanzo, 1932; Duclerc et al., 1973; Sarà, 1973; De la Serna & Alot, 1992; Susca et al., 2001; Medina et al., 2002; Corriero et al., 2003; García et al., 2005; Rooker et al., 2007; Alemany et al., 2010; Koched et al., 2013; Zarrad et al., 2013). This temporal variation is due to a progressive increase in the surface temperature from east to west, since spawning is triggered in waters with temperatures above 20ºC (Alemany et al., 2010; Reglero et al., 2018).

III. II. Aquaculture

At a global level, the ABFT is the most important tunid and one of the most appreciated species in the Mediterranean Sea (Majkowski et al., 2011), but its aquaculture is relatively recent (Mylonas et al., 2010; Benetti et al., 2016). For this species the separation between aquaculture (**Figure III.3**) and fishery (**Figure III.4**) is very complex, since its traditional culture is based on fattening extractive-fishing captured wild adults in sea cages during short-term periods previous to commercialization (De la Gándara et al., 2016). During these periods, various small defrosted pelagic species are used for feeding, including: anchovy (*Engraulis encrasicolus*), pilchard (*Sardina pilchardus)*, sardinella *(Sardinella aurita*), herring (*Clupea harengus*), mackerel *(Scomber scombrus*), horse mackerel (*Trachurus* spp.), chub mackerel (*Scomber* japonicus), bogue (*Boops boops)* and some cephalopods (Vita et al., 2004) like short-finned squid (*Illex s*pp).

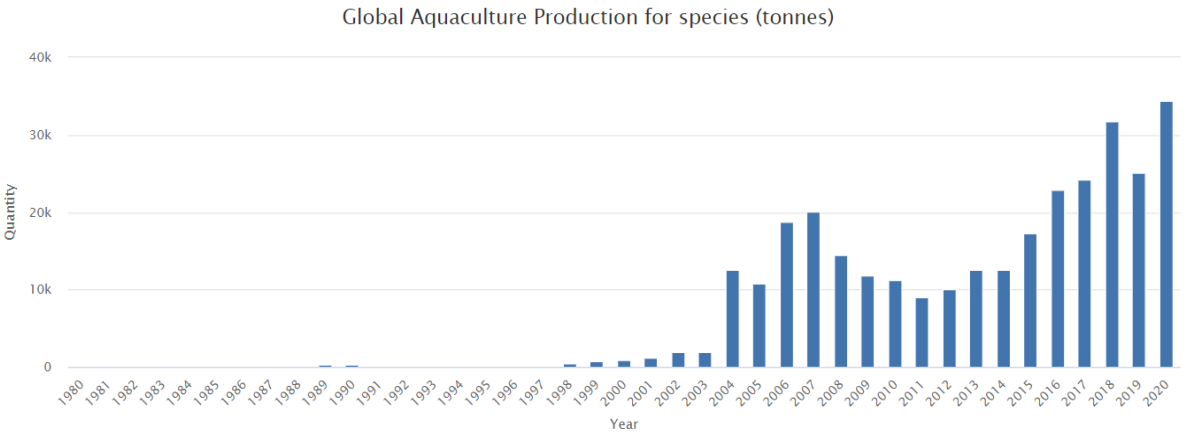


Figure III.3. Global ABFT aquaculture production per year. Source: FAO, 2023c

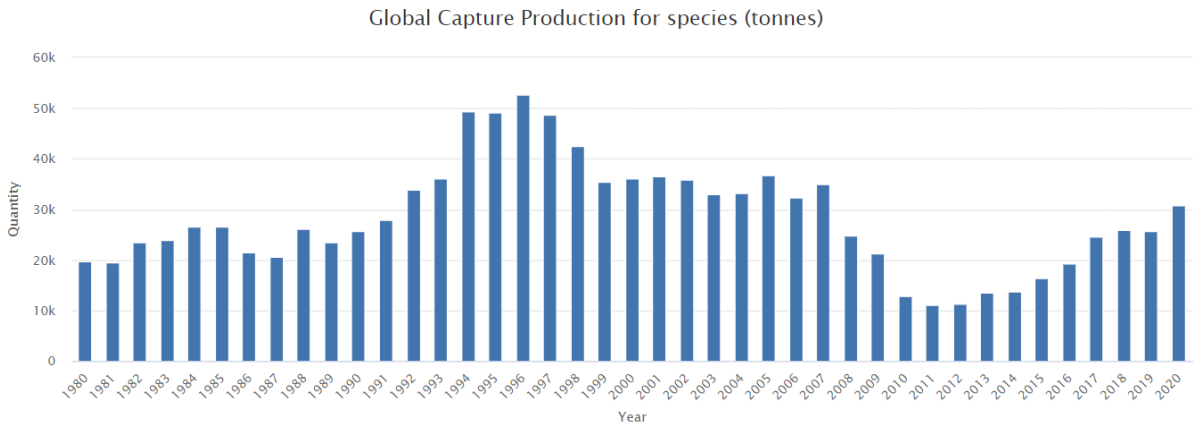


Figure III.4. Global ABFT captures per year. Source: FAO, 2023c

The ABFT tuna fattening first started as commercial activity in Spain and then, extended to all the Mediterranean Basin. ABFT is farmed exclusively in Portugal and the Mediterranean countries, where its culture has increased since 1997 (Chaabani, 2015) in the form of fattening. This activity became the principal destiny of the extractive-fishing captured tunas, and in the Mediterranean exist 63 bluefin tuna fattening structures (ICCAT, 2023a), being concretely the three main producers of ABFT using this method Spain, Croatia and Malta, followed by Portugal, Italy, Greece, Turkey, Morocco and Tunisia (**Figure III.5,** FAO, 2023a). The Spanish company 'Ricardo Fuentes e Hijos, S.A.' after making deals with some of Japan's top trading companies: 'Mitsui and Company, Ltd' and 'Mitsubishi Corporation', became the largest producer, processor and distributor of tuna products in the Mediterranean (Bregazzi, 2005).

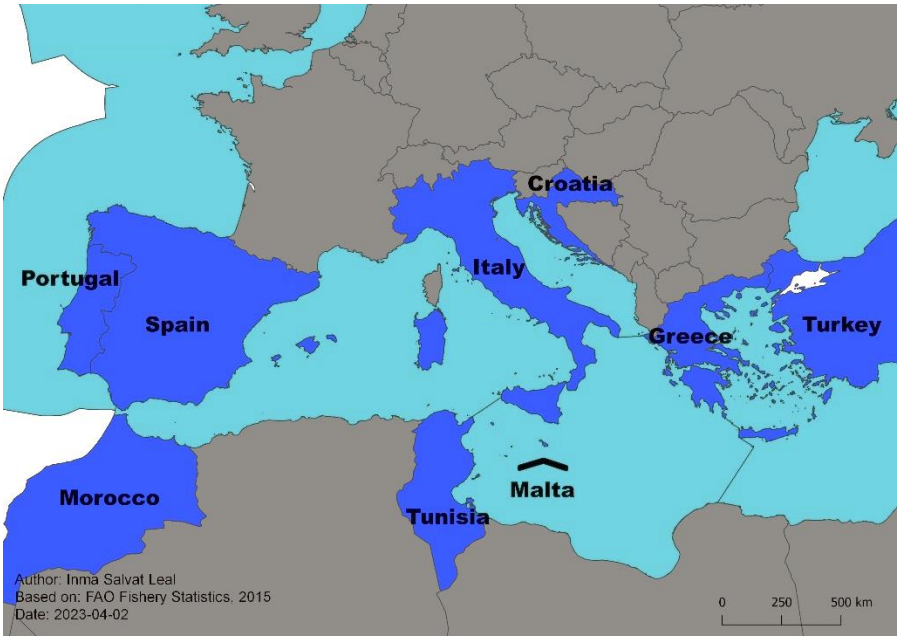


Figure III.5. ABFT productor countries in the Mediterranean and Portugal. Source: Inma Salva-Leal, based on FAO Fishery Statistics 2015.

Under these conditions, the aquaculture of ABFT remains an interesting point to develop, and surely the demand for hatcheries will grow rapidly. By covering the growing demand for ABFT in the markets through high performance and efficient aquaculture, the adequate protection and conservation of wild specimens are guaranteed and their migrations and breeding are free from human intervention on an excessive scale. For the long-term culture that entails the maintenance of breeding in captivity, the use of onshore tanks is necessary. Using onshore tanks allows to develop rearing techniques and to control the physical conditions, which is essential for tuna aquaculture (Wexler et al., 2003). In general, declines in fishery stocks worldwide incentivised the global expansion of aquaculture (Naylor et al., 2000; Deviller et al., 2004), growing rapidly the demand for hatcheries to supply aquaculture facilities with domesticated fish suitable for farm production (Warren-Myers et al., 2018). However, solving other problems that limit ABFT's onshore captivity breeding will be a key task to accomplish in the coming years (Mourente & Tocher, 2003, 2009).

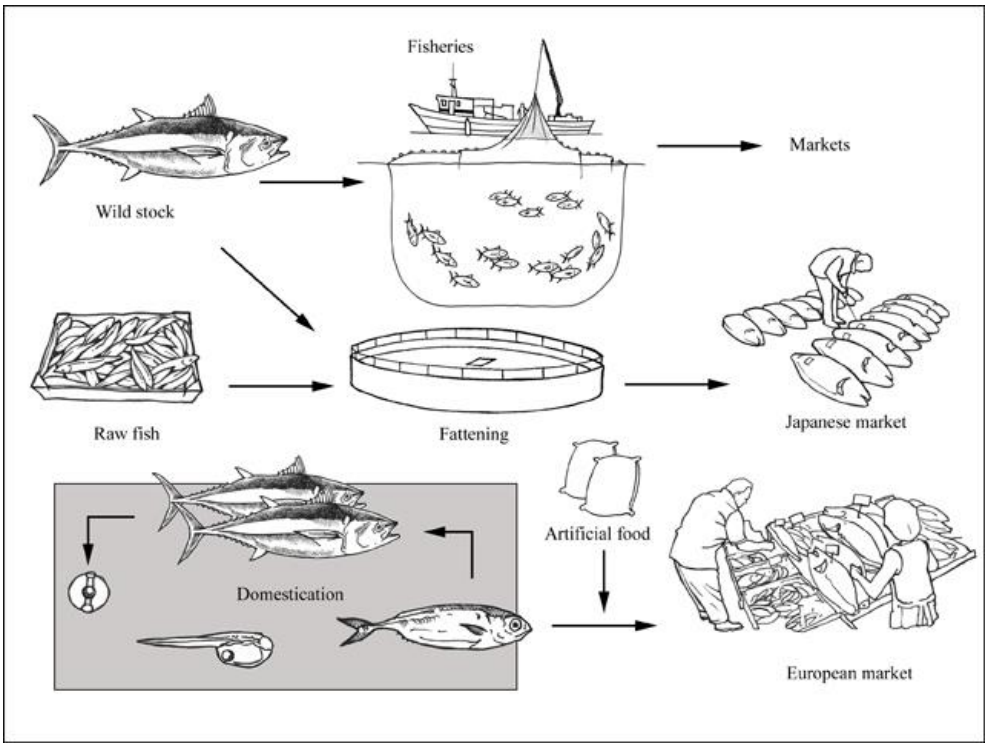


Figure III.6. Productive cycle of *Thunnus thynnus*. Source: FAO, 2023a.

The commercialization of aquaculture specimens opens a new sector in the ABFT trading (see **Figure III.6.** for the possibilities of ABFT production), which could lead to sell specimens below the minimum marketable size (30 kg), to offer products more nutritious and profitable than the current fattening or captured specimens, or sell products recognized as low in mercury and other substances with risk for consumers, *inter alia*. A controlled diet in relation to certain contaminants (i.e., mercury and other heavy metals), and a greater food and health control guarantees placing in the market products with superior food safety. Also, the better nutritional quality in terms of higher protein and healthy fat of these tunas would lead to a greater consumer acceptance. Regarding the rearing, in short-term, a controlled diet in relation to certain contaminants (i.e., mercury and other heavy metals), and a greater food and health control guarantees placing in the market products with superior food safety. This situation, can allow the offering of safer products, related with the decrease in risky substances, whose tolerable weekly intake is controlled by competent bodies decision. On the other hand, in long-term, the genetic improvement of the species is possible with

large-scale production, selecting breeders with higher yield, precocity or developing a domestic variety of the species with stress-resistance closer to those currently desired.

III.III. Cycle closure, life cycle and culture of ABFT

III.III.I. Cycle closure

In an effort to reduce the dependance on extractive-fishing (wild) adult individuals and to allow consistent supply of ABFT in the market, there has been an intensive effort on closing the ABFT biological life-cycle in Europe to farming purposes since the 2000's (Doumenge, 1996; Lioka et al., 2000; De la Gándara et al., 2016). In addition, the success with closing biological life-cycle in captivity of PBFT in Japan by 2002 (Sawada et al., 2005), raised the European interest in developing similar domestication protocols to support ABFT farming (Ottolenghi, 2008; Mylonas et al., 2010; De la Gándara et al., 2012). In 2016 this procedure was achieved in floating cages in San Pedro del Pinatar (Cartagena, Southeastern Spain), with tunas born in captivity in 2011 and 2012 (Ortega & De la Gándara, 2017). In addition, the largest land-based facility devoted to ABFT reproduction owned by Spanish Institute of Oceanography in Cartagena, Spain (**Figure III.7**), started to run in 2016, which represented a great advance for the reproduction control and the complete closed-cycle production of this species, as it will allow ABFT to spawn in captivity under controlled conditions (De la Gándara et al., 2016).

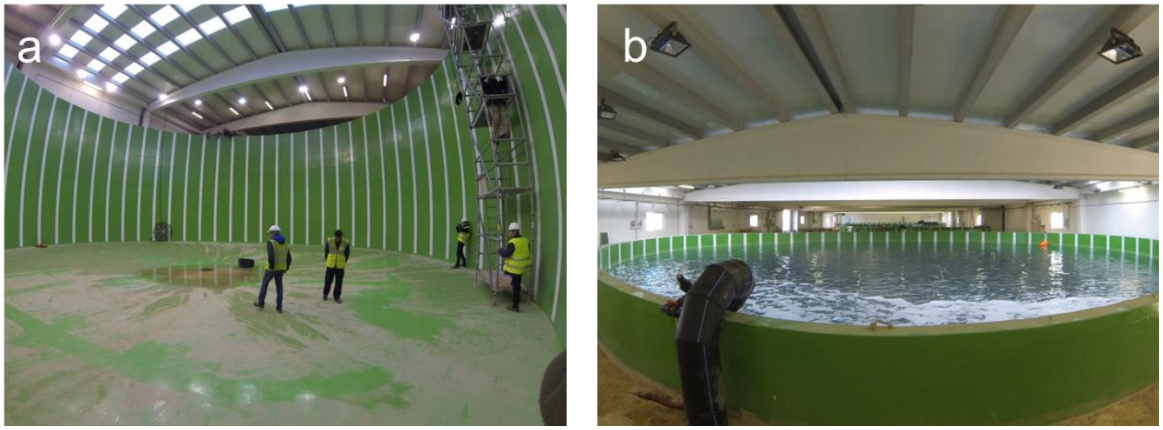


Figure III.7. Land-based tuna facilities in Cartagena, Southeastern Spain.

III.III.II. Life cycle

The ABFT spawning peak occurs around the summer solstice[1], is nocturnal (something that appears to be common in tuna), occurring from 2-5 am, with a mean interval of 1.2 days between spawning episodes (Medina et al., 2002). Salinity preferences are between 36.9 and 37.7 g L^{-1} and temperature between 21.5-26.5ºC (**Figure III.8**) (Sarà, 1964, 1973; Alemany et al., 2010).

Figure III.8. Ideal conditions for ABFT spawning. Source: Planet Tuna.

[1] from 15th-30th of June.

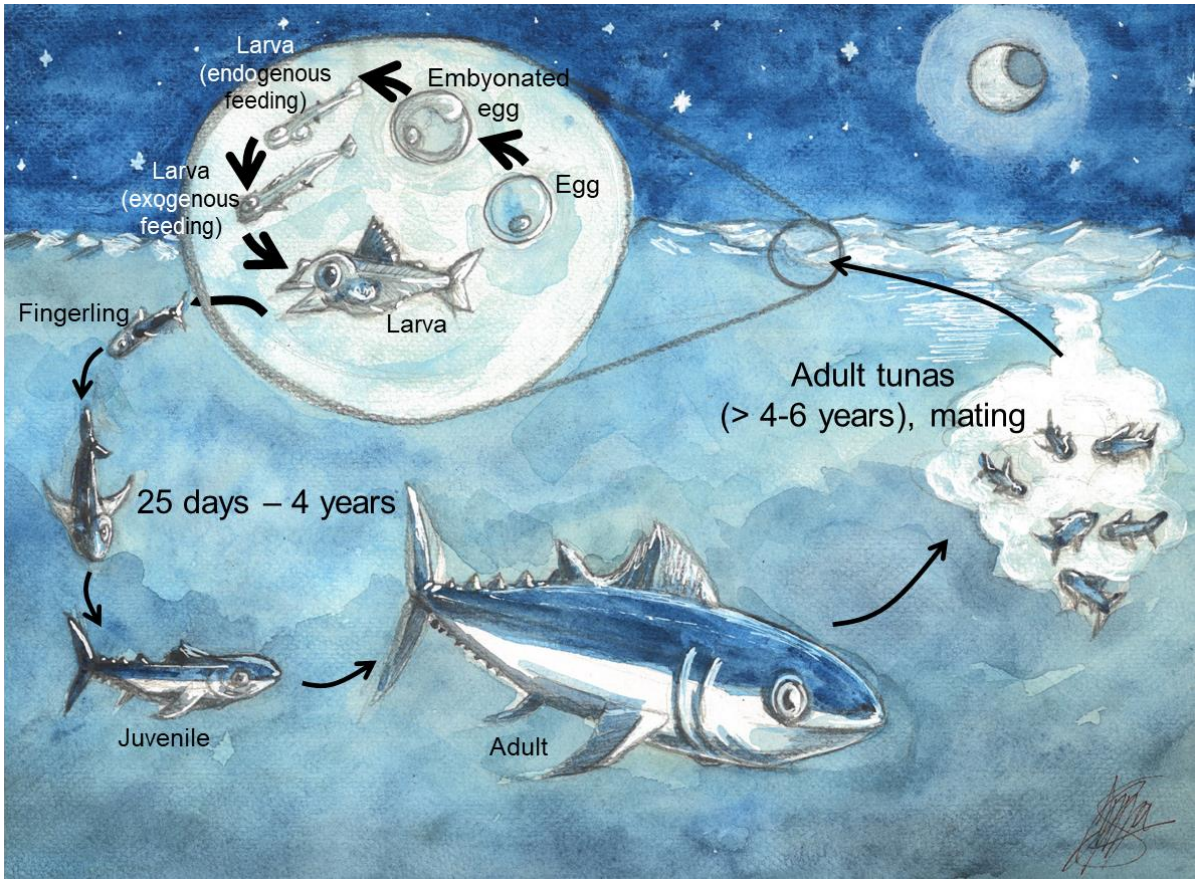


Figure III.9. ABFT lifecycle. Modified from: Planet Tuna. Drawing: Inma Salvat-Leal.

Once spawning has occurred (**Figure III.9**), the eggs, of pelagic nature, between 1 and 1.1 mm and generally one lipid droplet (**Figures III.10 and III.11a),** hatch within a few days, giving a yolk sac larva of 3-4 mm in length **(Figure III.11b)** (Ortega, 2015)**.** Then, the larvae undergo different stages until post-flexion, concretely Blanco and colleagues (2019) determined four developmental phases based on morphological characteristics of the caudal fin and the notochord. They followed a modified classification of the criteria of De la Gándara et al. (2013), and Kendall et al. (1984) and Kaji et al. (1996) for *Thunnus thynnus* (**Figure III.12**): i) larvae in pre-flexion (straight notochord), ii) larvae with development of the first caudal fin rays (straight notochord with some rays in the ventral side), iii) larvae in flexion (the notochord tip bends upwards with an increase in the amount of fin rays), iv) larvae in post-flexion (the final tip of the notochord disappears, the

hypural plate[2] and caudal fork are defined and the posterior margin of the upper hypural plate finishes at 90° from the notochord axis).

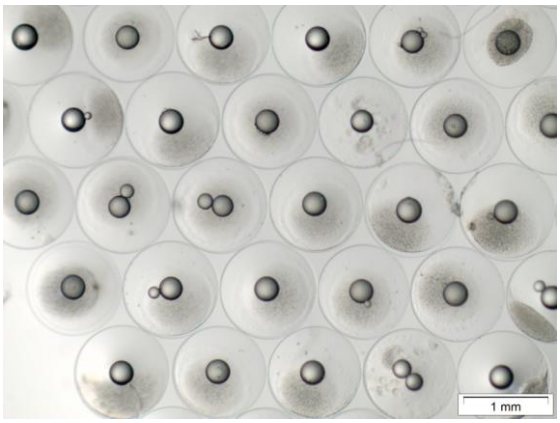


Figure III.10. ABFT eggs under stereomicroscope. Source: Inma Salvat-Leal.

ABFT has a very quick larval growth (Brothers et al., 1994). Overall, ontogenetic development in this species is similar to the one described for other altricial[3] larval teleosts (i.e., Sarasquete et al., 1995; Cobcroft & Pankhurst, 2003; Ortiz-Delgado et al., 2003; Gisbert et al., 2004; Papadakis et al., 2013), acquiring in two weeks a degree of development that allows efficient predation and digestion of more complex foods. ABFT is considered juvenile from 25 dph until 4-6 years old, when they reach the sexual maturity. As juveniles they are anatomically identical as the adults, but smaller and sexually inactive (Kendall et al., 1984). After the juvenile stage, the ABFT adults are sufficiently mature to start mating.

[2] Hypural plate: joint between the caudal fin and the last vertebrae of the column.

[3] Altricial species: the species unable to move on their own shortly after birth/hatching.

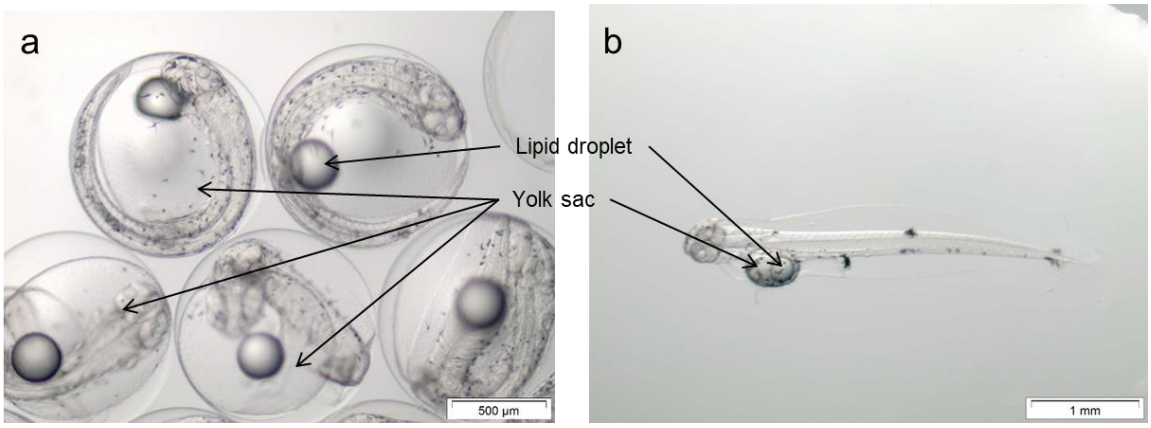


Figure III.11. Transition of the lipid droplet and the yolk sac between a) Embryonated ABFT eggs and b) Just hatched ABFT larva. Source: Inma Salvat-Leal.

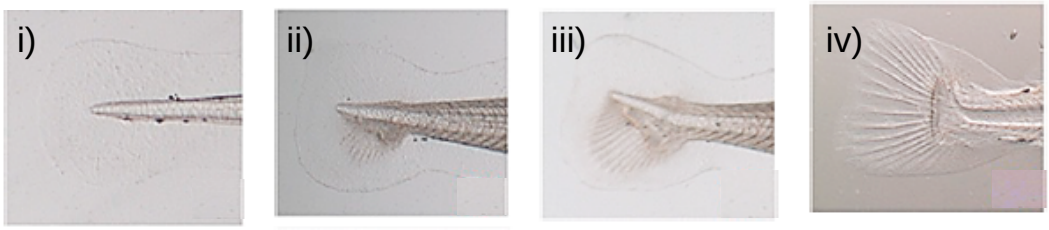


Figure III.12. Early-stage differential morphologies of ABFT larvae, i) larvae in pre-flexion, ii) larvae with development of the first caudal fin rays, iii) larvae in flexion, iv) larvae in post-flexion. Modified from: Blanco et al., 2019.

III.III.III. Culture

Under culture condition larval hatching occurs after 38-40 hours with temperatures of 22-24ºC, while at 27-28ºC the eggs hatch in less than 30 hours. During the first 48 hours the larvae have endogenous feeding, consuming their lipid droplet within the yolk sac.

According to De la Gándara and colleagues (2012, 2016) and Ortega (2015), the ulterior diet in ABFT culture is as follows: i. 2-14 dph, enriched rotifer (*Brachionus plicatilis)* or marine copepods (nauplius and copepodits of *Acartia tonsa*); ii. 12-18 dph enriched *Artemia salina* nauplii or adult copepods; iii. 16-30 dph, sea bream yolk-sac larvae *(*0-2 dph *Sparus aurata)*; iv. 25-30 dph (juveniles), dry food.

The transition from endogenous to exogenous feeding is considered one main bottleneck affecting larval survival, and successful “first-feeding” is a prerequisite for survival (Hjort,1914). Nearly all offspring produced (>99.9%) in most marine fish species in nature, will not survive their first year of life (Houde, 2008). After this critical period, as most of the marine fish, tunas need fed on zooplankton during early stages of development (**Figure III.13**). The first live preys used for ABFT feeding under cultured conditions is usually rotifer, however, the use of copepods increase larval growth and survival (Ortega, 2015; Betancor et al., 2019) and coincides with the wild specimens’ natural prey (Llopiz & Hobday, 2015). However, its cultivation is more expensive and less efficient than rotifers. The dietary change from invertebrates (zooplankton) to fish larvae is associated with the development of a functional digestive system (Kaji et al., 1999), and with the presence of intestinal folds for food retention in the anterior mid-stomach (Rønnestad et al., 2007). Concretely, ABFT has a very quickly larval growth (Brothers et al., 1994), as well as a highly voracious piscivorous behaviour from early ages (Hunter & Kimbrell, 1980; Young & Davis, 1990; Sabate et al., 2010; Catalán et al., 2011).

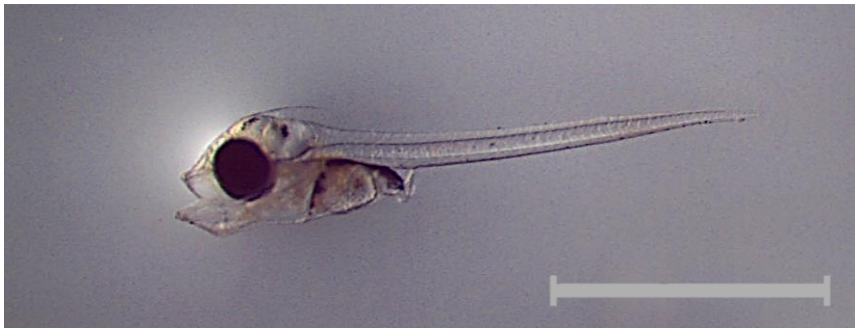


Figure III.13. ABFT larva with 8 dph (early stage of development), scalebar = 2 mm. Source: Inma Salvat-Leal

Once the ABFT are weaned onto inert diet and became juvenile, from 35-40 dph onwards, they are transferred to bigger tanks and/or sea cages (De la Gándara et al., 2016). When initially transferred, juvenile ABFT use to be fed on dry food, progressing to minced frozen small pelagic fishes (see a resume of the wild and

aquaculture tunas' usual diets in **Figure III.14**; De la Gándara et al., 2012, 2016; Ortega, 2015), including: sand-eel (*Gymnammodytes cicerellus*), sardine (*Sardina pilchardus*), mackerel (*Scomber scombrus*), and anchovy (*Engraulis encrasicolus*). Juveniles are fed to apparent satiation and the regime decrease from 6-8 meals per day at the beginning to 2-3 meals after some months. During these first 3-4 months after stocking, mortalities can range between 60-90%, mainly during the first month, being the main cause of mortality collisions, unbalanced nutrition and diseases, because the fight-or-flight response against perceived danger is biased to the latter (De la Gándara et al., 2016). After, from 5 months mortality can progressively decrease to less than 2% monthly (Ortega et al., 2014). Under these conditions, most juvenile ABFT reach 2 kg by month 6 (De la Gándara, et al., 2016).

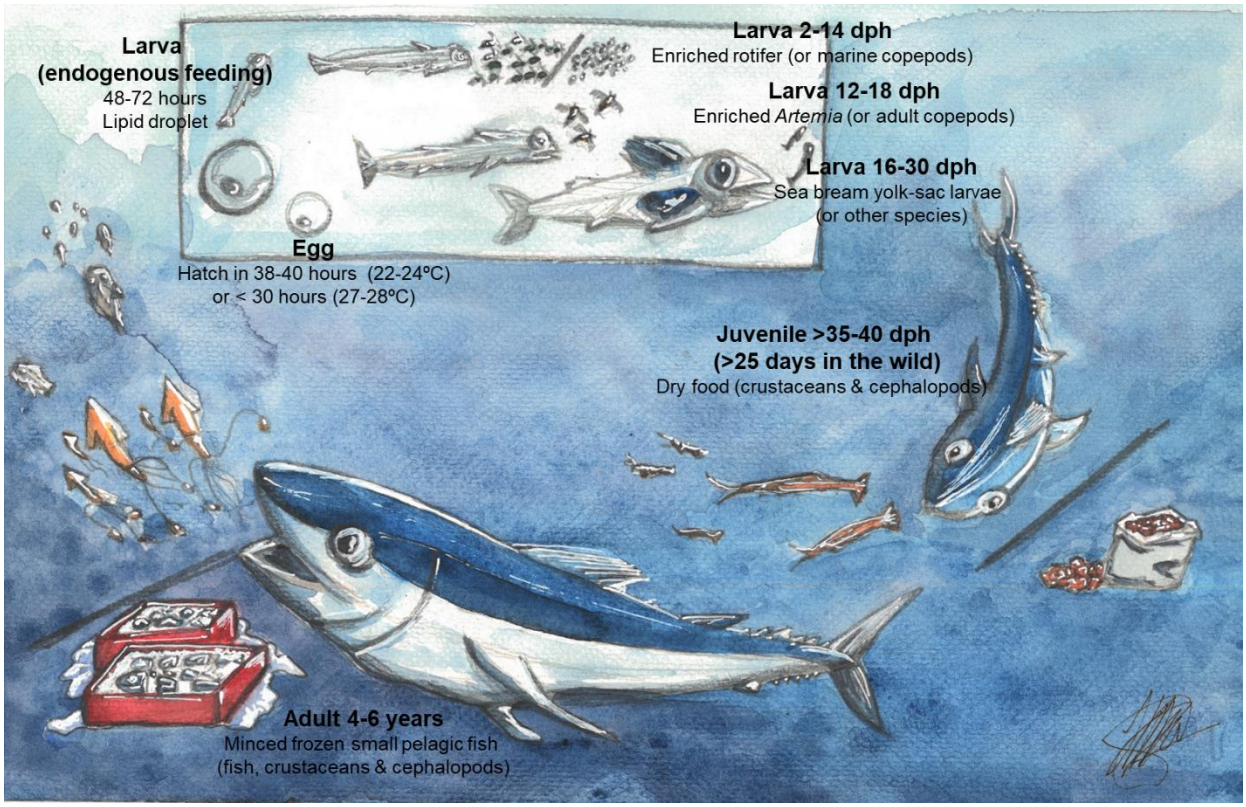


Figure III.14. Feeding by stage in aquaculture and wild tunas, the diet of wild tunas is described inside brackets. Drawing: Inma Salvat-Leal.

III.IV. Traceability. Marking and identification techniques.

The increasing complex marketing patterns claim efforts to regulate, monitor and control fisheries trade. In this context, traceability, as the ability to track the flow of products throughout the production process, constitutes a powerful tool to warrant product authenticity, but also to certify the compliance current rules and to fight fraud (Stockhausen et al., 2009). Traceability is based on a rigorous documentation procedure (i.e., labelling, certification) and supported by independent control measures that verify the documents required to fulfil the traceability outline, giving inspection authorities powerful control methods at the beginning of the market chain, and the consumers access to information and tools to verify and validate labels. Worldwide, traceability has gained importance in the fisheries sector (i.e., US has reinforced laws related to wildlife crimes). Advanced technologies based on chemistry, molecular biology, biotechnology and genetics show great potential for fisheries control and traceability (Stockhausen et al., 2009). However, quite different strategies are followed depending on the country. In the EU, the current legislative document core with respect to traceability and food safety is the Regulation (EC) 178/ 2002. This regulation refers explicitly to traceability as a means to ensure safety of food and consumer protection, and is defined as '*the ability to trace and follow a food, feed, food-producing animal or substance intended to be, or expected to be incorporated into a food or feed, through all stages of production, processing and distribution*'. Actually, the fisheries sector traceability in the EU relies essentially on primary information provided by the producer and other participants involved in the supply chain and production. For example, a traceability scheme based on data of the geographical origin assignment of fish can contribute to compliance the current stock and conservation management outlines, such as the catch quotas. Therefore, a qualified traceability scheme supports the credibility of fishery companies, thus having advantage in the fisheries market. Also, these companies are best

prepared for the potential removal of products in case of a detected issue (i.e., contamination, errors…). The support of independent (but validated) control technologies, like the use of natural chemical tracers (**Chapters I-III and VI** of the Thesis), natural morphometrical tracers (**Chapters IV-VI**) or artificial markings (**Chapters VII and VIII**), would be highly beneficial to fisheries and the multiple components of it, like fisheries management, aquaculture, conservation, and consumer protection.

In the ABFT future production context, the product traceability will be key in identifying extractive fishing (wild) specimens from those captive-reared (farmed), and the search of marking methods to differentiate these specimens will be an important challenge to be addressed in the coming years. Currently, different types of tagging methods exist in tuna, being the three main types (ICCAT, 2023b): conventional tags (streamers and spaghettis, Fromentin, 2002; also called traditional tags, Porch et al., 2001), archival tags and pop-up tags (being the last two electronic tags). These are mainly used for migration and movement patterns (i.e., Block et al., 2005). Lately, chemical signatures in hard structures (natural tags) have been developed. For example, Secor and colleagues (2002) measured the elemental concentration in otoliths and attained to distinguish nurseries from ABFT collected from 3 Mediterranean areas and the Gulf of Mexico. Also, alternative geochemical markers (especially stable ∂^{18} isotopes, could discriminate the otolith chemistry from medium and large ABFT. Finally, genetic signatures, another type of natural tags have been also explored, for example using microsatellite and mitochondrial DNA or isozymes (Broughton & Gold, 1997; Pujolar & Pla, 2000; Viñas et al., 2001, 2003; Ely et al., 2002; Carlsson et al., 2004), however results are controversial and not conclusive (Frometin & Powers, 2005). In resume, electronic studies alone will not be sufficient to get a comprehensive picture of ABFT population dynamics due to its complexity and mixture (Fromentin & Powers, 2005), and chemical and genetic signatures despite being promising should be further studied to avoid conflictive results. In this context and given the developing of the ABFT onshore captive-rearing, the search of new tracers and marking methods is pressing, therefore we decided to search new tools that could use the natural features of different groups

of ABFT as tracers, to avoid direct human handling, but also, to test some methods of artificial marking that could be used in hatcheries for early stages of ABFT. In the natural tracers Section, we decided to test both chemical and morphometrical features of some structures for group discrimination.

III.IV.I. Natural Tracers

There are non-invasive methods that can guarantee the traceability of products of different origins. Some tissues in which natural tracers can be found (and are used in this Thesis) are: gill, brain, liver, bone, muscle and kidney, also, a structure specific of teleost with unique characteristics was studied: the otolith. They can constitute an area-specific 'fingerprint' (Walther & Limburg, 2012), which makes them really interesting as natural tracers, especially due to their non-resorbable nature. On all these tissues and the otoliths, the inorganic elements were analysed and therefore the specific chemical profile could be detailed.

III.IV.I.I. Tissues

The internal natural features of fish are tools that have rarely been documented, even though they can guarantee traceability being non-invasive tracers, which is a must in ABFT newly aquaculture production. The size, age, gender, reproductive status, feeding habits, and habitats of the fish, in terms of being wild or captive-reared, modifies the elemental bioaccumulation among tissues (Licata et al., 2005; Percin & Sogut, 2010; Vizzini et al., 2010). Being this is a valuable tool for origin discrimination, for example, some ABFT batches have been differenced through their tissular chemical profile (i.e., Percin et al., 2011; Sogut & Percin, 2011; Sogut et al., 2011; Salvat-Leal et al., 2023, and unpublished data). In ABFT, their intense activity levels, rapid growth and metabolic rates, lead to a high food intake rate and to a trace elements exposure (Sogut & Percin, 2011). Therefore, like many fish they are reliable bioindicators for trace elements monitoring in aquatic ecosystems (Varol & Sünbül, 2019), and some composition studies in ABFT focused on the idea of an element profile regarding fish origin or

batch (i.e., Percin et al., 2011; Sogut & Percin, 2011; Sogut et al., 2011; Salvat-Leal et al., 2023, and unpublished data). Consequently, this is a valuable tool for origin discrimination. Concretely, certain inorganic elements in ABFT tissues have been proposed in the Turkish Mediterranean as non-invasive and natural tools for determining the origin of specimens weighing over 50 kg (Sogut et al., 2011). In this study they focused on trace elements, which have a wide range of vital roles in the functioning of animals (Hamilton & Hoffman, 2003).

III.IV.I.II. Otoliths

They are one of the structures most commonly used as natural tracers, because they are versatile (i.e., many methods can be applied on them with this purpose), grow continuously during the fish's life, and their mineral part remains unaltered after deposition (Campana & Thorrold, 2001). They are found in the inner ear of teleost fish, and are small calcium carbonate ($CaCO_3$) structures of biogenic origin, immersed in endolymph (Vinagre et al., 2014). These are bilateral structures, which like Panfili et al. (2002) described, in a homogeneous ambiance will develop symmetrically. Generally, fish otoliths are left-right symmetrical except for flatfish and catfish (Panfili et al., 2002), and three otoliths by side inside the otic capsule are found (**Figure III.15**): *sagitta* (which is the largest and most used otolith, located parallel to the medulla), *lapillus* (medially from the sagitta) and *asteriscus* (attached to the posterior ventral part of the *sagitta*). The acoustic nerve lays approximately in the middle part of the *sagitta (*Pavlov, 2019), this is why, otoliths have the function of stability maintenance, and perception of sound waves, angular movements, gravity and depth pressure (Blacker, 1974; Lundberg et al., 2015).

In a three-dimensional environment, such as the aquatic environment, spatial awareness and postural equilibrium are fundamental for locomotion. Sound perception in the water is also crucial for the detection of congeners, prey and predators (Vinagre et al., 2014). The otoliths have an exceptional feature: they grow continuously during the fish's life, remaining the mineral part unaltered after deposition, so they constitute permanent recorders of environmental exposure (Campana & Jones, 1992; Campana & Thorrold, 2001) and they register the individuals' life story. Therefore, their composition is highly complex, consisting on a matrix poor in proteins (3%), rich in calcium carbonate (96%) and completed

with different minor inorganic elements. The otoliths are embedded in a solution called endolymph inside the otic capsule, and attached to a gelatinous membrane over the sensory epithelium called macula (**Figures III.15 and III.16**) where sensory hair cells connect to the acoustic nerve (Hüssy et al., 2021). The elemental incorporation in the otolith is a complex biogeochemical process influenced by many factors, being elemental signatures the result of environmental and metabolic processes. In this sense, the separation of individuals in both time and space may induce different otolith chemical compositions (Kitchens et al., 2018). Some elements are absorbed primarily from the surrounding water, forming an area-specific “fingerprint” (i.e., Walther & Limburg, 2012). Meanwhile, other elements are under strong physiological control (Campana, 1999; Sturrock et al., 2015; Limburg et al., 2018; Hüssy et al., 2020). Specifically, in tropical tuna species, the otolith chemical composition seems to be a powerful tool for group discrimination, some examples are the use of otolith element:Ca (E:Ca) ratios with this purpose in PBFT and ABFT (Rooker et al., 2001a, 2003; Traina et al., 2021), the use of otolith stable isotopes in ABFT (Rooker et al., 2014), and the use of both otolith E:Ca ratios + stable isotopes in bigeye, yellowfin and skipjack tuna using (Rooker et al., 2016; Artetxe-Arrate et al., 2019, 2021).

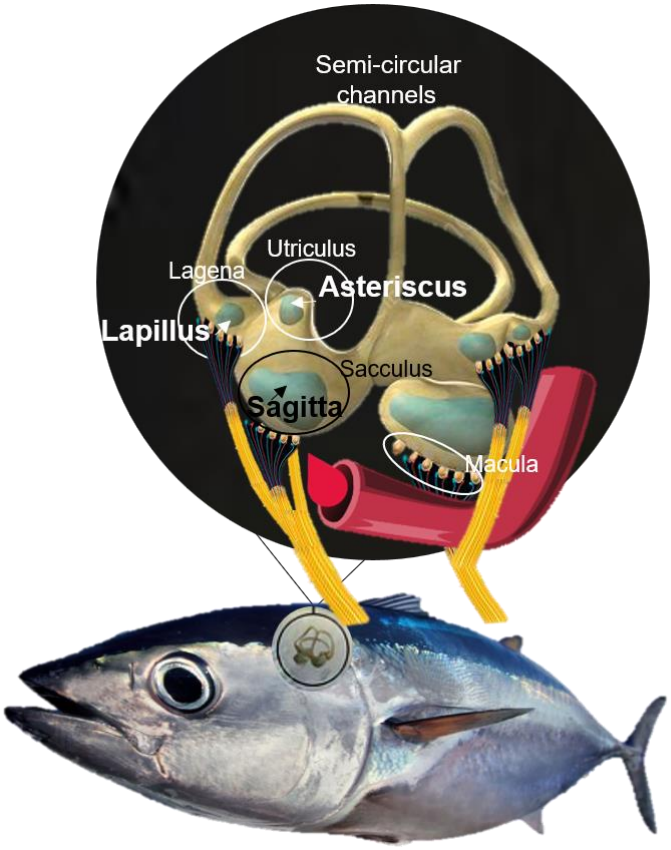


Figure III.15. Otoliths' location and anatomy in teleost fish. Source: Inma Salvat-Leal, modified from Saitô et al. (1989) and Ashworth (2016).

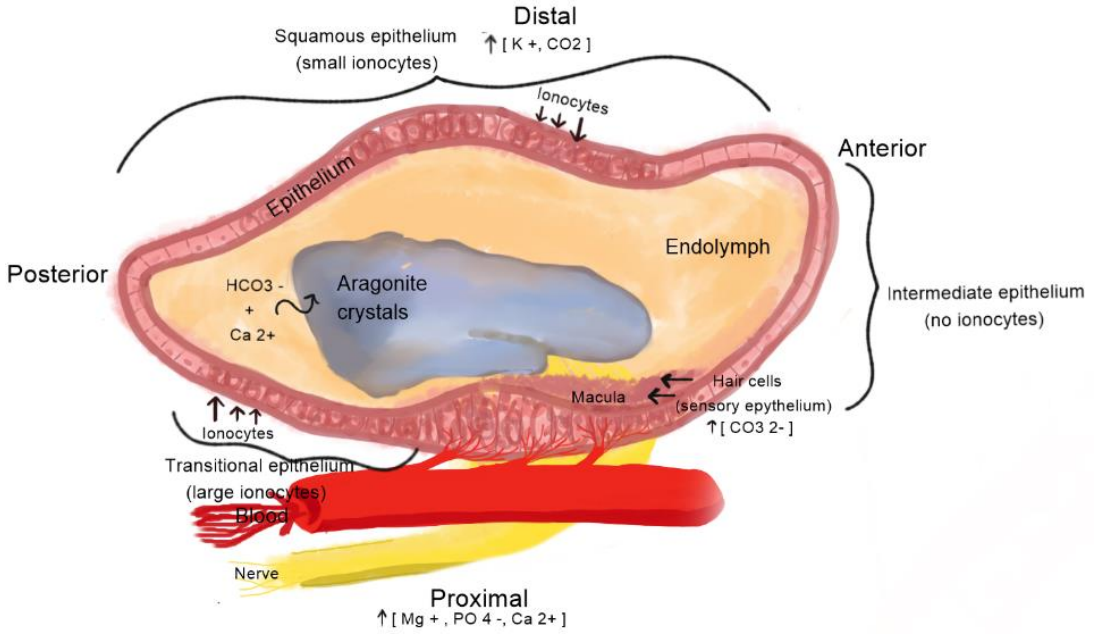


Figure III.16. *Sagitta* in the sacculus. Source: Inma Salvat-Leal, modified from Saitô et al., 1989; Pisam et al., 1998; Mayer-Gostan et al., 1998; Hüssy et al., 2021).

Apart from the elemental composition, morphological differences have also been described due to different crystal types (Carlström, 1963; Strong et al., 1986; Campana, 1999; Tomás & Geffen, 2003; Falini et al., 2005). This is because $CaCO_3$ can have three iso-morphologies with identical chemical formulas but different crystalline structures: aragonite, calcite and vaterite (Carlström, 1963; Falini et al., 2005). In the *sagitta* otoliths $CaCO_3$ crystals are normally arranged as aragonite (Carlström, 1963), being calcite and vaterite forms more frequent in captive-reared fish (Gauldie, 1986; David et al., 1994; Bowen et al., 1999; Sweeting et al., 2004; Reimer et al., 2016) and associated to anomalous otoliths (Strong et al., 1986). Different factors can modify the normal $CaCO_3$ deposition (Strong et al., 1986; David et al., 1994; Sweeting et al., 2004; Tomás et al., 2004; Reimer et al., 2017; Holmberg et al., 2019), because the otolith morphology (Strelcheck et al., 2003; Cardinale et al., 2004; Gagliano & McCormick, 2004; Vignon & Morat, 2010) and shape (Mérigot et al., 2007; Mille et al., 2016; Vignon, 2018; Mahé et al., 2021), depend on a mixture of genetic and environmental factors: like feeding habits, growth rate and habitat, and therefore the separation of populations induces divergent otolith shape (Messieh, 1972; Lombarte & Lleonart, 1993). Regarding the environmental factors, one of the main causes affecting the otoliths is environmental stress (Vinagre et al., 2014), which could be due to the water quality (Portz et al., 2006), specially to marine pollution (Bat et al., 2018; Pokazeev et al., 2021). This environmental stress is also one of the main causes of bilateral abnormalities in symmetrical structures like the otoliths (Morales-Nin, 1987; Ma et al., 2008; Vinagre et al., 2014; Manzadeh et al., 2018; Mahé et al., 2019; Yedier et al., 2022; Yedier, 2022), which is the base of the development of asymmetry. In a non-homogeneous environment, small random perturbations can deviate locally the development of the otolith, and is possible that these perturbations accumulate on right or left sides separately, leading to asymmetric phenotypes (Geladakis et al., 2020). Therefore, the analysis of some kinds of asymmetry, commonly acts as a biomarker for the individual's fitness and/or stress (Parsons, 1989; Dongen, 2006; Beasley et al., 2013; Sánchez-Chardi et al., 2013). In fish, several bilateral traits have been used to study this

(i.e.: eyes, dentition, fin rays and pharyngeal arches *inter alia*, Michaelsen et al., 2015; Leung et al., 2017), including the otoliths (Díaz-Gil et al., 2015).

III.IV.II. Artificial Marking

In order to properly evaluate the effectiveness of stocking management or traceability, fisheries managers often rely on some type of fish marking or tagging technique (Lü et al., 2019). In this sense, mass-marking techniques have received substantial attention and three different types have been mainly used: the external application of fluorescent pigment, the introduction of stock-specific genetic markers, and the use of chemicals to mark calcified tissues.

Through the last years, chemical markers have been suggested to be the most suitable tools for mass-marking of small juveniles (i.e., Simon & Dörner, 2005; Baer & Rösch, 2008), having broad applicability (i.e., in several fish stages) and being often used (Baras et al., 2000; Simon, 2007). Chemical marks mainly include: calcein, oxytetracycline hydrochloride (OTC), alizarin red S (ARS) and alizarin complexone. Most of them are fluorochromes, producing detectable fluorescent marks in bony structures (Eckmann, 2003), and therefore, fish exposed to them will incorporate marks later detectable under specialized equipment (Guy et al., 1996; Warren-Myers et al., 2018; Uglem et al., 2020). Several techniques for introducing these markers have been investigated in teleost (Gelsleichter et al., 1997): injection, dietary intake or immersion; but the choice of the technique depends on the environment, life history stage, and experiment condition (Lagardère et al., 2000).

For example, tetracycline antibiotics produce fluorescent marks in the fish bony parts (**Figure III.17**) (Weber & Ridgway, 1967). The most common formulation of antibiotics used is the OTC, which produces yellow-gold fluorescent marks (Odense & Logan, 1974; Brooks et al., 1994; Wells et al., 2013) and is most visible under UV-light. Marks with OTC are created by either the direct immersing of fish, by its combination with feed, or by direct injection. Concretely, marking fish via direct injection is only acceptable if they are already destined to be injected (i.e., during microchip identification or routine vaccination). However, the fluorochrome labelling dye with ARS is a feasible mass-marking alternative to

OTC marking (Bashey, 2004; Simon et al., 2009), and produce fluorescent marks ranging from yellow to red-violet (**Figure III.18**) depending on the light source (Beckman & Schulz, 1996; Lagardère et al., 2000; Liu et al., 2009). ARS is the most popular among the fluorochromes (Williamson et al., 2009; Smith et al., 2010; Wells et al., 2013; Warren-Myers et al., 2018), because is the most cost-effective, causes less negative effect on survival and has not availability limitation (Tsukamoto, 1988; Tsukamoto et al., 1989; Walt & Faragher, 2003; Taylor et al., 2005; Baer & Rösch, 2008). Internal marks in the otoliths are retained for years (Simon et al., 2009) and marking success rates of 100% are achievable with little to no effect on mortality in juvenile fish using the best concentration-time combination (Warren-Myers et al., 2018). In addition, one or two permanent marks in the otoliths are possible through two alizarin immersions, if they are separated in time. This is why in this Thesis it has been chosen to test two current but differing otolith chemical mass-marking methods: OTC and ARS.

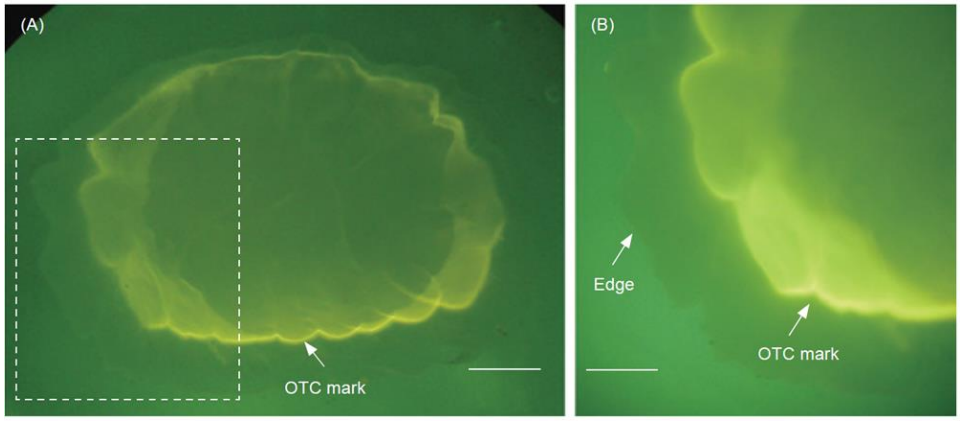


Figure III.17. OTC marks in Japanese eel (*Anguilla japónica)* juveniles, a) OTC mark and b) OTC mark magnified, showing the newly deposited increment after. Scale bars: A = 500 μm; B = 300 μm. Source: Lin et al., 2012

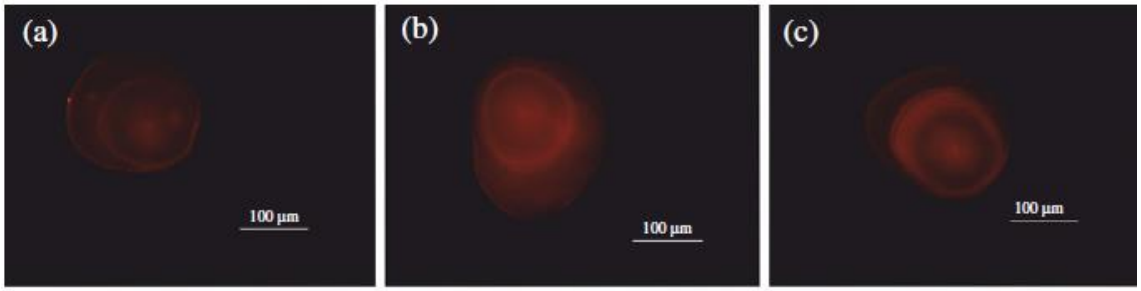
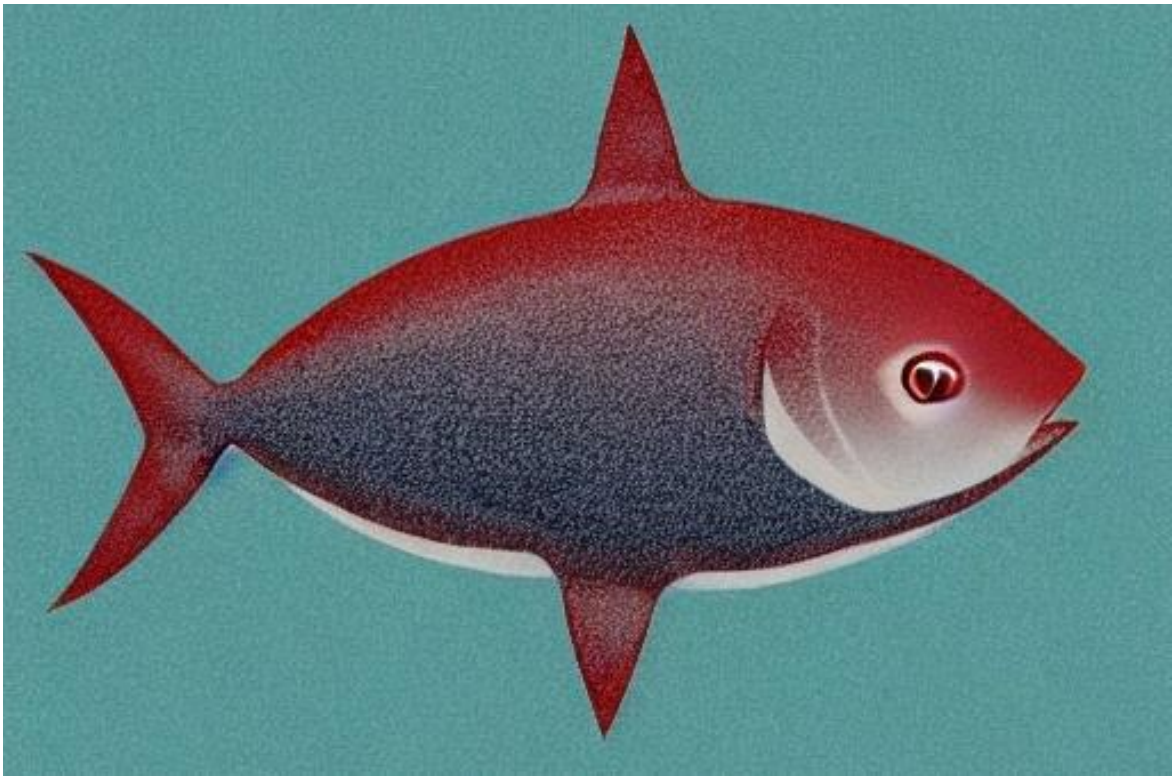


Figure III.18. Otoliths of ide (*Leuciscus idus)* larvae fed with *Artemia salina* nauplii immersed in ARS during (a) 1, (b) 2 and (c) 4 days of feeding (under filtered UV-light). Source: Stańczak et al., 2015.



IV. Aims of the study

IV.I. General objective of the thesis:

To identify useful markers for the discrimination among different ABFT groups, by various lines of research:

- Analysis of chemical composition and morphometrical characteristics from various tissues (including the otoliths), looking for natural differences derived from differing life conditions, habitats and diets (Chapters I-VI).

- Artificial marking of otoliths using different methods, in order to leave constancy of the aquaculture farms' rearing (Chapters VII and VIII).

IV.II. Specific objectives by chapters:

1. Chapter I: Elemental composition in soft tissues as a model for identifying batches of juvenile Atlantic bluefin tuna (*Thunnus thynnus*). In this study, we aimed to classify ABFT juvenile from three batches (wild tuna and two captive-reared batches raised in two environments) through differences in the main trace elements in four soft tissues (liver, kidney, brain and muscle).
2. Chapter II: Composition of inorganic elements in the hard tissues of juvenile *Thunnus thynnus*. This study evaluates the mineral concentrations in bones and gills of ABFT from three separate batches (wild tuna and two captive-reared batches raised in two environments) and then examines their differences in the main trace elements.
3. Chapter III: Otolith mineral composition as a model for identifying the batch of juvenile Atlantic Bluefin Tuna (*Thunnus thynnus*). In this study, we took advantage of the benefits of a multi-elemental study combined with multivariate analysis to discriminate young ABFT batches using their otolith chemical profile. For this purpose, otoliths from tunas across two different environments in the Mediterranean were examined: wild and farmed.

4. Chapter IV: Otolith morphology in juveniles of Atlantic bluefin tuna (*Thunnus thynnus*). We hypothesize that different morphometries can be found in the otoliths from ABFT juveniles regarding their provenance. With this purpose, we examined otoliths of specimens collected from two environments in the Mediterranean: wild and farmed. Our aim being to determine whether the tuna could be discriminated based on their early life otolith morphometry.
5. Chapter VI: Asymmetry study in otoliths from Atlantic bluefin tuna (*Thunnus thynnus)* from two different ambients. The aims of this study were to i) identify the types of asymmetry that occur in these specimens, and ii) quantify and compare the level of asymmetry among wild and farmed batches.
6. Chapter VI: Vaterite precipitation in Atlantic bluefin tuna (*Thunnus thynnus*) otoliths. Our objectives were to i) identify if ABFT otoliths present abnormal forms and/or vaterite, ii) if present, estimate the proportion of vaterite in both wild and farmed ABFT, and iii) describe and compare the otolith morphology depending on its composition (aragonitic or vateritic-otoliths).
7. Chapter VII: Is oxytetracycline useful for marking otoliths of juvenile Atlantic bluefin tuna? In this study, OTC marking was applied to ABFT to test its validity as a marking technique for the first time.
8. Chapter VIII: Is Alizarin red S useful for marking otoliths of Atlantic bluefin tuna eggs? Here, we aimed to determine if ARS mass-marking is a feasible method for ABFT eggs.

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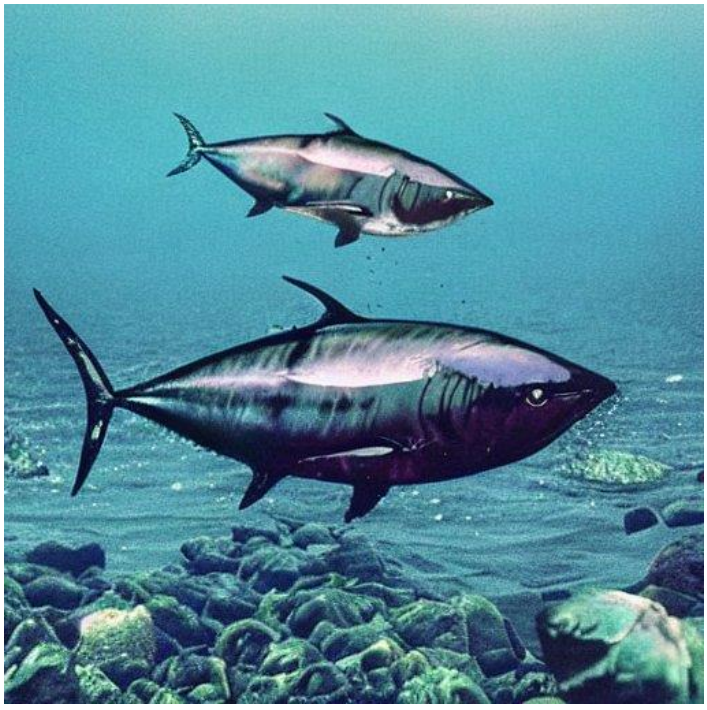

V. SCIENTIFIC BODY

FIRST SECTION, natural chemical tracers found in seven different tissues of ABFT: kidney, liver, brain, muscle, gill, bone and otolith.

CHAPTER I

Elemental composition in soft tissues as a model for identifying batches of juvenile Atlantic Bluefin Tuna (*Thunnus thynnus*)

Salvat-Leal et al., 2023

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Abstract

Integral Atlantic bluefin tuna (*Thunnus thynnus*) aquaculture will become a reality in the coming years and so, tuna batches will have to be clearly identifiable to avoid commercial fraud and ensure this species' conservation. Consequently, the objective of this study was to analyse the components of juvenile bluefin tissue to be able to discriminate between three tuna batches: specimens born in captivity and raised in inland facilities (onshore tanks), fish born in captivity and raised in the sea (sea cages), and wild tuna. Ten macro and trace elements (Ca, Fe, K, Mg, Na, P, S, Cu, Mn and Zn) were selected, and their concentrations were analysed in four soft tissues: liver, kidney, brain and muscle. Only one of the elements (Cu) showed statistically significant differences for fish batch in all tissues, so multivariate tests (Principal Component Analysis, PCA and Canonical Discriminant Analysis, DCA) were performed. In the PCA, there were partial batches separation in kidney and muscle. In DCA, the percentage of cases correctly classified using this validation were 60.8% (liver), 88.6% (kidney), 79.5% (muscle) and 82.2% (brain). Globally, muscle appear to be the best tissue for discriminating the batch of tunas, and wild specimens are the most readily identifiable.

Keywords: bluefin tuna, food analysis, food composition, soft tissues, trace elements.

Introduction

The Atlantic bluefin tuna (*Thunnus thynnus*, ABFT) is a species of great commercial importance and as such its capture is subject to rigorous controls to ensure its quality and compliance with international laws. In general, the aquaculture of tuna species is a relatively recent activity (Mylonas et al., 2010; Benetti et al., 2015) and capture-based aquaculture is a type of intensive production that in ABFT has only been practiced since the late 1990s (De la Gándara et al., 2016). However, production techniques have changed since the biological cycle of the ABFT was first fully disentangled in 2016 (Ortega & De la Gándara, 2017), thereby enabling the development of integral aquaculture for this species. In future years, juvenile specimens of ABFT will be bred in aquaculture facilities and, once established, new tools will be required to discriminate batches of these specimens and ensure correct adherence to sanitary regulations.

In recent years, studies using marking methods have been developed in aquaculture for various fish species (Canonico et al., 2005; Krkošek et al., 2006; Brooks & Jones, 2008; Glover et al., 2013). To identify captivity-born fish, some marking techniques can be applied, like external labels, intramuscular microchips, otolith marking, stable isotopes and genetic markers (Greene et al., 2009; Huelga-Suárez et al., 2012; Thorrold et al., 2001). However, techniques to mass tagging are difficult to implement and sometimes cause complications in growth and mortality due to handling remain (Gilderhus & Marking, 1987; Mohler, 2003). Thus, non-invasive methods that can guarantee the traceability of products of different batches are required. In fact, some techniques to differ fish stocks based on their origin, mainly by otoliths composition or by genetic markers are used. However, other techniques about the composition of different tissues of fish are tools rarely documented, even though it is known that, in the growth and development of both terrestrial and aquatic animals, growing conditions play a key role in tissue configuration (Jara & Chodyniecki, 1999; Brucka et al. 2009).

Recently, certain trace elements have been proposed in the Turkish Mediterranean as non-invasive and natural tools for determining the origin of ABFT specimens weighing over 50 kg (Sogut et al., 2011). As well, the use of this technique to identify and analyse biological variables is beginning to receive greater attention in stock identification (Kusznierz et al., 2008; Bektas & Belduz, 2009; Specziar et al., 2009). Multi-element studies are valuable tools for performing standardised chemical composition profiles and are potentially of great interest in food authentication and possibly for application in fisheries (Cubadda, 2006). However, several biotic and abiotic factors such as the age and weight of fish, place of capture, the tissues studied, and the statistical model employed could distort results.

In this study, differences in the main trace elements (Ca, Fe, K, Mg, Na, P, S, Cu, Mn and Zn) in four soft tissues (liver, kidney, brain and muscle) of juvenile ABFT (less than 1-year old) from different batches (wild, raised in onshore tanks and in sea cages) were investigated. Two multivariant statistical models were used to classify the origin of the fish: principal component analysis (PCA) and discriminant canonical analysis (DCA).

Material & Methods

i. Sample collection

Samples of ABFT weighing less than 1000 grams were taken in 2018 (for batch 1, 2 and 3: 24-22-28, liver; 15-13-9, kidney; 24-22-27, muscle, and 24-15-29, brain). The fish of batch 1 and 2 consisted of ABFT hatched from eggs from naturally spawning captive adults in sea cages and raised in the facilities of the Spanish Institute of Oceanography (Mazarrón, Spain). The larval culture was fed on rotifer and copepod in a 40-m^3 tank; weaned fish were fed an artificial diet (Magokoro S-3, Marubeni Nissin Feed Co., Ltd., Tokyo, Japan) and maintained at 24.9°C at a salinity of 37.5 g L^{-1} in a 20-m^3 tank. At 41 days post-hatching (individuals with suffisent body mass to be transported to the tanks and sea

cages), the specimens were split into two groups: fish of batch 1 was transferred to a 900-m^3 overflow system tank in the Infraestructura de Control de Reproducción del Atún Rojo (Cartagena, Spain) where they were fed with herring *Clupea spp.*, round sardinella *Sardinella aurita* and Atlantic mackerel *Scomber scombrus*; fish of batch 2 was placed in floating cages in the sea at Cartagena (37°34'39.2"N, 0°52'35.9"O). All fish dying due to traumatic events were collected soon after death and sampled. The batch 3 (wild tunas) were caught by the hook-and-line method (barbless hook) in October 2018 in Mazarrón Bay (Murcia, Spain) and sampled immediately after capture. In accordance with European legislation (Directive 2010/63/UE), the procedures employed did not require ethical permissions.

Tissue samples taken from liver, kidney, muscle and brain were frozen immediately and stored at -20°C until analysis. Muscle samples were taken from the front of the head, liver samples from the ventral and cranial regions, and kidney samples from the cranial region; the brain was removed whole (or as intact as possible).

ii. Sample preparation and elemental analysis

To determine the concentrations of Ca, Fe, K, Mg, Na, P, S, Cu, Mn and Zn, samples were analysed using inductively coupled plasma optical emission spectrometry (ICP-OES, *ICAP 6500 Duo, Thermo Scientific,* Waltham, USA). Samples (0.1–0.2 g) were treated with 4 mL of trace mineral grade HNO_3 (69% Suprapure, *Merck*, Darmstadt, Germany) and 1 mL of H_2O_2 (33% Suprapure, *Merck*, Darmstadt, Germany) in special Teflon reaction tubes and heated at 220ºC in a microwave digestion system (UltraClave-Microwave Milestone®, Sorisole, Italy) for 20 minutes, and then diluted with double deionised water to 10 ml. The detection limit (DL) was 10 µg g^{-1} for major constituents (Ca, K, Mg, Na, P and S) and 0.001 for the other elements. For every sample, two readings were made, the mean of which was used as the concentration value. To check for

possible metal contaminants, one blank sample for every 11 samples was also analysed.

Multi-element calibration standards (SCP Science, in 4% HNO^3, Québec, Canada) were prepared with specific concentrations for each element, taking as a reference UNE-EN ISO 11885 for the determination of elements by ICP-OES. Furthermore, intermediate patterns of all elements were prepared. The recovery percentages of standar reference material (1577b -National Institute of Standars & Technology- Dicoex, Bilbao, Spain) were 91.62 (Ca), 97.99 (Cu), 106.86 (Fe), 98.33 (K), 103.79 (Mg), 111.21 (Mn), 98.06 (Na), 97.48 (P), 99.48 (S) and 96.34 (Zn).

iii. Statistical analysis

The results obtained were subjected to statistical analysis using the SPSS software (*Statistical Package for the Social Sciences*, *IBM 24.0*, New York, USA). For the elemental concentrations, means and standard deviations were obtained. An ANOVA (Tukey and Games-Howell *post hoc* tests) test was used as a statistical method to study differences between specimens of different batches, while Levene's test was used to assess the homogeneity of variance. The significance levels for all tests were set at 0.05.

In order to classify the batch of the fish using the chemical data, two multivariate techniques were used: PCA and DCA. For the PCA, a threshold factor loading of 0.32 corresponding to an explained average variance of 56.6% was considered (Peterson, 2000). In addition, to evaluate the validity of the method, the Kaiser Meyer Olkin (KMO) index, a p-value lower than 0.05 (Bartlett's Test of Sphericity) and the eigenvalue criterion (greater than 1) were employed. For the DCA, Wilk's Lambda was used to test the significance of the discrimination ($p<0.05$). Two functions were created, and a split-sample validation (cross-validation testing procedure) was performed to assess the capacity of the selected variables to

predict different batches for the tested fish. In this validation, one individual is removed from the original matrix. The DCA is then performed using the remaining observations to classify the omitted individual; the number of misclassified individuals indicate the degree of intermingling, while the proportion of individuals correctly reallocated is taken as an integrity measurement for a group (Poulet et al., 2005; Yakubu & Osenbor, 2011). The formulas from the case classification were obtained to classify the new specimens of unknown batch. In these formulas, the constant and function coefficients were obtained for each of the tissues, batches and elements:

$$F(x) = a + (b * [X])$$

where a = a constant for the combination of a tissue and a batch; b = a coefficient of classification function for the combination of an element and batch; and X = the concentration of an element for a given tissue and batch (in a particular specimen). Once the formula has been applied, the result with the highest value indicates the possible batch of the fish.

Results

The concentrations of the trace elements detected in ABFT tissues are shown in **Table V.I.1**. Copper was the only element with statistical differences between groups for all tissues; no differences between batches for any tissue were found for Ca, Na and Zn. Of all the tissues, the liver had the fewest elements with significant differences.

Table V.I.1. Concentration of trace elements in tissues of ABFT. Data: mean ± standard deviation, µg g^{-1}, ww. For each element and tissue, the same superscript letter shows statistical differences between batches (1- tanks, 2- sea cages, 3- wild); superscripts in parentheses means marginally significant (p=0.05-0.1).

	Batch	n	Ca	Cu	Fe	K	Mg	Mn	Na	P	S	Zn
Liver	1	24	161±109	2..45±0.8^{b}	116±53	2647±292^{a,b}	260±88.6	3.25±1.2	1861±912	2433±423$^{(b)}$	2953±466$^{(b)}$	25±6
	2	22	174 ±129	3.37±2.3	103±46	3124±328^{a,C}	256±109	3.31±1	1774±832	2620 ±390	2952±476$^{(c)}$	27.5±4
	3	28	170±85.9	4±2^{b}	94.7±40.9	3627±770^{b,c}	306±45.5	3.36±0.7	2155±1104	2777±678$^{(b)}$	3272±560$^{(b)\ (c)}$	26.8±5.2
Kidney	1	15	244 ±125	12.7±10.9^{a,b}	182±62^{a,b}	3059±555^{a,b}	213±39.4$^{(b)}$	2.23±0.8	2002±884$^{(b)}$	3087±576	2820±482	31.4±7.2
	2	13	185±160	2.07±1.3^{a}	87.4±32.3^{a}	4142±510^{a}	272±100	2.49±1.02	1383±935	2974±660$^{(c)}$	3259±727^{c}	28.2±6.8
	3	9	243±56.4	1.53±0.4^{b}	66±21^{b}	4291±443^{b}	296±82.2$^{(b)}$	2.39±0.9	1181±465$^{(b)}$	3554±480$^{(c)}$	2407±377^{c}	27.1±8.4
Muscle	1	24	84.4±67.9	0.281±0.1^{b}	3.05±0.8^{a,b}	3657±494^{b}	278±52.1^{b}	2.34±0.5	807±564	2800±833	2415±423	5.28±2.2
	2	22	124±135	0.314±0.05^{c}	4.31±1.8^{a}	3733±479^{c}	285±44.8^{c}	2.59±0.4	998±657	2964±396	2464±534	5.09±1.1
	3	27	112±173	0.515±0.08^{b,c}	4.53±1.4^{b}	4365±734^{b,c}	346±56.9^{b,c}	2.32±0.7	677±410	3018±845	2613±478	4.56±1.3
Brain	1	24	142±35.8	0.98±0.2^{b}	32.9±16.3	2284±375	93.4±30.3^{b}	0.69±0.2^{a,b}	2462±713	2401±337	1768±243	11.2±7.8
	2	15	183±66.2	1.12±0.2^{c}	38.7±25.4	2338±485	121±34	1.34±0.4$^{a,(c)}$	2791±537	2737±679^{c}	1624±256	8.87±1.3
	3	29	185±145	1.508±0.4^{b,c}	28.8±13.7	2388±523	129±55.5^{b}	1.06±0.5$^{b,(c)}$	2906±1190	2201±470^{c}	1687±221	8.23±2.2

In the PCA, the integration of all 10 elements was represented by four (liver and muscle) and three (kidney and brain) principal components that explained 71–79% of the total variance in the original data set. The KMO index was low in all cases (liver=0.462, kidney=0.521, muscle=0.529 and brain=0.563). For kidney, the fishes from the batch 1 (onshore tank) were separated from the other groups in PC1 (Cu, Fe, Na and K; **Figure V.I.1a**); for muscle, fishes of batch 3 (wild tunas) were separated based on component 2 (Cu, K and Mg; **Figure V.I.1b**); and for liver and brain, no differentiation between groups was observed (**Figures V.I.1c** and **V.I.1d**).

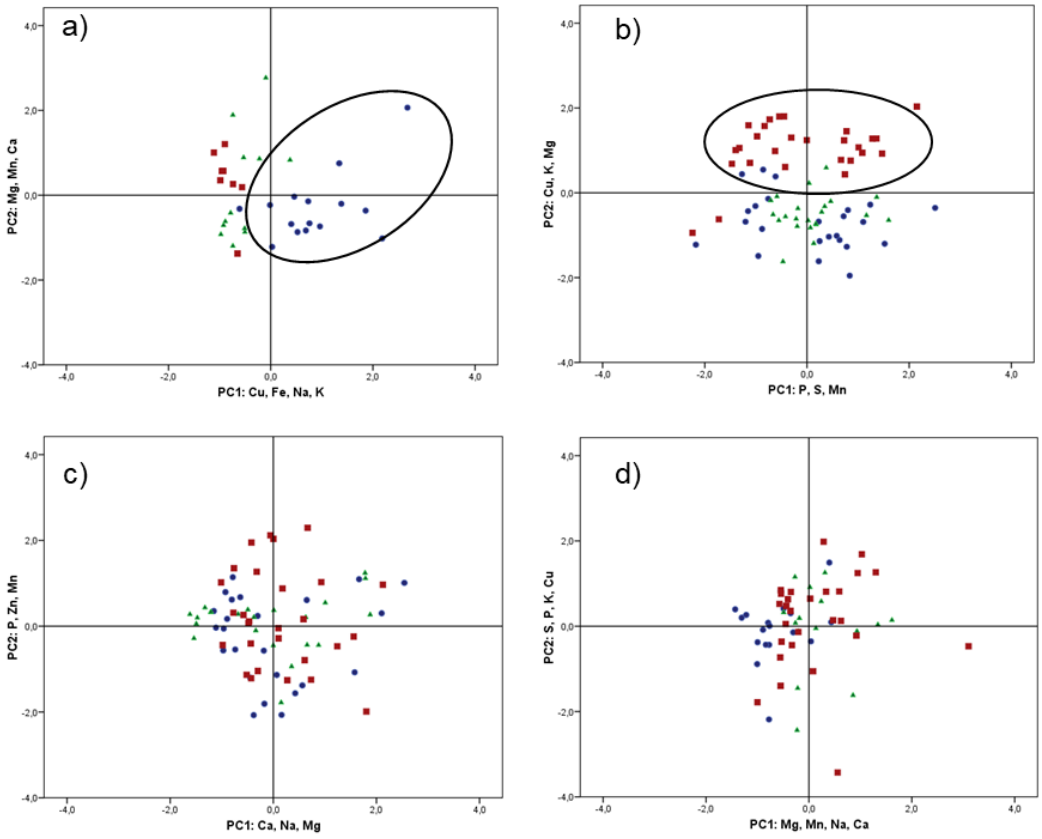


Figure V.I.1. PCA analyses carried out with 10 trace elements: Ca, Fe, K, Mg, Na, P, S, Cu, Mn, Zn. a) Kidney; b) Muscle; c) Liver; d) Brain. Two components (axis 1 and axis 2) explaining 45.3 (liver), 53.0 (kidney), 46.8 (muscle) and 53.8% (brain) of the total variance. The circle shows the exemplars grouped due to their similar characteristics. ● Batch 1; ▲ Batch 2; ■ Batch 3.

With the DCA, three elements (Ca, Na and Zn) were not considered in any tissues. Data from the canonical discriminant functions (CDF) are shown in **Table V.I.2**.

Of the three different fish batches, two CDFs were created for kidney, muscle and brain. Membership of the predicted groups in terms of cross-validation are shown in **Table V.I.3**, while the formulas for the case classifications are given in **Table V.I.4.** The percentage of cases correctly classified using this validation were 60.8% (liver), 88.6% (kidney), 79.5% (muscle) and 82.2% (brain). Differences between groups are shown in **Figure V.I.2** (the histogram for liver only shows one element, K) and **Figure V.I.3** (dispersion plot of CDF for kidney, muscle and brain).

Table V.I.2. Canonical discriminant functions and statistic data (DCA) from ABFT soft tissues.

	Function	Eigenvalue	% variance	Canonic correlation	Lambda of Wilks	Canonical Discriminant Function Coefficients (Standarized)
Liver	1	0.615	100.0	0.617	0.619, p<0.001	K (1.0)
Kidney	1	4.02	78.4	0.895	0.095, p<0.001	Fe (0.69), K (-1.06), P (0.151), S (0.475)
	2	1.11	21.6	0.725	0.475, p<0.001	Fe (-0.004), K (0.223), P (-1.09), S (1.07)
Muscle	1	5.05	95.9	0.914	0.136, p<0.001	Cu (1.23), Fe (0.041), Mn (-0.517), Zn (-0.4)
	2	0.215	4.1	0.421	0.823, p<0.05	Cu (-0.217), Fe (0.914), Mn (0.74), Zn (-0.738)
Brain	1	2.68	75.4	0.853	0.145, p<0.001	Cu (1.03), Mg (1.61), Mn (-1.86), P (-0.851), S (0.164)
	2	0.873	24.6	0.683	0.534, p<0.001	Cu (0.629), Mg (-0.196), Mn (0.791), P (0.204), S (-0.792)

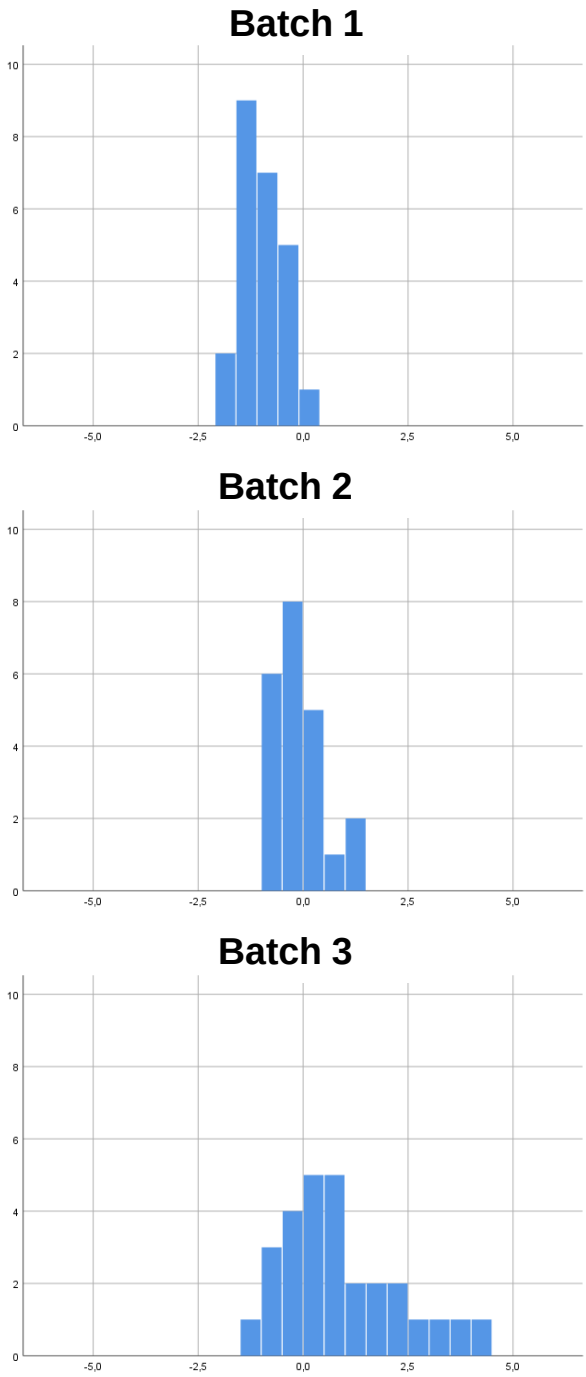
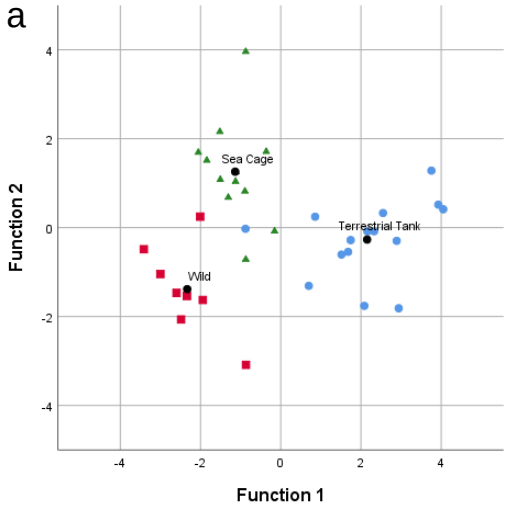
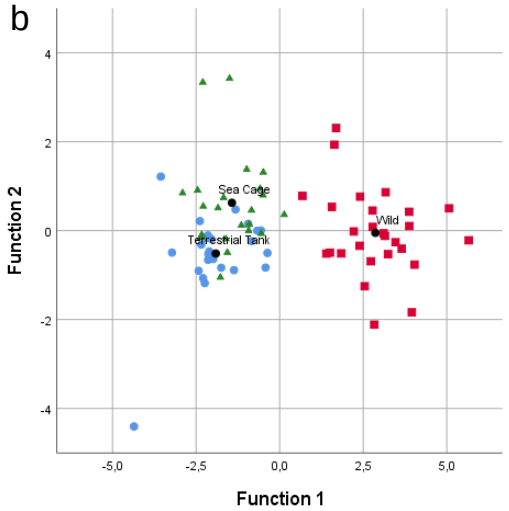


Figure V.I.2. Canonical Discriminant Funcions of the liver from the three different batches (for the element K). Batch 1 = Onshore tanks, Batch 2 = Sea cages; Batch 3 = Wild.

a


Sea Cage
Terrestrial Tank
Wild
Function 2
Function 1
4
2
0
-2
-4
-4
-2
0
2
4

b


Sea Cage
Wild
Terrestrial Tank
Function 2
Function 1
4
2
0
-2
-4
-5,0
-2,5
0,0
2,5
5,0

c

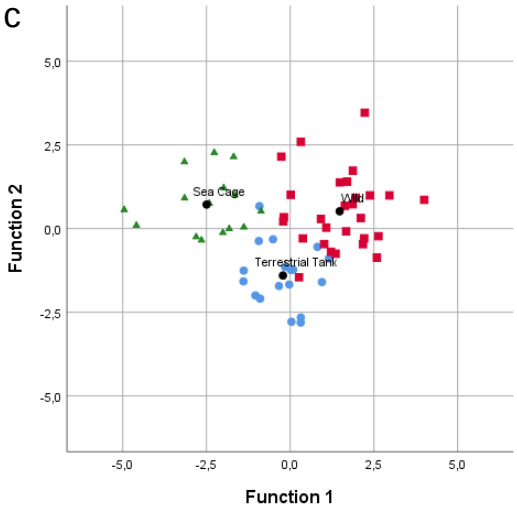

Sea Cage
Wild
Terrestrial Tank
Function 2
Function 1
5,0
2,5
0,0
-2,5
-5,0
-5,0
-2,5
0,0
2,5
5,0

Figure V.I.3. Batch spatial distribution based in functions outcoming from the DCA analysis and group separation by tissue. a= kidney; b= muscle; c= brain. The small point in the middle shows the group centroid. ● Batch 1; ▲ Batch 2; ■ Batch 3.

Table V.I.3. Pronosticated belonging groups: DCA classification accuracy (*) or missclasification (remaining) by batch and tissue from the cross-validation test. Data=percentage.

	Batch	1	2	3
Liver	**1**	75.0*	25.0	0.0
	2	31.8	36.4*	31.8
	3	14.3	17.9	67.9*
Kidney	**1**	86.7*	13.3	0.0
	2	0.0	91.7*	8.3
	3	0.0	12.5	87.5*
Muscle	**1**	70.8*	29.2	0.0
	2	31.8	68.2*	0.0
	3	0.0	3.7	96.3*
Brain	**1**	83.3*	5.6	11.1
	2	0.0	93.3*	6.7
	3	10.7	7.1	82.1*

Table V.I.4. Classification case formulas; [element]= element concentration for the case to be classified.

	Batch	Formula
Liver	1	-13.4+(92.8*[K])
	2	-18.3+(110*[K])
	3	-24+(127*[K])
Kidney	1	-25.1+(0.061*[Fe])+(66.3*[K])+(22.8*[P])+(35.7*[S])
	2	-34.6+(0.012*[Fe])+(140*[K])+(-14.1*[P])+(37*[S])
	3	-37.1+(-0.005*[Fe])+(153*[K])+(32*[P])+(-23.6*[S])
Muscle	1	-13.6+(42.7*[Cu])+(0.418*[Fe])+(4.54*[Mn])+(0.207*[Zn])
	2	-17.5+(48.2*[Cu])+(1.18*[Fe])+(5.64*[Mn])+(-0.432*[Zn])
	3	-35.4+(133*[Cu])+(0.862*[Fe])+(0.631*[Mn])+(-1.18*[Zn])
Brain	1	-30.3+(1.27*[Cu])+(-578*[Mg])+(4.32*[Mn])+(-35.3*[P])+(384*[S])
	2	-30+(-2.23*[Cu])+(-1495*[Mg])+(19.6*[Mn])+(12.3*[P])+(297*[S])
	3	-30.9+(11.5*[Cu])+(-48.9*[Mg])+(0.12*[Mn])+(-56.4*[P])+(331*[S])

Discussion

Elemental composition in tuna tissues is commonly used to determine the concentration of pollutants – mainly Hg – in commercial-size specimens for reasons of food safety (i.e., Annibaldi et al., 2019). However, trace element composition has attracted interest in recent decades in the food industry (Percin et al., 2011) and some studies have been performed on ABFT (i.e., Sogut and Percin, 2011; Sogut et al., 2011; Ugarte et al., 2012; Belmonte et al., 2021) to detect elements such as Cu, Mn, Ni, Zn, Fe, Mg and Se in tissues including muscle, liver, kidney, heart, brain, bone, gill and the first dorsal spine. However, to date these studies have only provided data for wild and fattened tuna weighing over 50 kg, and not for juvenile fish.

i. Trace element concentrations

In our study, only one of the elements (Cu) showed statistically significant differences for fish batch in all tissues (**Table V.I.1**). This essential trace element is required for cellular functioning (Lall & Kaushik, 2021) and previous studies of ABFT have reported similar concentrations to those we found in muscle (Di Bella et al., 2015; Vizzini et al., 2010; Milatou et al., 2015; Ugarte et al., 2012). In this tissue, Percin et al. (2011) reported significantly higher concentrations in wild than in farmed tuna, which agrees with our results. In kidney, other authors have reported statistical differences between wild and farmed tunas, with greater concentrations in wild fish (Sogut and Percin, 2011), which contradicts our results, probably due to the high coefficient of variation detected in kidney from tuna from the onshore tanks (batch 1). In liver and brain, a similar pattern was found for Cu concentrations, with statistical differences observed between wild (batch 3) and onshore tank tunas. Vizzini et al. (2010) report similar Cu concentrations in the liver of wild and farmed tunas. To the best of our knowledge, no data regarding significant differences in Cu in brain of this fish have ever been reported.

The remaining elements (K, Mg, Fe, S, Mn and P) are essential for fish health (National Research Council, 1993, 2011). Potassium is an important cation involved in the acid:base balance and osmoregulation (Lall, 2002), and we found statistical differences for K between the batches of fish in three tissues (liver,

kidney and muscle, **Table V.I.1**). No data on K in tunas could be found in the literature. Magnesium is an important macroelement present in soft tissues such as muscle (Knox et al., 1981). Our data reveal greater concentrations ($p<0.05$) in wild specimens, similar to those reported by Ugarte et al. (2012). In addition, there were statistical differences for this element between brain tissue from tuna from the onshore tank and the wild tuna, although, once again, no previous references in the literature to Mg in brain could be found. Iron is an essential trace element for vertebrates (Lall & Kaushik, 2021) and is used mainly in the production and functioning of enzymes including haemoglobin, myoglobin and cytochromes. Iron concentrations were also higher ($p<0.05$) in muscle in wild tuna than in onshore tanks tuna (4.53 vs. 3.05 μg g^{-1}, respectively). Even though the statistical differences between these two batches agree with those reported by other authors (Percin et al., 2011), the levels we detected were lower than those reported by these and other authors (Di Bella et al., 2015; Milatou et al., 2015; Ugarte et al., 2012; Girolametti et al., 2021); in general, tunas are deemed a good source of Fe (HealthLinkBC, 2020). In the studied batches, the Fe differences could be related to the distinct feeding conditions, a fact also observed in Percın et al. (2011). For consumers, Fe intake would be slightly lower in the case of farmed tunas' muscle, but the edible part of both groups would be considered good for the intake of this element. In kidney, a tissue that eliminates Fe (Bury et al., 2012), the inverse situation was found (lower concentrations in wild tuna), which contrasts with the results of Sogut and Percin (2011). These authors also reported lower concentrations than those found in this study in kidney (10.4–14.02 vs. 66–182 μg g^{-1}, respectively), which could be due to the different weights of the tunas studied (54–57 kg vs. 0.3–1.0 kg). Sulphur, Mn and P are all relevant elements in biochemical processes and are constituents of amino acids or nucleotides (Leach et al., 1997; Aschner et al., 2005; National Research Council, 2011; Lall & Kaushik, 2021). We only found statistical differences between onshore tanks and wild tunas in Mn in brain (**Table V.I.1**), a result that differs from the results reported in muscle by Percin et al. (2011) and kidney by Sogut and Percin (2011) in tunas weighing approximately 50 kg. Interestingly, we found high levels of Mn in all tissues, higher than those reported in muscle, liver and kidney

by other authors (Percin et al., 2011; Sogut and Percin, 2011; Di Bella et al., 2015; Licata et al., 2005; Ugarte et al., 2012).

Another noteworthy finding was that most wild tuna had greater ($p<0.05$) concentrations of elements than tuna reared in onshore tanks (**Table V.I.1**). According to Percin et al. (2011), differences in tissue accumulation might be related to factors such as weight, feeding profile or habitat. In our study, although fish were chosen specifically with similar weights, there were small differences between the specimens kept in onshore tanks and wild tunas. Only three correlations with weight were detected: S in kidney in tuna from the sea cage (batch 2) and Mg in muscle and brain in tuna from the onshore tanks (batch 1). Therefore, elements such as Cu, K, Fe, Mn and P could be used to study the batches and origin of fish.

In relation with food safety, none of the analysed elements in this study is described in the Regulation 1881/2006 (and posterior modifications), which control the maximum allowed concentrations for some metals in the UE (Pb, Cd, Hg, As and inorganic Sn). Nevertheless, the studied elements could be considered as toxic if found at high levels in the edible parts of tuna (i.e., Cu and Fe; Tietz et al., 1990; Watanabe et al., 1997; Olsson, 1998; Percin & Konyalioglu, 2008; Vizzini et al., 2010). In addition, the specific legislation to food safety is complex and perpetually evolving (Bondoc, 2016) and the levels of trace element have attracted interest in recent decades in the food industry, developing more strict regulations (Percin et al., 2011). Specifically, ABFT has a wide food spectrum and a long-life span (Santamaria et al., 2009) with a life cycle of 20 years (Chase, 2002). Therefore, the (bio)accumulation of trace elements in soft tissues is quite important (Licata et al., 2005; Storelli et al., 2005; Kojadinavic et al., 2007; Tuzen & Soylak, 2007; Yildirim et al., 2009; Vizzini et al., 2010; Cammilleri et al., 2017). However, the standards of trace elements from fish muscle that we could consider (USEPA, 1989; MAFF, 2000; USDA, 2009) offer differing ranges of concentration (i.e., for Cu: 20.0 $\mu g\ g^{-1}$ in MAFF, 120 $\mu g\ g^{-1}$ in USEPA, and 0.86 $\mu g\ g^{-1}$ in USDA; see Percin et al., 2011 for detailed information)

and it could not signal unequivocable if the concentrations found are or not above these limits.

ii. Principal Component Analysis

In order to obtain an overall picture of elemental composition in tuna of different batches, all the trace elements were integrated into a PCA for each tissue (**Figure V.I.1**). Bartlett's Test of Sphericity, the eigenvalue criterion and explained variance were appropriate in all tissues, although the KMO indices were low (0.462–0.563). According to Shrestha (2021), if the KMO is below 0.5, the results are not suitable for data analysis and so the PCA for liver was not taken into account. For kidney, while batch 1 tunas were clearly different, batch 2 and 3 specimens were not (**Figure V.I.1a**). In fish, the kidney has both exocrine and endocrine functions, i.e., hormone production and haematopoietic functions (Hyttel et al. 2009; Zapata & Amemiya 2000; Press & Evensen, 1999), and play a vital role in osmoregulation and homeostasis (Davidson, 2014). Therefore, differences in the characteristics of the water (batch 1, onshore tank tunas) could have affected the results. In addition, Cu and K (two elements with statistical differences for their origins) were part of the principal component that separated the groups (PC1). Finally, a clear separation between the batches was found for muscle (**Figure V.I.1b**). In this case, specimens from the batch 1 and 2 were mixed in both components (PC1 and PC2); meanwhile Cu, K and Mg (forming the PC2) separated the batch 3 from the other groups (Cu, K and Mg were elements with statistical differences between batch 3 and the other two batches). Several authors have reported that diet is the main source of Cu and Mg (Lall, 2002; Cowey et al., 1977; Bury et al., 2003; Kamunde et al., 2002). For these elements, the wild specimens (batch 3) had higher concentrations than those found in remaining groups, being the tendance wild > sea cages ≥ tanks. Tank and sea cage individuals (batch 1 and 2, respectively) were fed on defrosted bait *ad libitum*, but wild juveniles have an opportunistic diet with the presence of shrimps, cephalopods and crustaceans (Uotani et al., 1990; Sarà & Sarà, 2007; Sinopoli et al., 2004), especially in the Mediterranean Sea (Karakulak et al., 2009; Van Beveren et al., 2016). In cephalopods and crustaceans, microelements play essential roles in biological functions (Rjeibi et al., 2015, cephalopods; Jacobo et

al., 2016, crustaceans). This diet could explain the higher concentration in wild's muscle of Cu and K. However, as stated above, data for Mg should be viewed with caution due to the correlation found in muscles in fish of batch 1 (onshore tank). In summary, this statistical model does not allow us to discriminate these groups for muscle.

iii. Discriminant Canonical Analysis

The DCA enables differences between populations to be maximized or made more evident (Balzarini et al., 2015). In this test, for each tissue a pair of functions describing the differences between the three batches was created. These functions are composed of some of the elements selected by the analytical software as the most discriminant (Yakubu & Okunsebor, 2011). Wilk's lambda was used to test the significance of the discrimination, which were significant for all functions (**Table V.I.2**).

Calcium and Na are key elements involved in functions such as the development and maintenance of the skeletal system, the osmotic balance, and the acid:base equilibrium (Lall, 2002; Zimmer et al., 2019; Lall & Kaushik, 2021). Nevertheless, these elements showed non-validity for this method of analysis (**Table V.I.2**) and so no data for Ca and Na were taken from the tuna tissue.

Only one function was found for liver (K), which had the lowest classification value (60.8%); the remaining elements were used to build two functions for kidney, muscle and brain (**Table V.I.2**). Again, good separation in kidney was found (88.6%, cross-validation) with no confusion between specimens from the batches 1 and 3 (**Table V.I.3**). In terms of functions, there were two elements (Fe and K) in kidney that also showed differences between groups in the ANOVA test and PCA, so *a priori* these tissues probably could be used to ascertain the batch of fish. However, the batch 2 (sea-cage fish) was the best identified group (91.7%), in contrast to the PCA results (batch 1, onshore tanks).

Muscle was the third-best tissue for identifying the batch of fish (79.5%), although a number of factors should be borne in mind. First, specimens of batch 3 were discriminated in 96.3% of cases and there was no confusion with tunas of batch 1, and only in 3.7% of cases was there confusion with specimens from batches 2

and 3 (**Table V.I.3**). This is a very interesting result that could be used to differentiate different batches as wild and reared tuna. Second, the batch 3 was a separate group in the PCA for muscle, which adds to the potential of this tissue for discriminate the origin of fish. Finally, Cu was conclusive in all three statistical analyses. According to a number of authors (Bury et al., 2003; Kamunde et al., 2002), diet is the major source of the Cu required for physiological functions, growth and fish development. Myoglobin (an abundant protein in muscle) is one of the most important compounds containing Fe (Lall & Kaushik, 2021). Percin et al. (2011) state that Mn and Zn (also included in functions of DCA) are useful indicators of the origin of tuna due to their intensive feeding regimes and transport, and to their migration routes and alimentation, respectively. Thus, the control of these two elements in tuna diets could help improve this information and identify the batches and thus the origin of specimens. However, a disadvantage of the use of this tissue is the confusion between batch 1 and 2 specimens (**Table V.I.3**), probably due to their similar diet of small frozen pelagic species (De la Gándara et al., 2010).

Finally, for brain (a small organ in tunas), there was a high percentage of discrimination (82.2%), which thus allows us to identify specimens from the batch 2 (sea cage tunas, 93.3% of success), and a low percentage of uncertainty between fish of batches 1 and 2 (**Table V.I.3**). However, the inherent difficulties involved in obtaining this tissue and the possibilities of confusion between reared and wild tuna make this tissue of little use for analysing the batch of specimens.

Conclusion

The essential elemental composition in soft tissues in ABFT could be used to discriminate different tuna batches. For some elements, ANOVA tests using results for all tissues reveal differences between batches of fish, while a PCA can differentiate groups of specimens using tissue from kidney and muscle. A DCA can generate formulas for identifying the possible batch of specimens. Muscle appears to be the best tissue to be used with this tool and wild specimens can be readily identified (PCA and DCA), although in reared specimens the differences

between fish are more complicated. In muscle, the elements selected for analysis are the essential, present in high enough concentrations to guarantee good analytical results. Future research into the elemental composition of tuna diet and different origins of fishes could provide fresh data that can be used to identify juvenile tunas.

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CHAPTER II

Composition of inorganic elements in the hard tissues of juvenile *Thunnus thynnus*

Abstract

Atlantic bluefin tuna aquaculture has developed quickly in the last years, and it is important to be able to distinguish between tunas coming from aquaculture and fisheries. In this study we establish a novel discrimination method based on the chemical composition of discard tissues (gills and bone). Three tuna batches were studied: wild, cultured-reared in onshore tanks, and culture-reared in sea cages. Eleven macroelements were analyzed and their concentrations were checked using ANOVA and two multivariate tests: Principal Component and Discriminant Canonical Analysis. Gills were the best tissue for discriminating between these batches, from which wild tuna were the easiest to identify. Mg, Mn and S are the best elements for differentiating tuna groups. Both the mean concentration comparisons and the Discriminant Canonical Analysis were the most successful methods for discrimination.

Keywords: bluefin tuna, bone, discrimination, gill, inorganic element, juvenile.

Introduction

In vertebrates, hard tissues are key structures that give support to the organism and at the same time store minerals. Inorganic elements such as calcium (Ca), phosphorous (P), magnesium (Mg), manganese (Mn) and boron (B) are important in these tissues as they participate in bone mineralization and indirectly affect other physiological functions such as the bioavailability of zinc (Zn) (Aschner & Aschner, 2005; Lall & Kaushik, 2021). Also, the Ca:P ratio in bones changes as fish develop and some elements are mobilized from bones when dietary Mg intake is low (Cowey et al., 1977; Lall & Kaushik, 2021). On the other hand, gills are a vital route for mineral uptake including elements such as copper (Cu), sodium (Na), iron (Fe), Mn, selenium (Se), Zn, chromium (Cr), cobalt (Co), Ca, P and Mg (Miller et al., 1980; Hodson & Hilton, 1983; Pedersen et al., 1998; Dabrowska et al., 1991; Shearer & Åsgård, 1992; Rouleau et al., 1995; Baudin et al., 2000; Bury & Grosell, 2003; Bury et al., 2003; Taylor et al., 2003; Evans & Clairbone, 2009; Blust, 2012; Hogstrand, 2012; Grosell, 2012; Lall & Kaushik, 2021). Potassium (K) is important for the osmotic balance and acid-base equilibrium (Lall, 2002), while sulphur (S) is considered to be an indispensable nutrient for all living organisms (Kormarnisky et al., 2003) given its role as an essential component in amino acids, proteins, enzymes, vitamins and other molecules. Finally, strontium (Sr) has been shown to have key biological functions including increasing bone mineral density (Siccardi et al., 2010) in certain species.

Although the aquaculture of the Atlantic bluefin tuna (*Thunnus Thynnus*) (hereafter, ABFT) is still currently based on intensive production after capture (see review in De la Gándara et al., 2016), there are improvements since the closure of their life cycle in captivity in 2016 (Ortega & De la Gándara, 2017). European regulations prohibit the catch of ABFT under 30 kg in weight or with a fork length of less than 115 cm (Regulation (UE) 2016/1627). However, in the future, ABFT juveniles born in captivity will be commercialized, so it will be necessary to establish methods for discriminating tuna batches (i.e., wild or captivity-reared tuna). Thus, a natural method for identifying batches, based on

the influence that culture conditions exert on tissue configuration during fish growth and development can be an extremely useful tool (Jara & Chodyniecki, 1999; Brucka et al. 2009). In this sense, hard tissues are rich in minerals and so their chemical composition profile could be of future application in the fisheries sector (Cubadda et al., 2006) as differential tools. Thus, the aim of this study was to evaluate mineral concentrations in bones and gills of ABFT from three separate batches (wild tuna and two captive-reared batches raised in different environments) and then to evaluate their relationship by multivariant statistical models.

Material & Methods

i. Sample collection

Samples of 74 ABFT weighing less than 1000 g with three different locations were taken in 2018: onshore tanks (batch 1), sea cages (batch 2) and wild tunas (batch 3) (n=24-22-29 for gill, and n=24-22-28 for bone). The fish of batch 1 and 2 consisted of ABFT hatched from eggs from naturally spawning captive adults in sea cages and raised in the facilities of the Spanish Institute of Oceanography (Mazarrón, Spain). The larval culture was fed on rotifer and copepod in a 40-m^3 tank; weaned fish were fed an artificial diet (Magokoro S-3, Marubeni Nissin Feed Co., Ltd., Tokyo, Japan) and maintained at 24.9°C at a salinity of 37.5 g L^{-1} in a 20-m^3 tank. At 41 days post-hatching (individuals with suffisent body mass to be transported to the tanks and sea cages), the specimens were split into two groups: fish of batch 1 was transferred to a 900-m^3 overflow system tank in the Infrastructure for Atlantic bluefin tuna aquaculture (Infraestructura de Control de Reproducción del Atún Rojo, Cartagena) where they were fed with herring *Clupea spp.*, round sardinella *Sardinella aurita* and Atlantic mackerel *Scomber scombrus*. Fish of batch 2 was placed in floating cages in the sea at Cartagena (37°34'39.2"N, 0°52'35.9"O). The specimens were collected soon after their natural death and then sampled. The batch 3 were caught by the hook-and-line method (barbless hook) in October 2018 in Mazarrón Bay (Murcia, Spain) and sampled immediately after capture. In accordance with European legislation (Directive 2010/63/UE), the procedures employed did not require ethical permissions.

Bone and gill samples were collected and washed with purified water (MilliQ), then with nitric acid (2%) and again with MilliQ water, before being dried at room temperature. All samples were stored at -20˚C until analysis. Gill samples were collected from the bony denticles of the branchial arch.

ii. Sample preparation and mineral analysis

Tissue samples were pre-treated as described elsewhere (Salvat-Leal et al., 2023). Briefly, 0.5–1.0 g of samples was submitted to acid digestion using trace mineral grade HNO_3 (69%) and H_2O_2 (33%) in a microwave digestion system (UltraClave-Microwave Milestone®, Sorisole, Italy) at 220°C for 20 min. Then, samples were diluted with 10 mL of Type 1 purified water (Milli-Q®) and inorganic element (Ca, Fe, K, Mg, Na, P, S, Cu, Mn, Zn and Sr) concentrations were determined using an Inductively Coupled Plasma Optical Emission Spectrophotometer (ICP-OES, *ICAP 6500 Duo Thermo Scientific*, Waltham, USA). The recovery percentages of standar reference material (1577b -National Institute of Standars & Technology- Dicoex, Bilbao, Spain) were 91.62 (Ca), 106.86 (Fe), 98.33 (K), 103.79 (Mg), 98.06 (Na), 97.48 (P), 99.48 (S), 97.99 (Cu), 111.21 (Mn), 96.34 (Zn) and 73.73 (Sr). All concentrations are expressed in microgram per gram dry weight. The detection limit (DL) was 10 µg g^{-1} for major constituents (Ca, K, Mg, Na, P and S) and 0.001 µg g^{-1} for the remaining elements.

iii. Statistical analysis

The results obtained were analyzed using SPSS software (*Statistical Package for the Social Sciences*, *IBM 24.0*, New York). For the mineral concentrations, geometrical means and standard errors were obtained. The Kolmogorov-Smirnov was used to test the normality and Levene's test to assess the homoscedasticity of the data. A General Linear Model (GLM), with HSD Tukey and Scheffe as *post-hoc* tests, was used to analyze the relationship between weight, elemental concentrations and the fish batch, meanwhile Kruskall-Wallis ANOVA test was used as a statistical method to study differences between batches. The significance levels for all tests were set at 0.05.

In order to classify the group of the fish using the chemical data, two multivariate tests were used: Principal Component Analysis (PCA) and Discriminant Canonical Analysis (DCA). For the PCA, a threshold factor loading of 0.32 corresponding to an explained average variance of 56.6% was considered (Peterson, 2000). In addition, to evaluate the validity of the method, the Kaiser Meyer Olkin (KMO) index, a *p-value* lower than 0.05 (Bartlett's Test of Sphericity) and the eigenvalue criterion (greater than 1) were employed. For the DCA, Wilk's Lambda was used to test the significance of the discrimination ($p<0.05$). Two functions were created, and a split-sample validation (cross-validation testing procedure) was performed to assess the capacity of the selected variables to predict the batches of the tested fish. In this validation, one individual is removed from the original matrix. The DCA is then performed using the remaining observations to classify the omitted individual; the number of misclassified individuals indicate the degree of intermingling, while the proportion of individuals correctly reallocated is taken as an integrity measurement for a group (Poulet et al., 2005; Yakubu & Osenbor, 2011). The formulas from the case classification discriminate new specimens of unknown batch. In these formulas, the constant and function coefficients were obtained for each of the tissues, groups and elements:

$$F(x) = a + (b * [X])$$

where a = a constant for the combination of a tissue and a batch; b = a coefficient of classification function for the combination of an element and batch; and X = the concentration of an element for a given tissue and batch (in a particular specimen). Once the formula has been applied, the result with the highest value indicates the possible group of the fish.

Results

Mean weights were 592.96 ± 131.69 g (onshore tanks), 520.74 ± 98.06 g (sea cage) and 517.44 ± 104.19 g (wild tunas). No direct influence of fish weight on elemental concentrations was detected (GLM, $p>0.05$). The detected concentrations of inorganic elements in the gills and bones of juveniles of ABFT

are shown in **Table V.II.1**. The element with statistical differences between groups for both gills and bones was Cu; by contrast, no statistical differences were found for Ca, K, Na or P in any tissue. Bones were the tissue with the highest number of elements with statistical differences (ANOVA of Kruskall-Wallis, $p<0.05$); wild and sea cage were the batches with highest number of elements with statistical differences in gills and bones, respectively. For most elements, the highest concentrations were found in gills. In bones, only two elements (Cu and Zn) had higher ($p<0.05$) concentrations in one of the batches (wild) than those found in the remaining groups.

Table V.II.1. Concentrations of inorganic elements in tissues of ABFT. Data: geometric mean ± standard error, µg g^{-1}, ww. For each element and tissue, the same superscript letter shows statistical differences between batches; superscripts in parentheses means: marginally significant (p=0.05-0.1). Batch 1= Onshore tanks, Batch 2= Cage, Batch 3= Wild.

	Batch	Ca	Cu	Fe	K	Mg	Mn	Na	P	S	Sr	Zn
Gill	1	53292.2 ± 3573.23	3.483 ± 0.208	625.319 ± 155.825	1553.36± 311.7	3163.4 ± 304.9	35.948 ± 3.218 [a]	3111.7 ± 641.9	35664.6 ± 2879.4	5781.8 ± 248.9[a]	282.810 ± 24.609 [(a)]	67.581 ± 6.439
	2	43544.0 ± 4834.26	2.641 ± 0.221 [b]	515.572 ± 69.632	968.1 ± 162.78	2341.4 ± 390.3	25.267 ± 3.671	1414.1 ± 336.4	27427.4 ± 3348.0	3442.7 ± 253.7[a, b]	255.543 ± 31.864	50.214 ± 5.165
	3	40192.2 ± 4737.08	4.222 ± 0.420 [b]	505.607 ± 163.909	1080.3 ± 157.5	2219.7± 245.11	19.529 ± 2.248 [a]	1618.0 ± 421.2	25827.4 ± 2403.623257	4719.6 ± 234.3[b]	182.008 ± 22.128 [(a)]	58.186 ± 5.356
Bone	1	83262.1 ± 4181.1	0.388 ± 0.066 [a]	29.454 ± 3.352 [a]	1747.0 ± 264.2	1607.794353 ± 96.44690405	16.980 ± 1.323	3248.64 ± 285.4	43914.02727 ± 3605.353438	2461.5 ± 145.8	223.708 ± 12.283	37.173 ± 2.187 [a]
	2	83866.9 ± 5108.3	0.455 ± 0.119 [b]	48.731 ± 9.080 [a]	1437.3± 273.94	1459.666417 ± 91.72397445[b]	16.449 ± 1.121	2758.2 ± 291.2	37547.23297 ± 2278.174719	2264.83 ± 152.52	260.750 ± 17.094	40.282 ± 2.764 [b]
	3	89290.1 ± 6046.1	0.763 ± 0.117 [a,b]	43.766 ± 84.587	1596. ± 150.7	1871.3 ± 111.4[b]	16.185 ± 1.424	3468.3 ± 216.8	43755.8 ± 2641.9	2623.6 ± 162.1	237.594 ± 14.833	54.536 ± 3.821 [a,b]

In the PCA (**Figures V.II.1** and **V.II.2**), the 11 elements were represented by three principal components in both gills and bones, which explained 88.4% and 73.7% of the total variance, respectively. The KMO indices in both tissues exceeded 0.7 (0.766 in gills and 0.731 in bones), although the eigenvalues were higher in gills (>0.771) than in bones (>0.551). In gills, the principal component 1 (PC1) consisted of P, Sr, Ca, Mn, Mg and Zn, while in PC2 it consisted of K, Na and S; no separation between groups were found (**Figure V.II.1a**). Nevertheless, a slight grouping did appear for sea cage batch tunas when we introduced the PC3 (Cu and Fe, 1c). In bone, the PCs consisted of Ca, Mg, Sr, Mn, P, Zn and Na (PC1), S and K (PC2), and Cu and Fe (PC3), with no clear differentiation between groups (Fig. 2a, 2b and 2c). Meanwhile, the PCs were the same in both tissues, except for Na (in PC2 for gills and in PC1 for bones).

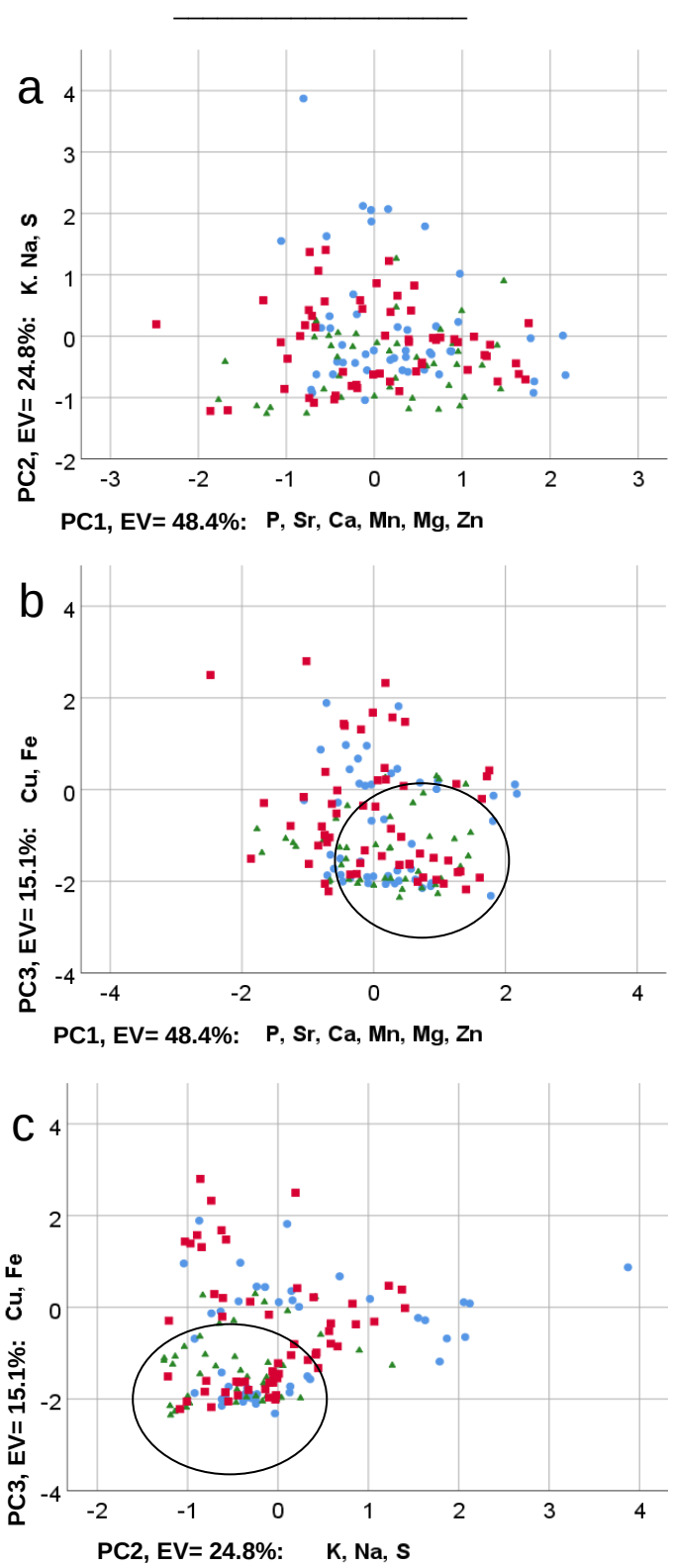

Figure V.II.1. Spatial distribution of the batches of tuna based on components from the PCA analysis for gills, the circles points the high superposition of batches. ● Batch 1 = Onshore tanks; ▲ Batch 2= Sea cages; ■ Batch 3 = Wild. A= PC1/PC2, B= PC1/PC3, C= PC2/PC3.

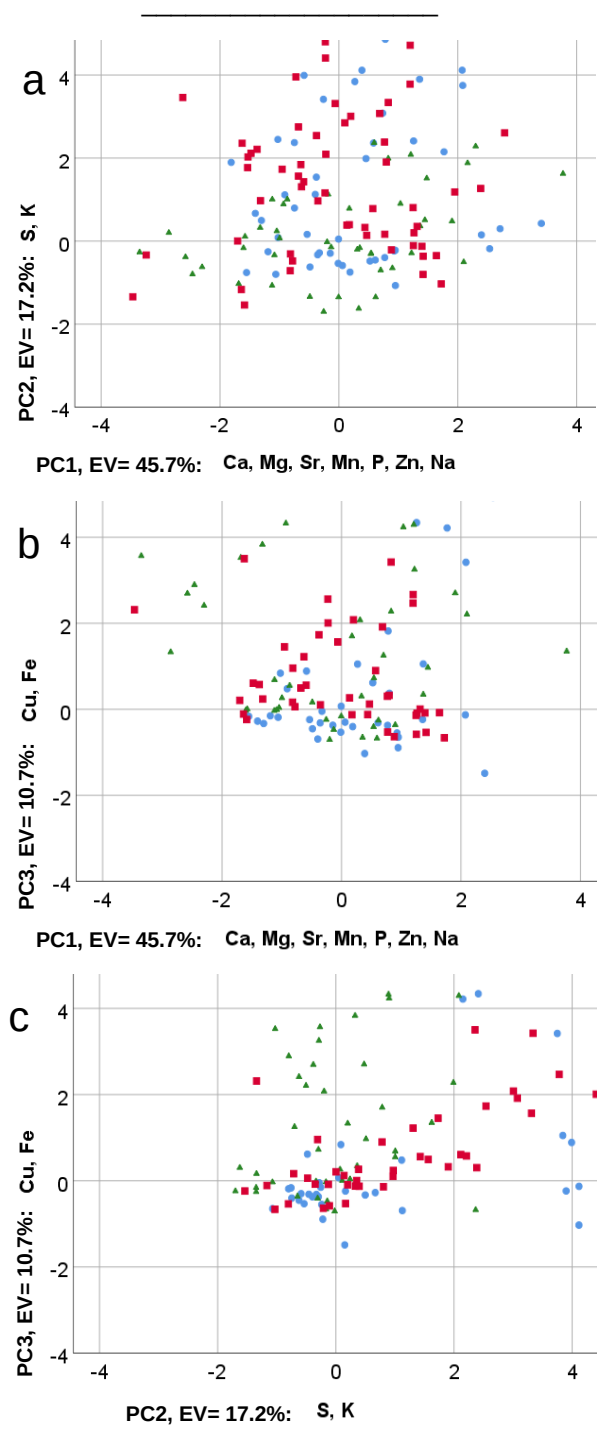

Figure V.II.2. Spatial distribution of the batches of tuna based on components from the PCA analysis for bones. ● Batch 1 = Onshore tanks; ▲ Batch 2= Sea cages; ■ Batch 3 = Wild. A= PC1/PC2, B= PC1/PC3, C= PC2/PC3.

For the DCA test, four elements (Mg, Mn, S and Zn) were considered for the functions in both tissues, and three elements (Ca, K and Na) were not considered in any tissue. Canonical Discriminant Function (CDF) data are given in **Table V.II.2**. Two CDF were created for both gills and bones. The percentage of cases correctly classified after the implementation of the cross-validation method in the DCA were 80.0% in gills and 77.0% in bones. For both tissues, the wild tuna group was the best discriminated, followed by the onshore tank and sea cage batches (**Table V.II.3**). The formulas for the case classification are given in **Table V.II.4**. The differences between the three groups are shown in **Figures V.II.3a** and **V.II.3b**.

Table V.II.2. Elemental-based canonical discriminant functions (CDF) as outcomes of the DCA analysis, their contribution to the discrimination between groups (%), and the overall accuracy (%) by tissue.

	Function	Eigenvalue	% variance	Canonical correlation	Lambda of Wilks	Canonical Discriminant Function Coefficients (Standardized)
Gill	1	1.45	72.2	0.769	0.262	Cu (0.933), Mg (1.92), Mn (-2.647), S (-0.872), Zn (0.809)
	2	0.557	27.8	0.598	0.642	Cu (0.129), Mg (-0.042), Mn (-0.610), S (0.908), Zn (0.378)
Bone	1	1.91	79.7	0.810	0.232	Fe (0.816), Mg (2.042), Mn (-2.20), P (0.634), S (-0.611), Sr (-1.164), Zn (0.978)
	2	0.486	20.3	0.572	0.673	Fe (0.246), Mg (-0.172), Mn (-0.688), P (-1.22), S (0.133), Sr (1.45), Zn (0.522)

Table V.II.3. DCA classification accuracy * and misclassification (%) by batch and tissue. Batch 1= Onshore tanks, Batch 2= Cage, Batch 3= Wild.

	Batch	1	2	3
Gill	1	79.2*	8.3	12.5
	2	13.6	77.3*	9.1
	3	0.0	17.2	82.8*
Bone	1	75.0*	12.5	12.5
	2	31.8	63.6*	4.5
	3	7.1	3.6	89.3*

Table V.II.4. Classification formulas; [element]= elemental concentration for the case to be classified. Batch 1= Onshore tanks, Batch 2= Cage, Batch 3= Wild.

	Batch	Formula
Gill	1	(-14.2) + (-0.607 x [Cu]) + (-22.8 x [Mg]) + (0.333 x [Mn]) + (44.0 x [S]) + (-0.037 x [Zn])
	2	(-6.44) + (0.059 x [Cu]) + (-5.04 x [Mg]) + (0.154 x [Mn]) + (21.86 x [S]) + (-0.019 x [Zn])
	3	(-10.2) + (1.00 x [Cu]) + (12.3 x [Mg]) + (-0.162 x [Mn]) + (23.3 x [S]) + (0.042 x [Zn])
Bone	1	(-12.0) + (-0.007 x [Fe]) + (-29.8 x [Mg]) + (0.592 x [Mn]) + (-0.322 x [P]) + (48.9 x [S]) + (0.033 x [Sr]) + (-0.054 x [Zn])
	2	(-14.2) + (-0.008 x [Fe]) + (-69.7 x [Mg]) + (0.701 x [Mn]) + (-2.11 x [P]) + (58.6 x [S]) + (0.079 x [Sr]) + (-0.051 x [Zn])
	3	(-14.1) + (0.001 x [Fe]) + (58.2 x [Mg]) + (-0.293 x [Mn]) + (-0.286 x [P]) + (32.8 x [S]) + (0.019 x [Sr]) + (0.129 x [Zn])

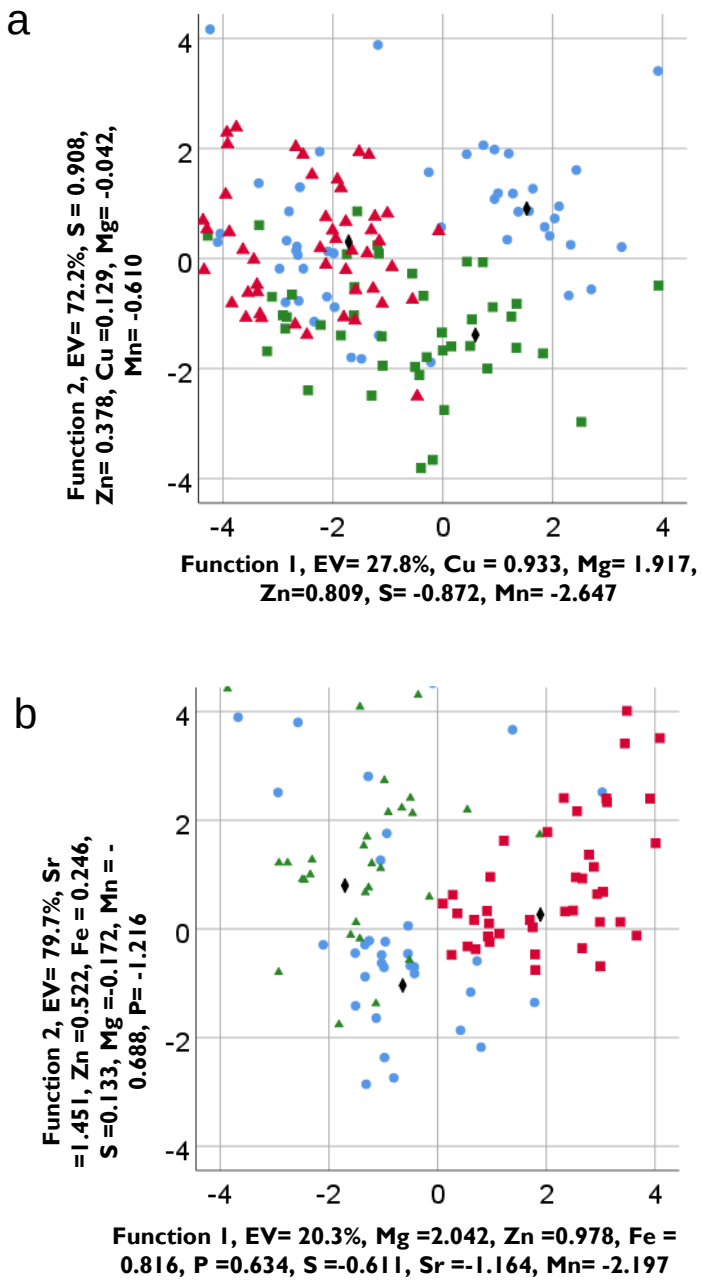

Figure V.II.3. Spatial distribution of batches of tuna based on functions from the DCA analysis and group separation by tissue. A: for gill, B: for bone.

Discussion

Usually, studies on inorganic elements' composition of marine species are mainly used to emphasize major concentrations of toxic pollutants in adult fish (> 50 kg) muscles. Thus, concentrations of mercury (Hg), cadmium (Cd) and lead (Pb) (Cabañero et al., 2005; Morgano et al., 2011, Burger & Gochfeld, 2013; Annibaldi et al., 2019) are analyzed, although only a few authors ever develop element profiles as a means of determining fish batches (i.e. Percin et al., 2011; Percin & Sogut, 2010; Sogut et al., 2011; Salvat-Leal et al., 2023). With this in mind, we envisaged to explore this uncharted differentiation method in juveniles of ABFT, even though other methods have been already proved (i.e., artificial tagging, Block et al., 2005, Fromentin, 2010; molecular markers, Boustany et al., 2008, Riccioni et al, 2010; genetic markers, Rodríguez-Ezpeleta et al., 2019). In addition, gills and bones composition profiles could be used as for discriminating batches, given that their chemical composition may depend on their rearing conditions and that they are accessible and commercially worthless tissues (Brucka et al., 2009).

i. Inorganic elemental concentrations

Only one element (Cu) showed statistically significant differences between groups for both tissues (batch 3, wild specimens, higher than batch 1 and 2, Table 1). This agrees with a previous study of soft tissues (brains, livers, muscles and kidneys, Salvat-Leal et al., 2023, Chapter I), in which Cu was the only element for all the tissues with statistical differences between batches (higher concentrations in wild tunas). Copper is an essential element for cellular functioning (Lall & Kaushik, 2021) and is always present in fish. These greater concentrations of Cu in wild specimens could be due to their richer and more varied diet. Juvenile tuna fed on small pelagic fish, shrimps, cephalopods and crustaceans (Saitô et al., 1990; Sarà & Sarà, 2007; Sinopoli et al., 2004). In this sense, Cu plays a part in oxygen transport through the respiratory pigment hemocyanin in crustaceans (Jacobo et al., 2016), and metals as Cu and Zn play an essential role in biological functions in cephalopods (Rjeibi et al., 2015). In the Mediterranean Sea, tuna is an active predator of cephalopods, *inter alia* (Karakulak et al., 2009; Van Beveren et al., 2016), and have high feeding rates,

so their diet represents an important exposure pathway for these elements (Bustamante et al., 2002, 2004, 2006).

For the remaining elements, higher concentrations were mainly found in the gills of the onshore tank specimens, but only statistically significant in Mn and S (marginally from Sr). In bones, elemental concentrations showed no clear patterns, despite higher concentrations of three out of four elements (Cu, Mg and Zn) in wild tuna. To the best of our knowledge, no data regarding elemental concentrations in juveniles of ABFT gills or bones have ever been published, although several studies of soft tissues have reported significantly higher concentrations in wild as opposed to farmed tuna (Vizzini et al., 2010; Salvat-Leal et al., 2023; Percin et al., 2011; Sogut & Percin, 2011; Milatou et al., 2015). This partially agrees with our findings for Cu and for bones, but not for gills. This could be due to the differing physicochemical water properties from onshore tanks, which had a closed-circulation system in which the elements can concentrate in higher proportions in the water and therefore, in the gills, meanwhile wild and sea cages batches had an open water-system. In other freshwater species, Brucka et al. (2009) reported similar Cu concentrations in gills to those found in our study. Thus, it seems that Cu could be an important element for discriminating batches, with similar concentrations present in gills and bones to those found in livers and muscles, respectively (Salvat-Leal et al., 2023).

Nevertheless, the higher levels of most elements (Cu, Fe, K, Mg, Mn, Na, S and Sr) in gills than in bones could be due to the unusually large surface area and thin blood-water barrier that characterize tuna gills (Hughes, 1984), which permits greater transference than in other teleost species (Bushnell & Brill, 1992). In tuna, gills are important organs for exchange and have a huge surface area, much greater than in other fish (Bernal et al., 2001), and it is in direct contact with the surrounding environment. Specifically, tuna need to actively pump water over their gills by swimming continuously, a method known as “ram ventilation” (Roberts, 1978; Bernal et al., 2001). In addition, differences in tissue accumulation between groups could be related to factors such as weight, feeding

profile and habitat, including the water chemistry (Percin et al., 2011; Kennedy et al., 2005; Khan et al., 2012; Miyan et al., 2016; Wright et al., 2018). Thus, some authors have reported that older fish accumulate more trace elements than younger fish (Olsson, 1998; Licata et al., 2005). However, the effects of body weight alone were not sufficient to explain the differences in concentrations between groups and, consequently, no weight-related accumulation can explain the higher storage of elements in wild tuna bones and in onshore tank tuna gills. Therefore, differences in the characteristics of the water (tank and wild tunas) could have affected the degree of accumulation in both tissues as the three batches had differing water circulation systems: the wild group (batch 3) had the highest water renewal, sea cages (batch 2) had a lower renewal and onshore tanks (batch 1) had water recirculation.

The remaining elements with statistical differences between batches (Fe, Mg, Mn, S and Zn) have important functions in fish. They participate in many physiological functions, biochemical processes and metabolism, and are crucial as components or co-factors in different enzymatic systems (Lall, 2002; National Research Council, 2011; Lall & Kaushik, 2021). However, different concentration patterns were found including higher levels of several elements in the gills of onshore tank tuna, as well as higher levels in wild tuna bones, but with significant differences only for Mg and Zn. To date, no study has ever examined the gills and bones of reared and wild tuna and so, further work on the characteristics of water and tuna feeding behaviour are necessary if these differences and patterns are to be disentangled.

Finally, no differences in Ca, K, Na and P (marginally for Sr) between batches were found. These elements are key in functions such as the osmotic balance, the acid-base equilibrium, and the development and maintenance of the skeletal system (Lall, 2002; Zimmer et al., 2009; Lall & Kaushik, 2021). No data on these elements in hard tissues in tuna could be found in the scientific literature, the exception being a study of freshwater species in which Ca concentrations were higher than in our study (Brucka et al., 2009).

ii. Principal Component Analysis

The eleven elements were combined in a PCA for each tissue and distributed amongst three PCs that coincided in gills and bones (except for a component change in Na). Their explanation of the variance, KMO, Bartlett's test of Sphericity and eigenvalue criterion were appropriate for all tissues. The KMO, accounted for over 0.5 in both cases and so the results were suitable for data analysis (Shrestha, 2021). Nevertheless, no clear differences in the three batches in the PCA of the individuals' spatial distribution were found. Gills were the only tissue in which PC3 (Cu and Fe) showed a slight distinction for sea-cage specimens. This agrees with the importance of Cu concentrations in this tissue, so when this element enters into the statistical analysis, the sea cage group separation is clearer. By comparison, in the bone PCA the introduction of PC3 (Cu and Fe) did not clarify the situation, even when both Cu and Fe showed significative statistical differences between tanks and sea cages. As noted above, the important filtering function of gills could highlight variances in the nature of the water (sea cage tunas), which could have affected the differences between the two tissues.

Comparing these results with previous studies in soft tissues (Salvat-Leal et al., 2023), better performance indices were found for hard tissues: KMO > 0.7 in gills and bones, *vs* soft tissues: KMO < 0.57 in livers, kidneys, muscles and brains, with total explained variance higher in gills (88.5%) than in soft tissues (71.0–79.0%). However, the differentiation of groups in gills was not as clear as that reported in some soft tissues (kidneys and muscles), so it seems that these hard tissues cannot be used to distinguish the fish group using this particular statistical test.

iii. Discriminant Canonical Analysis

In gills and bones, the DCA created two functions that describe the differences between the batches, agreeing with Balzarini et al. (2015) results for the ability of this analysis to maximize differences between populations. These functions are composed of elements selected as the most discriminant by the analytical software (Yakubu & Okunsebor, 2011). Significant discrimination was found for

all tissues (Wilk's lambda, $p<0.05$), and the correlation coefficients showed that the functions created were useful, especially the CDF1, which explained 59.1% of the total variance in gills and 65.6% in bones (when values higher than 45% are expected; Torrado-Fonseca, 2013).

For both tissues, Ca, K and Na were not included in the DCA functions, coinciding the Ca and Na usefulness with ABFT studies in soft tissues (Salvat-Leal et al., 2023). Nevertheless, they are all important elements that maintain inner homeostasis in fish. On the other hand, two functions were found for both tissues, with wild as the best identified group. Four elements (Mg, Mn, S and Zn) were selected for CDFs in both tissues, while Cu was also selected in gills, and Fe and Sr in bones. Surprisingly, Cu does not appear in the elemental selection of the DCA in bones. Thus, there were elements in the functions of DCA with (Cu, Mn and S in gills; Fe, Mg and Zn in bones) and without (Mg and Zn in gills; Mn, P, S and Sr in bones) significant differences between the means of the batches. Finally, the elemental composition for CDFs of gills was similar than those reported by Salvat-Leal et al. (2023) for brains; bones were the tissue with highest number of elements for CDFs. In terms of the identification of the tuna batches, gills had the highest percentage of success (80.0% accuracy), with the lowest percentages of confusion between the wild and onshore tank groups. This percentage was higher than those reported for juvenile ABFT livers and muscles but lower than for kidneys and brains (Salvat-Leal et al., 2023).

Conclusion

The chemical profile has demonstrated its utility for three batches under different conditions (fed and environment). Of the elemental concentrations, mostly Cu (if we look for an inter-tissular element of choice) but also Fe, Mg, Mn and S were found to be the most useful elements for comparing batches in bones and gills from juveniles ABFT. The gills were the best tissue for comparing batches, due to their tissular concentration, since (1) they had greater elemental concentrations than bone, and (2) their function as a thin blood-water barrier increases the possibility of detecting elemental differences. This could make it

better short-term marker; hovewer, in the bone, a deposition tissue, the chemical profile could be more stable, acting as long-term marker.

Regarding multivariant analysis, even though the criteria for the PCA were fine for both tissues, no clear differences were found between the three groups. In the DCA, all the created functions in both tissues were useful, especially the first CDF that explained most of the total variance in the analysis. For this analysis, four elements were selected (Mg, Mn, S and Zn) for both gills and bones; however, gills had the highest discriminating success, and wild was the easiest group to differentiate.

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CHAPTER III

Otolith mineral composition as a model for identifying the batch of juvenile Atlantic Bluefin Tuna (Thunnus thynnus)

Abstract

In this study, the suitability of otolith chemistry as a tool to Atlantic bluefin tuna (ABFT, *Thunnus thynnus*) batch discrimination was examined. The chemical composition of otoliths, which are the teleost ear stones, have been proved to allow the accurate classification of a random fish to their area of origin if the chemical signatures between groups are strong. Thus, the otolith chemical composition of principal elements (Al, Ca, Fe, Mg, Na, P, Rb, S, Sr, Ti and Zn) of juvenile ABFT (less than 1-year old, 0+) from two different ambiances (extractive fishing, and culture-reared tunas) were determined, and MANOVA and Discriminant Canonical Analysis (DCA) were applied. Concentrations of Mg, Na, P, Rb and Sr significantly differed among batches, having farmed tuna higher concentration of these elements. In addition, P and Sr separated both batches (DCA), achieving a maximum overall discrimination success of 78.4%. This is the first study of the chemistry of captive born and bred ABFT otoliths, and their ability to discriminate against wild tunas.

Keywords: bluefin tuna, chemistry, aquaculture tuna, otolith, wild tuna

Introduction

The demand on Atlantic bluefin tuna (ABFT, *Thunnus thynnus*) meat has increased sharply in the past decades, encouraged by the extremely high profits from the global sushi market (Chaabani, 2015). This exploitation inevitably led to great fishing efforts (Rodríguez-Roda, 1964; Rey, 1999; Fromentin & Powers, 2005; Morais et al., 2011), and in the 90's, the development of protection measures after the reduction of breeding populations in the Western Atlantic (National Research Council, 1994; Sissenwine et al., 1998). Given this situation, the aquaculture of the species remains an interesting point to develop, both trying to compensate these problems encountered on industrial fishing and to cover the increased tuna meat demand. However, the commercialization of aquaculture specimens brings the necessity to develop accurate and verifiable methods to distinguish culture-reared (farmed) from extractive fishing (wild) specimens in the near future. From 2016, the largest stock of captive breeders worldwide has been concentrated in the Spanish Institute of Oceanography (IEO, 'Instituto Español de Oceanografía', Mazarrón, Southeastern Spain), and the largest stock of ABFT farmed juveniles can only be found in their facilities.

In order to be able to discriminate wild and farmed ABFT, a natural tracer would be very useful, these are non-invasive, not requiring human handling, which is a must in ABFT aquaculture production. Lately, certain inorganic elements in ABFT tissues have been proposed in the Turkish Mediterranean as non-invasive and natural tools (Sogut et al., 2011). Also, according to Zitek et al. (2010) the otolith chemistry is a valuable technique for discriminating between wild and farmed individuals without having to perform mass-marking, remaining their mineral part unaltered after deposition, and elements like Magnesium (Mg) and Strontium (Sr) have been concretely signalled as good markers (Limburg, 1995; Secor et al., 1995; Chang et al., 2019; Doubleday et al., 2014; Thomas et al., 2017). In the otoliths, some elements are absorbed primarily from the surrounding water and therefore provide a record of the environmental concentrations (Watanabe et al., 1997; Campana, 1999; Milton & Chenery, 2001), like Sr (Limburg, 1995; Secor et al., 1995), forming an area-specific "fingerprint" (Walther & Limburg, 2012),

and thus, serving as natural tags for marine fish (Kennedy et al., 1997; Thorrold et al., 1998a, b). Meanwhile, other elements (i.e., Sodium (Na), Potassium (K), Phosphor (P), Sulfur (S) and Mg; Thresher et al., 1994; Proctor et al., 1995; Dorval et al., 2007; Hamer & Jenkins, 2007) are under strong physiological control (Campana, 1999; Sturrock et al., 2015; Limburg et al. 2018; Hüssy et al. 2020). The divergent behaviour of these elements, coming from diverse sources and being metabolised differently, makes multi-element screening a valuable tool for fisheries (Cubadda, 2006). In aquaculture, farmed fish is grown in different water conditions, feeding regimes and stocking densities than wild fish. Then, the method based on the otolith composition could be a very successful tool for their discrimination (Campana et al., 2000; Campana & Thorrold, 2001; Arechávala-López et al., 2016).

In some tropical tuna species, the otolith chemical composition seems to be a powerful tool for wild groups discrimination. In this sense, the otolith element:Ca (E:Ca) ratios in *Thunnus orientalis* and *T. thynnus* (Rooker et al., 2001a, 2003; Traina et al., 2021), stable isotopes in *T. thynnus* (Rooker et al., 2014) and E:Ca ratios + stable isotopes in *T. obesus*, *T. albacares* and *Katsuwonus pelamis* (Rooker et al., 2016; Artetxe-Arrate et al., 2019, 2021) have shown their usefulness. However, for discriminating wild and farmed specimens, the otolith chemistry has only been pursued in other species (*Salmo trutta fario* and *Onchorynchus mykiss* - Zitek et al., 2010; *O. tshawyzscha* - Marklevitz et al., 2011; *O. mykiss* – Watson et al., 2018; *Dicentrarchus labrax* and *Sparus aurata* - Arechávala-López et al., 2016; *Acanthopagrus schlegelis* - Chang et al., 2019). In ABFT some research about chemical composition as tool for discriminate batches has been done in other tissues (kidney, Sogut & Percin, 2011; muscle and liver, Sogut et al., 2011; muscle, liver, brain and kidney, Salvat-Leal et al., 2023), but as far as we know it hasn't been studied in the otoliths.

Therefore, the aim of this study was to develop otolith chemical signatures to discriminate young ABFT batches. For this purpose, otoliths from tunas across two batches in the Mediterranean were examined: wild and farmed. It is the first

time that ABFT juvenile otoliths from farmed individuals are used with this purpose.

Material & Methods

i. Sample collection

In October 2018, 35 wild ABFT age-0 juveniles (Batch 1) were captured in their nursery area in Mazarrón Bay (Murcia, Spain, Western Mediterranean) by the hook-and-line method (barbless hook) and sampled the same day of capture. On the other hand, a group of 66 farmed ABFT age-0 juveniles (Batch 2) hatched from eggs from naturally spawning captive adults in sea cages and raised in the facilities of the Spanish Institute of Oceanography (Mazarrón, Spain), were sampled in October-November 2018. These farmed fish were fed the first 12 dph on rotifer (*Brachionus plicatilis*) and copepod (*Acartia tonsa*) in tanks of 40-m^3, then, with artemia and sea bream yolk sac larvae. Weaned fish were fed an artificial diet (Magokoro S-3, Marubeni Nissin Feed Co., Ltd., Tokyo, Japan), and at 41 dph were identified and transferred to tanks in a recirculated aquaculture system in the Infrastructure for Atlantic bluefin tuna aquaculture (Infraestructura de Control de Reproducción del Atún Rojo, ICRA, Cartagena, Spain), where they were maintained at 23.5- 24.9°C at a salinity of 37.5g L^{-1}. Hereafter, they were fed with round sardinella (*Sardinella aurita)*, pilchard *(Sardina pilchardus)* and Atlantic mackerel (*Scomber scombrus)*. The samples were collected from individuals soon after their natural death. For each individual, otoliths were extracted by a frontal section on the head which permitted to localise the inner ear from above following the removal of the brain. After extraction, the otoliths were washed using purified water to remove adhering otic tissue. Finally, the otoliths were placed in polyethylene microtubes where they dried at room temperature before storage. The equipment used for the otolith extraction was cleaned carefully, by immersion in 96% ethanol, followed by rinsing in purified water.

ii. Mineral analysis

The concentrations of 11 elements (Al, Ca, Fe, Mg, Na, P, S, Sr, Rb, Ti and Zn) were analysed through inductively coupled plasma optical emission spectrometry (ICP-OES, *ICAP 6500 Duo, Thermo Scientific, with One Fast System*). For this analylsis only the right otoliths were used, they were dissolved with trace mineral grade H_2O_2 (33% Suprapure, *Merck*) and HNO_3 (69% Suprapure, *Merck*) in special Teflon reaction tubes and heated at 220ºC in a microwave digestion system (UltraClave-Microwave Milestone®) during 20 minutes, and then diluted to 10 mL using double deionised water. The elemental concentration detection limits (DL) were 10 μg g^{-1} for major constituents (Ca, Mg, Na, P and S) and 0.001 μg g^{-1} for the rest of the elements. For every sample, two readings were made, using the mean as concentration value. To avoid possible contamination, one blank sample for every 11 samples was also analysed.

Multi-element calibration standards (SCP Science, in 4% HNO^3) were prepared for each element with specific concentrations, taking as a reference UNE-EN ISO 11885. Furthermore, intermediate patterns of the elements were prepared. The calibration device was set for each batch, with a minimum of three points for every lot. The wavelengths for each element analysed were: Aluminium (Al, 167.089/ 396.15), Calcium (Ca, 184.01/ 315.89), Iron (Fe, 238.20/ 259.94), Magnesium (Mg, 202.03/ 279.55), Sodium (Na, 589.59), Phosphor (P, 185.94/ 214.91), Sulfur (S, 180.73/182.03), Strontium (Sr, 421.55), Rubidium (Rb, 780.03), Titanium (Ti, 336.12), Zinc (Zn, 206.20).

iii. Statistical treatment

The statistical analyses were performed using the SPSS software (*Statistical Package for the Social Sciences*, *IBM 24.0*, New York). The Kolmogorov-Smirnov ($n > 40$, Vigneau et al., 2000) was used to test the normality and Levene's test to assess the homoscedasticity of the size-corrected data. The tuna weight was compared between batches (wild *vs*. farmed) using T-test. Overall differences in elemental concentration between two batches were tested using multivariate analysis of variance (MANOVA), with the otolith weight as co-variable, to

eliminate the possible distortion of the differences. Then, to perform a Discriminant Canonical Analysis (DCA), the effect of size (otolith weight used as a proxy for fish size) was removed from the data to ensure that differences in fish size among samples did not confound any site-specific differences in otolith chemistry. Concentrations were weight-detrended by subtracting of the common within-group linear slope from the observed concentration (from Campana et al., 2007):

$$Residual = observed\ value - (a + b\ x\ otoW)$$

where *a* is the constant and *b* is he slope for the otolith weight (*OtoW*).

DCA was used to identify the elements driving the most differences between batches and estimate their ability to correctly classify individuals into the correct group. The significance levels for all tests were set at 0.05. The important output in the DCA is the Wilks' lambda in which a lower number means higher performance ($p< 0.05$), and the eigenvalue and canonical correlation in which a higher number imply better performance (Tatsuoka, 1971; Grimm & Yarnold, 1995; Stevens, 2002; Huberty & Olejnik, 2006). In this output one function is created, which is called Canonical Discriminant Function (CDF), and a split-sample validation (cross-validation testing procedure) was performed to assess the capacity of the selected variables to predict different origins for the tested fish. In this validation, one individual is removed from the original matrix. The DCA is then performed using the remaining observations to classify the omitted individual (Poulet et al., 2005; Yakubu & Osenbor, 2011). The formulas from the case classification were obtained to classify new specimens from the same background but of uncertain batch. In these formulas, the constant and function coefficients were obtained for each of the batches and elements:

$$F(x) = a + (b * [X])$$

where a = a constant for the combination of the otolith and a batch; b = a coefficient of classification function for the combination of an element and batch; and [X] = the concentration of an element for a given tissue and batch (in a

particular specimen). Once the formula has been applied, the result with the highest value indicates the possible batch of the fish.

Results

There were no statistically significant differences in fish weight between batches (T-test, p>0.05), being the average weight of wild juveniles 400.4 ± 165.5 gr (31.1 ± 3.7 cm of total length), and of farmed juveniles 512.9 ± 299.4 gr (31.9 ± 5.3 cm of total length). Of the 11 elements analysed, differences between the two batches for Na, Mg, P, Sr and Rb (MANOVA, $F(12, 69) = 4.72$, $p < .0005$; Wilk's $\Lambda = 0.65$) were found: batch 2 (farmed tunas) showing higher concentrations of these 5 elements (**Table V.III.1** and **Figure V.III.1**).

Table V.III.1. Concentrations of elements (mean ± standard error, mg kg^{-1}) in juvenile ABFT from two batches (wild, batch 1, and farmed tuna, batch 2). * Significant statistical differences between batch 1 (wild) and batch 2 (farmed) for each element are shown with * (T-test, p<0.05).

Batch	1 (Wild)	2 (Farmed)
Al	136.7 ± 11.6	143.6 ± 15.4
Ca	375065.5 ± 5162.9	365789.3 ± 3148.9
Fe	16.3 ± 3.0	20.9 ± 2.8
Mg*	46.1 ± 4.7	64.5 ± 7.6
Na*	3525.3 ± 55.7	3648.5 ± 39.8
P*	269.7 ± 25.8	383.4 ± 33.4
Rb*	17.2 ± 1.0	16.2 ± 1.5
S	3272.6 ± 52.1	3306.2 ± 42.3
Sr*	1316.6 ±17.2	1368.4 ± 20.1
Ti	9.9 ± 1.3	10.2 ± 1.2
Zn	11.9 ± 1.6	14.1 ± 1.1

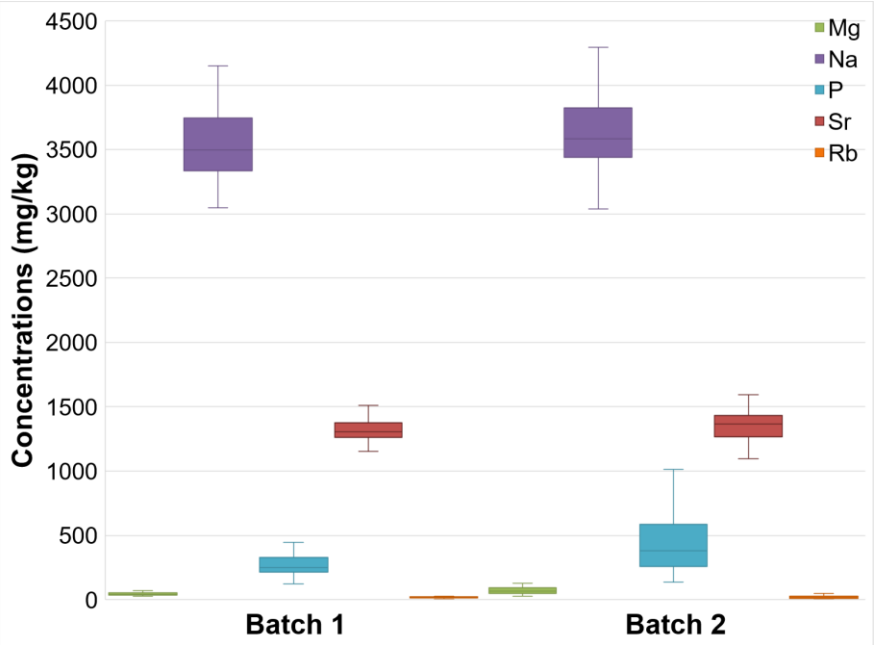

Figure V.III.1. Box-plots (median, 10th, 25th, 75th, 90th percentiles) of Na, Mg, P, Sr and Rb, elements in which significant statistical differences were found. Batch 1 = wild, Batch 2 = farmed.

The DCA selected two elements, P and Sr, which constituted a unique CDF, explaining the 100% of the total variance of the dataset. Wilk's Lambda and the canonical correlation were intermediate. The CDF and statistic data are given in **Table V.III.2**, the coefficient values for P and Sr permit to evaluate the importance of the variables (a higher value means higher importance). In total, 78.4% of the ABFT juveniles were successfully classified, being 87.3% of the farmed, and 63.6% of wild well distinguished, however 36.4% of the wild individuals were misclassified with farmed and 12.7% of the farmed with wild (**Table V.III.3**). The **Figure V.III.2** shows the information from the DCA, plotted as discriminant scores, formed by the addition of the DCA coefficient for each element. The formulas generated permit to identify the fish batch substituting the elemental concentrations, the groups with the higher number would be the probable batch of the fish, with a probability of 85.5% of being farmed if that is the result or 78.8% of probability of being wild if that is the result (showed in **Table V.III.4**).

Table V.III.2. Elemental based Canonical Discriminant Function outcoming from the DCA analysis information. TEV = total explained variance, sig = signification level.

CDF1 (coeficients)	Eigenvalues (TEV %)	Lambda Wilks (sig)	Canonical correlation	% Success (cross-validated)
Sr (0.652), P (0.864)	0.291 (100)	0.775 (0.000)	0.475	78.4

Table V.III.3. Percentages of classification accuracy* for the Discriminant Canonical Analysis. Batch 1 = wild and Batch 2 = farmed tuna.

	Batch 1	Batch 2
Batch 1	63.6*	36.4
Batch 2	12.7	87.3*

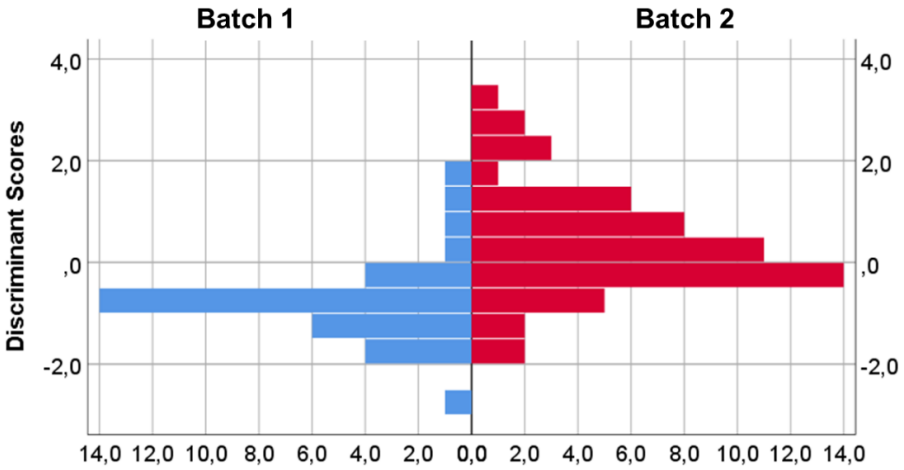

Figure V.III.2. Graphic representation from the DCA with elements' concentrations. The axes represent the residual values (x) and the number of them (y) for each batch. Batch 1 = wild and Batch 2 = farmed tuna.

Table V.III.4. Classification case formulas. Batch 1 = wild and Batch 2 = farmed tuna. [element]= element concentration for the case to be classified.

Batch 1 (Wild)	Batch 2 (Farmed)
Z = ((-0.003) x [P mg/kg]) + ((-0.004) x [Sr mg/kg]) + (-1.22)	Z = (0.002 x [P mg/kg]) + (0.002 x [Sr mg/kg]) + (-0.56)

Discussion

The chemical composition of the otolith has been used to difference batches of scombrids based on their breeding areas, sampling years or ages (**Table V.III.5**). In this structure, Ca is the major element and the concentration of other elements related to Ca have shown their usefulness in various tuna species (*Thunnus thynnys, T. orientalis*, *T. obesus*, *T. albacares* and *Katsuwonus pelamis:* Rooker et al., 2014; Rooker et al., 2001a, 2003; Rooker et al., 2016; Artetxe-Arrate et al., 2019, 2021; Traina et al., 2021). Various authors indicate that the otolith chemistry is determined by complex interactions between genetics, physiology, the environment, ontogenetic changes, and even post-mortem handling of otoliths (Campana, 1999; Thresher, 1999; Rooker et al., 2001b). Therefore, no element should be ruled out, especially if it appears in all the specimens studied. Thus, in our study, apart from the most studied elements (i.e., Sr and Mg, **Table V.III.5**), we included elements that have not been included in other studies, such as Al, Fe, P, S, Rb and Ti, as well as others poorly mentioned in the literature, such as Zn and Na (Rooker et al., 2001b; Secor et al., 2002; Wang et al., 2009; Rooker et al., 2021; Traina et al., 2021). This was the first time that the otolith composition from wild and farmed ABFT juveniles could be compared. As pointed in previous studies using other tissues (Salvat-Leal et al., 2023 – muscle, liver, kidney and brain; Percin et al., 2011- muscle; Sogut & Percin, 2011 – kidney) we hypothesized that the chemical signatures would differ between those two batches. In this study, otolith Mg, Na, P, Rb and Sr concentrations of ABFT varied significantly among batches. Specifically, only otolith Rb was higher in wild tunas, while the rest of the elements (Mg, Na, P and Sr) were higher in farmed. In terms of discrimination, only P and Sr were selected as group tracers, with a signature over 78% for the presented batches and conditions (**Tables V.III.3** and **V.III.4**), being the farmed individuals the best differenced (87.3% farmed vs. 63.3 % wild), probably due to more homogeneous developmental conditions. In this sense, P was the element primarily driving discrimination (CDF1 coefficient = 0.864). Elemental differences between both batches are likely linked to diet and ambient water chemistry.

Regarding the elemental differences (see **Figure V.III.3** for a review), first the Sr uptake is probably related to surrounding water concentrations. Sr is often incorporated into otoliths in direct proportion to ambient conditions (Secor & Rooker, 2000) since it has a similar ionic radius and valence to Ca (Izzo et al., 2016; Thomas et al., 2017; Hüssy et al., 2020). Therefore, Sr is dependent on salinity and temperature (Kalish, 1989; Secor & Rooker, 2000; Elsdon & Gillanders, 2002), two factors constantly controlled and regulated in the tuna facilities, and highly variable in wild fish. In our study, Sr differences would be related to the completely different water conditions in which the two batches were raised: wild specimens grew in open waters and farmed tunas were maintained in a recirculated aquaculture system (RAS). As mentioned above, in the tuna facilities the water parameters are constantly controlled and in addition, the RAS provides stable aquaculture production enabling more intensive fish breeding procedures (Deviller et al., 2005). However, dissolved substances including Sr and other elements can gradually accumulate in the facilities and thus in the specimens, depending on factors like the water source, which in our case was the deep Mazarron Bay waters, its elemental levels, the renewal rate, the performance of the system and the feeding operations (Pagand et al., 2000; Leonard et al., 2002; Sönmez et al., 2016). Therefore, higher concentration of substances in farmed tuna otoliths could be expected. Second, regarding otolith Mg, Na and P, they have been suggested to be physiologically regulated in fish (Mg: Dorval et al., 2007; Hamer & Jenkins, 2007; Na and P: Thresher et al., 1994; Proctor et al., 1995). From these elements, Mg is not considered a reliable environmental indicator, given that many studies report none or negative influence of water on otolith Mg (see review in Woodcock et al., 2012), and Na and P are none or poorly used in fish otolith studies. In relation to this, physiologically related elemental differences in our batches could be mostly explained by the divergent diets: farmed specimens were fed on defrosted bait *ad libitum* composed of small pelagic fish, which are rich, oily and highly nutritive preys (Ben Rebah et al., 2010; Šimat et al., 2020). Their diet was therefore constant and plentiful, which could explain the higher otolith concentration in elements (except for Rb). Meanwhile wild at this age (juveniles of less than 6

months) are characterized to have an opportunistic diet. Last but not least, regarding the otoliths Rb concentrations, they were higher in wild tunas. Rb is an element poorly studied, essential ultra-trace element for many organisms (Campbell et al., 2005) that can be also toxic due to its physiological interference with K^+ and Na^+ (Kosla et al. 2002). It also seems to be related to diet, given that there is a transfer of Rb between predator and prey (Johnson & Reeves, 1995; Nyholm & Tyler, 2000), so this is an element that biomagnifies throughout the trophic chain (Campbell et al., 2005). In this sense, the already mentioned opportunistic diet of wild tunas, give them greater variability of preys that could explain these differences. In resume, Sr is signaled as good natural tracer, coinciding with other otolith composition studies, but a wider range of elements, presumably physiologically controlled (like Mg, Na, P and Rb) could also be considered of interest, at least in juvenile ABFT, especially diet differences between batches are observed. Therefore, in future work, both water and feed samples should be taken to test what kind of element concentrations both wild and farmed tunas are exposed to.

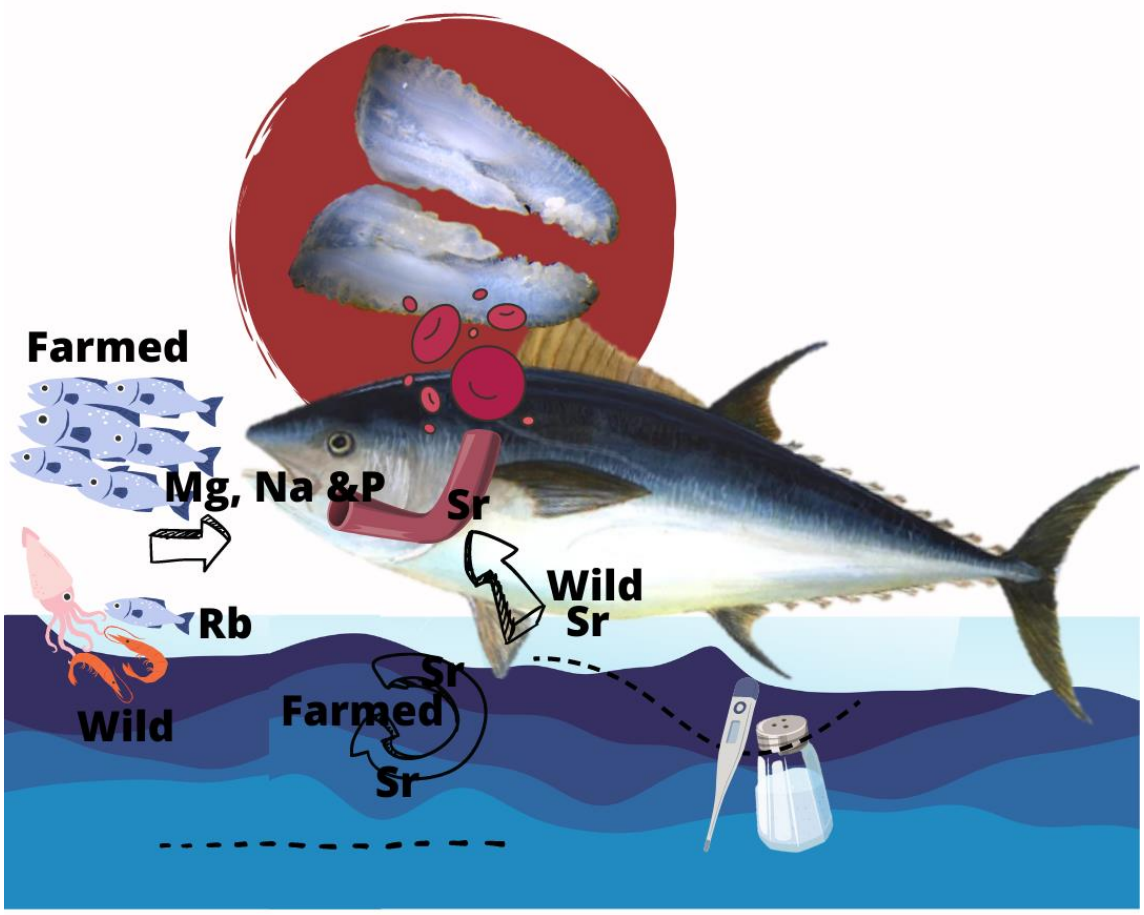

Figure V.III.3. Iconography from the possible causes of the otolith concentration differences between batches (Mg, Na, P, Sr and Rb): Mg, Na, P and Rb due to diet, Sr depending on the water chemistry.

The found concentrations of Mg, Sr, Ca and Na in the otoliths of our study have been compared with those reported in *T. orientalis* from the North Pacific Ocean and North-western Gulf of Mexico (Rooker et al., 2001a and 2001b, respectively), *T. Thynnus* and *T. albacares* from the Mid Atlantic Ocean (Rooker et al., 2001b), *K. pelamis* from the Equatorial Indian Ocean (Artetxe-Arrate et al., 2021), and *X. gladius* from the North Pacific Ocean (Wells et al., 2021). In general, these specimens had similar or greater length than our tunas (**Table V.III.6**), but lower concentration of Mg and Sr in their otoliths (except *K. pelamis*, Artetxe-Arrate et al., 2021), being our Ca concentration similar and our Na concentration slightly lower than those found in *T. orientalis* and *T. thynnus* (Rooker et al., 2001b). If the diet of fishes is excluded, these differences could be explained due the differing oceanographic features between the oceans and a marginal sea like the

Mediterranean, which is likely to result in considerable differences in ambient water chemistry (Rooker et al., 2001a). According to Desboeufs et al. (2005), marginal seas are typically richer in trace element concentration than ocean waters because of their proximity to continental sources of metals flowed as fluvial or atmospheric inputs. On the other hand, the riverine discharges in the western Mediterranean are higher than in the rest of this sea (Guerzoni et al., 1999), and rivers are likely sources of anthropogenic and lithophilic elements (Rooker et al., 2001a). In addition, metal-enriched inputs from the south-west coast of Spain (Tinto and Odiel rivers) are transported through the Strait of Gibraltar, and mix with waters of the western Mediterranean. This region is also well fertilized by upwelling (Minas & Minas, 1993; Dafner et al., 2001), and concentrations of elements displaying nutrient-type distributions (i.e., Ba, Mg, Mn) may be higher in these nutrient-rich waters. Also, Sr concentrations are often higher in marginal seas, characterized by high evaporation or low freshwater input (Talley et al., 2011). In relation to this, it would be expected that ABFT from the western Mediterranean like the ones from this study had higher contents of trace elements like Mg and Sr, specially comparing with ABFT from the Mid Atlantic Ocean (Rooker et al., 2001b).

Regarding the DCA results, only P and Sr were selected as group tracers (see **Figure V.III.4** for a resume). As mentioned in the paragraph above, P is poorly documented in fish otoliths' literature, probably due to the assumption that it is physiologically controlled (Hüssy et al., 2020). However, food is the main source of P in seawaters, which displays low phosphate concentration (Coloso et al., 2003), and therefore the concentration differences found in our batches could be explained by their divergent diets. In other studies, P usefulness in chemical composition studies to discriminate batches and the ambient water have been highlighted (Fengqin et al., 2011; Swanson et al., 2020). Finally, as mentioned previously, Sr is often identified as an important element to discriminate batches (Secor et al., 2002; Rooker et al., 2003; Wang et al., 2009; Rooker et al., 2016; Artetxe-Arrate et al., 2021; Traina et al., 2021). In resume, Sr have already been

signaled as good indicator in other otolith composition studies, but P could also be considered of interest, at least in juvenile ABFT.

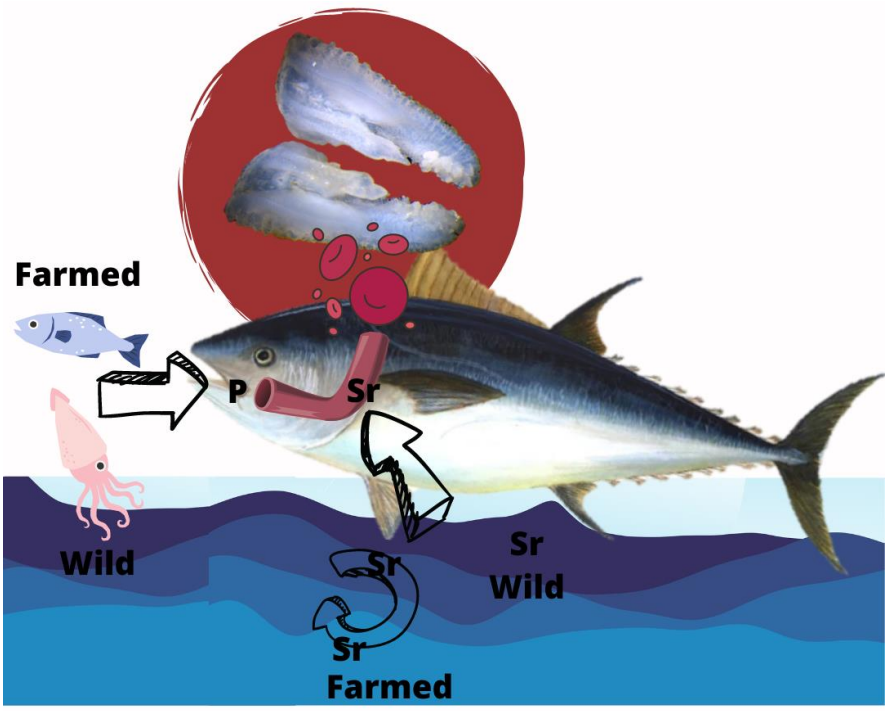

Figure V.III.4. Iconography from the possible causes of the otolith differences found within the DCA results between batches: P due to diet, and Sr depending on the water chemistry.

Despite the uncertainty around how the otolith chemistry may be affected by the ambient environment, trace elements within the ABFT's otolith cores promise as a natural marker for retrospectively examining the individuals' environmental histories and retrieve their origin in adult ABFT. Specifically, the elements Mg, Na, P, Sr and Rb have appeared to be of special interest discriminating farmed and wild ABFT. In the future, wider otolith sampling, coupled with water and feed sampling would need to be conducted to render these regional results broadly applicable.

Conclusion

In summary, already known ambient natural tracers within the otoliths like Sr, and some presumably physiologically controlled elements (Mg, Na, P and Rb) could be considered of interest discriminating two batches of juvenile ABFT from the Mazarrón Bay (Mediterranean Sea). Therefore, this study suggests that the otolith elements reflect the ambient water but are also affected by physiological processes in early development and can be used to develop otolith chemical signatures to discriminate young ABFT batches. However, in order to use this signature as a baseline for developing robust discrimination models using the otolith elemental profile, larger sampling should be done, including ambient sampling (water and feed).

Table V.III.5. Otolith elemental concentration and discrimination studies in species of Family Scombridae. Data: mean ± standard deviation, mg kg^{-1} dry weight. Statistical test: DCA = Discriminant Function Analysis; LDFA = Linear Discriminant Function Analysis; QDFA = Quadratic Discriminant Function Analysis; RF = Random Forest Analysis; ANN = Artificial Neural Network (ANN); JK-C = Jack-Knife Classification; CVC = Cross Validation Classification. Morphometrics data (cm for length and grams for weight, except $^{\delta}$ = kilograms): mean ± S.D. and/or range (minimum - maximum); TL = total length; FL = fork length; EFL = eye to fork length. Batches: w = wild; f = farmed. na = non analyzed; nr = non reported; ^{ß} Range of means.

Species	Place	n	Age	Lenght	Weight	Elements	Test	Discrimination (%)	Reference
T. thynnus	Mediterranean	35^{w}	0+	31.1 ± 3.7^{TL}	394.5 ± 167.8	Al, Ca, Fe, Na, Mg, P, S, Sr, Rb, Ti, Zn	DCA	78.4	This study
T. thynnus	Mediterranean	66^{f}	0+	31.9 ± 5.3^{TL}	516.3 ± 306.2				
T. orientalis	Pacific Ocean	32	0+	38.3 ± 9.5 $(23\text{-}54)^{FL}$	500 – 1000	Li, Mg, Mn, Ba, Ca, Sr	LDFA, JK-C	75 to 100	(1)
T. thynnus	Mediterranean Sea	15+9	0+, 1+	$31\text{-}39^{FL}$ (0+)	*nr*	Li, Mg, Sr, Ba, Na, K, Ca, Mn	DCA	68 and 81	(2)
	Atlantic Ocean	2+30	0+, 1+	$60\text{-}72^{FL}$ (1+)					
T. thynnus	Mediterranean Sea	43+9	0+, 1+	$25\text{-}42^{FL}$	*nr*	Li, Mg, Mn, Ba, Ca, Sr	LDFA, JK-C	71 and 62-80	(3)
	Atlantic Ocean	12	1+	$66\text{-}70^{FL}$					

T. maccoyii	Indian Ocean	18 15	3-21 13-25	127.8±20.5[FL] 172.9±8.6[FL]	30.3±12.8[δ] 98.9±20.6[δ]	Na, Mg, Mn, Ca, Sr, Ba	DFA, JK-C	74	(4)
T. obesus	Pacific Ocean	189	0+	27.7 to 54.4[FL,ß]	*nr*	Li, Mg, Ca, Mn, Sr, Ba	QDFA, CVC	46	(5)
T. albacares	Pacific Ocean	268	0+	26.0 to 53.9[FL, ß]	*nr*	Li, Mg, Ca, Mn, Sr, Ba		62	
T. albacares	Indian Ocean	56	0+ to 1+	Age 0: 29-39[FL] Age 1+: 52-64[FL]	*nr*	Ba, Mn, Mg, Sr, Ca	RF, ANN QDA, LDA	91	(6)
K. pelamis	Indian Ocean	128	0+	24.5 – 35[FL]	*nr*	Li, Sr, Ba, Mg, Mn, Ca	RF, CVC	44	(7)
T. orientalis		40 56	0+ 1+	27.8 to 45.5[FL,ß] 227.9±21.2[FL]	nr 230.8±68.0[δ]	Li, Mg, Mn, Zn, Sr, Ba, Ca	CDA, QDFA, CVC	87	(8)

T. thynnus	Mediterranean Sea	60	0+	20-46^{FL}	*nr*	Li, Mg, Mn, Zn, Sr, Ba, Ca	RF	60.3 (45-75)	(9)
X. gladius	Pacific Ocean	109 65	0+ Adults	< 100^{EFL} >100^{EFL}	*nr*	Ca, Mg, Sr, Ba	QDA	72.2	(10)

(1) Rooker et al., 2001a; (2) Secor et al., 2002; (3) Rooker et al., 2003; (4) Wang et al., 2009; (5) Rooker et al., 2016; (6) Artetxe-Arrate et al., 2019; (7) Artetxe-Arrate et al., 2021; (8) Rooker et al., 2021; (9) Traina et al., 2021; (10) Wells et al., 2021.

Table V.III.6. Mg, Sr, Ca and Na concentrations in otoliths from Scombridae species. Data: mean ± standard deviation, mg kg^{-1} dry weight. Morphometrics data (cm for length and grams for weight, except $^{\delta}$ = kilograms): mean ± S.D. and/or range (minimum - maximum); TL = total length; FL = fork length; EFL = eye to fork. Batches: w = wild; f = farmed. na = non analyzed; nr = non reported; ^{ß} Range of means

Species	n	Age	Lenght	Weight	Mg	Sr	Ca	Na	Reference
T. thynnus	35[w]	0+	31.1 ± 3.7[TL]	394.5 ± 167.8	46.1 ± 4.7	1316.6 ± 17.2	375065.5 ± 5162.9	3535.3 ± 55.7	This study
T. thynnus	66[f]	0+	31.9 ± 5.3[TL]	516.3 ± 306.2	64.5± 7.6	1368.4 ± 20.1	365789.3 ± 3148.9	3648.5 ± 39.8	
T. orientalis	32	0+	38.3 ± 9.5 (23-54) [FL]	500 – 1000	17.26 to 50.27[ß]	1181 to 1298[ß]	36.70 to 38.13[ß]	*na*	(1)
T. orientalis	69	0+ to 3+	58 – 70[FL]	*nr*	40.50 ± 14.72	1266 ± 72	373000 ± 11000	3892 ± 666	(2)
T. thynnus	92	0+ to 3+	101-102[FL]	*nr*	31.67 ± 7.61	1180 ± 151	378000 ± 13000	3668 ± 348	
T. albacares	56	0+ to 3+	56-120[FL]	*nr*	15.74 ± 3.95	1415 ± 98	385000 ± 13000	2960 ± 121	
K. pelamis	128	0+	24.5 – 35[FL]	*nr*	50.2 ± 13.4[§]	1615.5 ± 207.5[§]	383000[¥]	*na*	(3)
X. gladius	109	0+	< 100[EFL]	*nr*	(0+) 13.0 ± 3.7[č]	(0+) 914 ± 121.5[č]	(0+) 380000[2]	*na*	(4)
	65	Adults	>100[EFL]						

(1) Rooker et al., 2001a; (2) Rooker et al., 2001b; (3) Artetxe-Arrate et al., 2021; (4) Wells et al., 2021.

§ Mg and Sr are presented in E:Ca ratios in the study (µmol:mol), converted to mg/kg using the raw data given by Artetxe-Arrate et al., 2021 in their supplementary material.

čMg and Sr are presented in E:Ca ratios in the study (µmol:mol), converted to mg/kg by the equation used by Wells et al., 2021 from Baumann et al., 2015 to convert between element concentrations ([E], mg/kg) and E:Ca (µmol:mol) using the molar mass of each element (M_E, g mol $^{-1}$):

$$E{:}Ca = \frac{[E]}{1000}\left(M_E \frac{0.38}{M_{Ca}}\right)^{-1}$$

The considered molar masses of the elements were ^{40}Ca, ^{24}Mg and ^{88}Sr (PubChem, 2022a, b, c)

¥ Assumed according Sturgeon et al., 2005; ƨAssumed according Rooker et al., 2001b

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SECOND SECTION,

natural morphometrical tracers in the otoliths

CHAPTER IV

Otolith morphometry in juveniles of Atlantic bluefin tuna (*Thunnus thynnus*)

Abstract

The Atlantic bluefin tuna (*Thunnus thynnus*, ABFT) is a species of great commercial value, which aquaculture is in constant development. To know the morphometric characteristics of the otoliths of juvenile ABFT from two different batches (wild and farmed), four morphological parameters (area, perimeter, length and weight), three shape indices (circularity, eccentricity and compactness), and other shape parameters (three contour irregularity or Moments of Region Boundaries, and one Fournier Descriptor for high frequency of contour irregularities) were studied, and a multivariate technique (Discriminant Canonical Analysis, DCA) was used to discriminate the tuna groups. Between batches, differences were obtained for weight, area, length, perimeter, eccentricity and one of Moments of Region Boundaries in left otoliths, and for weight and perimeter in the right otolith, being larger wild batch specimens otoliths. The DCA correctly assigned 63.4% and 57.4% (right and left otoliths, respectively) of the specimens to their procedure. The use of the otolith morphometry through shape indices and Moments of Region Boundaries showed their utility discriminating two batches of juvenile ABFT.

Keywords: morphometry, otolith, tuna, discrimination

Introduction

The currently European legislative document essential in traceability and food safety is the Regulation (EC) 178/2002, which highlights the necessity of tracking a product at any step of the supply chain, in order to ensure food safety, support sustainable fish farms and fisheries and to fight illegal activities and fraud. Implementing seafood traceability has always been fraught with difficulties and a low traceability result in lacking knowledge about the source and mislabeling. Aquaculture and wild fisheries are facing the same problems for traceability because processors and retailers often handle the same type of products. In this context, batch discrimination methods are essential, since failure in group differentiation may lead to non-optimal exploitation or even over-fishing (Begg et al., 1999; Heath et al., 2013). The support of independent and validated control technologies, like natural tracers' tools, would be highly beneficial to fisheries and its multiple components (Stockhausen et al., 2009).

In recent years, diverse natural marking methods have been developed in aquaculture for various fish species (Canonico et al., 2005; Krkošek et al., 2006; Brooks & Jones, 2008; Glover et al., 2013). Among them we find the use of otoliths as a tool to identify species batches (Messieh, 1972; Lombarte & Lleonart, 1993). The otoliths, are calcified and bilateral structures, with important functions in balance and hearing (see a review in Schulz-Mirbach et al., 2019). Moreover, they grow continuously during the fish's life, remaining the mineral part unaltered after deposition (Campana & Thorrold, 2001). The otolith morphometry, which is the morphology and shape altogether, is considered a useful tracer in stock discrimination (Campana & Casselman, 1993), because it is species specific and depends on a mixture of genetic and environmental factors (Gagliano & McCormick, 2004; Mérigot et al., 2007; Vignon & Morat, 2010; Mille et al., 2016; Vignon, 2018; Mahé et al., 2021).

The otolith morphometry is easy to analyze through image analysis software tools that allow the obtention of data with great precision, avoiding the bias of human

observation and incorporating additional measurements to the classical morphology (weight, area, length, width and perimeter), such as the shape indices (circularity, eccentricity and compactness), the Moments of Region Boundaries and the Fourier Descriptors, some of which have helped to discriminate fish populations (i.e., Wang et al., 2019 and Geladakis et al., 2021). In as iconic species in the Mediterranean as the Atlantic bluefin tuna (ABFT, *Thunnus thynnus*), reliable traceability tools are also being demanded since two types of batches are currently handled in the market: extractive-fished (wild) and captive-reared (farmed). In previous studies, the combined use of measurements and multivariate statistical techniques in otoliths' morphology have discriminated wild ABFT adult stocks with different provenance (Brophy et al., 2016) and ABFT juvenile age groups (Megalofonou, 2006), but no studies have been done to discriminate juvenile batches from different provenance.

In this context, we assess the otolith morphometry in two different batches of juvenile ABFT, wild specimens collected in Mediterranean Sea and specimens born in captivity and raised in inland facilities, to determine whether these specimens could be discriminated based in morphological characteristics of their otoliths.

Material and Methods

i. Tuna sampling

101 ABFT specimens (from 100 to 1500 grams) from two batches were sampled. Specimens from batch 1 (wild, n=36) were caught by the hook-and-line method (barbless hook) in October 2018 in Mazarrón Bay (Murcia, Spain) and sampled immediately after capture. Fish from batch 2 (farmed, n=65) were cultured in land-based facilities of the Spanish Institute of Oceanography (IEO, Mazarrón, Spain). For that, fertilized eggs coming from spawning captive adults kept in sea cages belonging to Ricardo Fuentes Group, were reared in a 40 m^3 tank. Hatched larvae were fed on rotifers (*Brachionus plicatilis*), copepods (*Acartia tonsa*), artemia, sea bream (*Sparus aurata*) yolk sac larvae and then weaned with an artificial diet (Magokoro, Marubeni Nissin Feed Co., Ltd., Tokyo, Japan). Overall temperature

was between 23.5 - 24.9°C and salinity of 37.5 g L^{-1}. At 41 days post-hatching (dph), they were transferred to a 900 m^3 tank with a recirculated aquaculture system in the Infraestructura para el Cultivo del Atún Rojo (ICAR Cartagena, Spain). Here, they were fed with European anchovy, *Engraulis encrasicolus*, round sardinella, *Sardinella aurita,* and Atlantic mackerel, *Scomber scombrus*. Fish were collected soon after death and sampled, so in accordance with European legislation (Directive 2010/63/UE), the practices employed did not required animal experimentation permission.

ii. Otolith extraction

The materials used for the otolith extraction were prepared carefully, using two phases to cleansing, consisting in the immersion in 96% ethanol, then in Milli-Q water. For each specimen, both right and left otoliths were extracted realizing a frontal section of the tunas' head and extracting the brain, which permitted to localise the inner ear from above. After the extraction, the otoliths were washed using Milli-Q water to eliminate the entouring otic tissue. Finally, the otoliths were placed in small polyethylene tubes where they dried at room temperature previously to their storage.

iii. Morphometric analysis

The two otoliths from each specimen were placed in a dark field, following the positioning recommendations from the Report of The ICCAT GBYP International Workshop on ABFT growth (Rodríguez-Marín et al., 2020). The right otolith was placed on top of the image field; the left on the bottom, with the anti-rostrum side up and towards the interior of the pair (**Figure V.IV.1**).

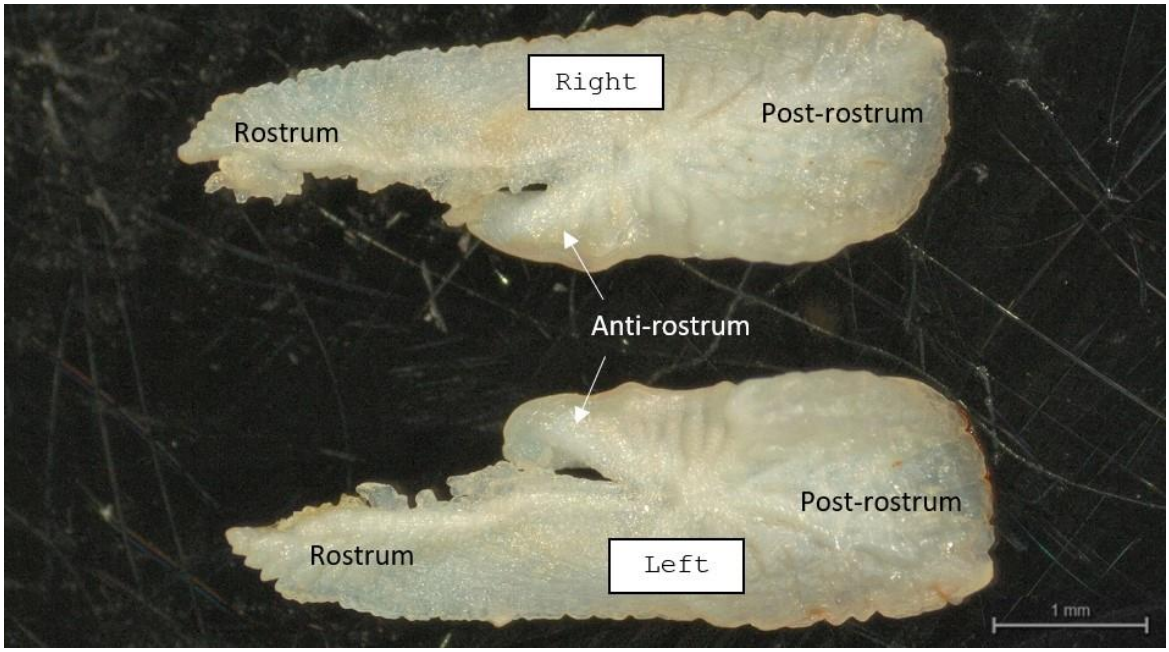
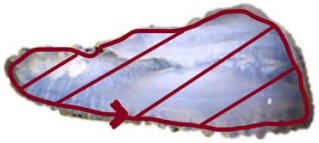


Figure V.IV.1. Both sides of otoliths from juvenile of ABFT.

Each otolith pair was observed (x1 and x2 magnification) under a stereomicroscope (Leica, Greenough Stereo Microscopes S9 Series, www.leica-microsystems.com) connected to a computer. Acquisition, image processing and analysis were performed using image software analysis Otolab (Nava et al., 2018). Seven structure parameters or traits were measured directly by the software in both right and left otoliths from each specimen: morphology parameters (area – OA -, perimeter -OP-, length -OL -, width -OW-), shape indices (circularity -OCI-, eccentricity -OE-, compactness -OCO-), shape in the form of four Moments of Region Boundaries (OF1, OF2, OF3, OF13) and a Fourier Descriptor (OFF) (**Table V.IV.1**).

Table V.IV.1. Measures of traits in ABFT juvenile otoliths according the software Otolab (Nava, 2018), their concepts and definitions.

Type of trait	Parameter	Concept	Definition
Morphological	Area (mm^2)		Number of pixels multiplied by the area of each of them (this must be calibrated previously[4]).
	Perimeter (mm)		Defined as the contour of an object, here the *Freeman chain code* with conectivity to 4 was used[5].
	Length & Width (mm)		Also called Feret diameters. Measured by the distance in pixels multiplied by their length[6]

[4] In most cameras, the pixels are square, so the size of the pixel can be estimated measuring one length. This must be done in the center, to avoid optical aberration of the entire lens (the pixels are deformed in at the outer edges).

[5] See a deeper explaination in Luengo (2010).

[6] The major and minor axes are defined by the direction of the two major eigenvectors of the object.

Shape indices (Dimensionless)	Circularity 		Similarity to a perfect circle, being 1 is a perfect circle Calculated from the area and perimeter following Eddins (2023) formula[7].
	Eccentricity 		Measures how elongated an object is, it has a value between 0 and 1. Related to a quotient between width and length[8].
	Compactness 		Roughness. Inverse to circularity.
Moments of Region Boundaries (Dimensionless)	OF1	$F1 = \frac{\sigma}{mean}$	The coordinates of all contour pixels are determined and their distances to the geometric center of the object (center of mass) are calculated. These coordinates are stored in two vectors (x, y), and new vectors can be calculated with the distances of each pixel from the center of mass. This vector of
	OF2	$F2 = \frac{\sqrt[3]{skewness}}{mean}$	
	OF3		

[7] C = $\frac{4\pi a}{p^2}$, where *a* is the area of a shape and *p* is its perimeter.

[8] Takes values of 0 for a circle and 1 for a very elongated ellipse.

		$F3 = \frac{\sqrt[4]{kurtosis}}{mean}$	distances has classical statistical parameters (mean, skewness, kurtosis…). OF1 measures irregularity, OF2 surface asymmetry and OF3 dispersion of this vector. OF13 measures the global irregularity in a simple way (i.e., otoliths with many lobulations should have higher values)[9]
	OF13	$F13 = F3 - F1$	
Fourier Descriptor (Dimensionless)	OFF		Measures the noise of the contour, how smooth the edges are. It is larger in more irregular objects, and grows larger when more fractal appearances, which the Moments of Region Boundaries do not (Shen et al., 1994).

[9] These parameters were popularized in a work by Shen and colleagues (1994).

Finally, the otoliths were weighted (the weight of the otolith -WO-) to the nearest 0.001 mg using an electronic micro-balance Sartorius CP2P, balanced using a material reference tested following the normative (ISO 9001) with four decimals attached.

iv. Statistical treatment

The traits results were subjected to statistical analysis using the SPSS software (*Statistical Package for the Social Sciences*, *IBM 24.0*, New York). For these eleven parameters, median, minimum and maximum values were obtained.

To ensure that differences in fish size among batches did not alter the results, the weight possible difference among batches was tested using U-Mann-Whitney, and a General Linear Model (GLM, with MANOVA as mean comparison test) with the fish weight as covariable was performed, and the means of each trait (corrected means) were calculated removing the differences due to the fish weight.

In order to discriminate the tuna batches using its morphometry, the multivariate technique DCA was used. Previously, the residuals were calculated to remove the effect of size (using the tuna weight as a proxy for fish size) in the observed value (raw data). The residuals data are obtained as follow:

$$Residual\ data\ = raw\ data - (a + b\ x\ TunaW)$$

where *a* is the constant and *b* is he slope for the fish weight (*TunaW*). In addition, OF1, OF2, OF3, OF13 and OFF data were excluded due to their redundancy. For DCA, Wilk's Lambda was used to test the significance of the discrimination ($p<0.05$), two functions were created, and a split-sample validation (cross-validation testing procedure) was performed to assess the capacity of the selected variables to predict different groups for the tested fish. The discriminant function is written as:

$$D = b_0 + b_1X_1 + b_2X_2 + \cdots + b_kX_k$$

where, D is the discriminant score, and b represents the coefficients or weights for the predictor variables X.

The proportion of specimens correctly reallocated is taken as an integrity measurement for a group (Poulet et al., 2005; Yakubu & Osenbor, 2011). The formulas from the case classification were obtained to classify same species but of uncertain background. In these formulas, the constant and function coefficients were obtained for each of the batches and variable:

$$F(x) = a + (b * [X])$$

where a = constant for the combination of an otolith trait and a batch; b = coefficient of classification function for the combination of an otolith trait and a batch; and X = the value of a trait measure for a given batch (in a particular specimen). Once the formula has been applied, the result with the highest value classifies the background of the fish.

For all tests, the significance levels were set at 0.05.

Results & Discussion

i. Morphometry comparison

Descriptive data of the otolith morphometric traits (raw data) are show in **Table V.IV.2**. Between batches, significant statistical differences were only found for OF2 in left otoliths (GLM, MANOVA, $p < 0.05$).

Regarding the tuna weight, no statistical differences between batches were found (U-Mann-Whitney, $p < 0.05$): 328.2 (255.4-758.6 grams) and 417.0 (100.6-1455.0 grams) for batch 1 and 2, respectively. Fish size and otolith size are generally strongly correlated (Hüssy, 2008), so the relation between the fish size and the

otolith morphometry was tested (GLM, with MANOVA for differences among batches, $p< 0.05$). This test showed correlation between the tuna weight and WO, OA, OL, OW, OE, OP, OF1 y OF3, as well as differences among batches for WO (right), and for WO, OA, OL, OE y OF2 (left), being these differences marginally significant for OP (right and left) (**Table V.IV.3, and Figure V.IV.2**). For these data with significant statistical differences, the values were higher in specimens from batch 1 (wild tunas).

The majority of the traits with statistically significant differences among batches were classic morphology measurements (i.e., WO, OA, OL, OW and OP). This is in accordance with other authors that suggest the traits such as area, perimeter and shape indexes as an easier way to discriminate groups from other more sophisticated methods such as Fourier series analysis (Bölles & Begg, 2000; Tuset et al., 2003). In fact, OE and OF2 are not classical morphology measurements, but OE is a shape index, and it determines the position of the centre of mass in reference to a perfect circle (Russ, 1990); meanwhile OF2 is a Moment of Boundaries, which measures the otolith surface asymmetry. These last traits should also be bear in mind in future otolith morphometry studies. In addition, four variables showed differences among batches only in left side otoliths (OA, OL, OE and OF2). These right and left side differences could be explained by the existence of some pathologies (i.e., calcification abnormalities, asymmetry, etc.) that result in larger otoliths that may be biased towards the left side (Tomás & Geffen, 2003; Reimer et al., 2016). Furthermore, the otolith shape depends both on fish genotype and on environmental influence (Mérigot et al., 2007; Vignon & Morat, 2010; Mille et al., 2016; Mahé et al., 2021), so the growing conditions could play a key role in the tissue configuration of both terrestrial and aquatic provenances (Jara & Chodyniecki, 1999; Brucka-Jastrzêbska et al., 2009). In this sense, the stressful environmental conditions suffered in open waters, like strong and abrupt shift in water composition (i.e., dissolved minerals or pollutants), temperature, salinity or composition (Vinagre et al., 2014), may affect the otolith crystalline growth through the calcification process. During this calcification, the otolith conformation is dependent on the organic matrix and endolymph chemistry, and therefore alterations in its homeostasis may generate

different forms of crystals (Gauldie, 1986; Shivkumara et al., 2006). This calcification disruption is often described as a cause of 'abnormal' otoliths (otoliths with unexpected shapes) and sometimes, asymmetry between the two sides of an individual (Browning et al., 2012; Jawad et al., 2016; Jawad & Adams, 2021; Yedier, 2022; Yedier et al., 2022). Therefore, future studies on fluctuating asymmetry could shed some light on these results.

Table V.IV.2. Descriptive original data (median, minimum and maximum) of fish weigh and the otolith morphometric traits. [a] Statistically significant differences between two batches. R= right, L= left. Batch 1= wild, batch 2 = farmed.

	Side	Batch 1	Batch 2
Tuna weight (gr)		328.20 (225.40- 758.60)	417.00 (100.60 – 1455.00)
WO (mgr)	R	3.82 (2.93-5.41)	4.04 (1.80-7.62)
	L	3.81 (3.11-5.53)	4.06 (1.71-7.69)
OA (mm^2)	R	5.55 (4.48- 7.15)	5.59 (2.32-25.81)
	L	5.61 (4.59 – 7.30)	5.34 (2.26-19.44)
OL (mm)	R	4.78 (3.93- 5.61)	4.74 (3.52-9.69)
	L	4.68 (3.61-5.64)	4.72 (2.83-9.65)
OW (mm)	R	1.65 (1.53-1.93)	1.65 (0.76-3.65)
	L	1.66(1.52-1.88)	1.69 (0.98-3.41)
OE	R	0.94(0.90-0.95)	0.94 (0.89-0.96)
	L	0.93(0.86-0.95)	0.94 (0.78-0.96)
OP (mm)	R	12.38(10.34-15.68)	12.22 (9.95-25.69)
	L	12.38(10.57-15.12)	12.06 (8.07-25.44)
OCI	R	0.47(0.33-0.55)	0.46 (0.23-0.61)
	L	0.47(0.32-0.58)	0.47 (0.20-0.69)
OCO	R	26.71(23.06-38.41)	27.31 (20.76-54.99)
	L	27.03(21.55-40.15)	26.86 (18.17 – 62.35)
OF1	R	0.37(0.33-0.47)	0.38 (0.30-0.50)
	L	0.36(0.28-0.44)	0.37 (0.21-0.47)
OF2	R	0.15(-0.22- 0.23)	0.14 (-0.23-0.23)
	L [a]	0.15 (0.37-0.53)	0.10 (-0.27 – 0.26)
OF3	R	0.41(0.37-0.53)	0.42 (0.33-0.57)
	L	0.40(0.32-0.50)	0.41 (0.24 – 0.56)

OF13	R	0.04(0.04-0.07)	0.04 (0.03-0.09)
	L	0.04(0.04-0.06)	0.04 (0.03-0.09)
OFF	R	0.39 (0.10-0.53)	0.41 (0.06-0.60)
	L	0.41(0.18-0.57)	0.41 (0.08-0.68)

Table V.IV.3. Corrected means coming from the GLM. [a] Statistical significant differences between batches (MANOVA, P < 0.05); ms marginally significant differences (MANOVA, p=0.05-0.100); R= right, L= left; batch 1= wild, batch 2 = farmed.

Trait		Batch 1	Batch 2
WO (mgr)	R[a]	4.345	3.973
	L[a]	4.380	3.984
OA (mm^2)	R	6.104	5.648
	L[a]	6.146	5.417
OL (mm)	R	4.923	4.723
	L[a]	4.919	4.463
OW (mm)	R	1.711	1.631
	L	1.714	1.668
OE	R	0.937	0.936
	L[a]	0.937	0.919
OP (mm)	R[ms]	13.034	12.406
	L[ms]	12.958	12.219
OCI	R	0.455	0.453
	L	0.463	0.459
OCO	R	28.060	28.708
	L	27.439	28.954
OF1	R	0.375	0.377
	L	0.372	0.355
OF2	R	0.118	0.078
	L[a]	0.119	0.030
OF3	R	0.421	0.424
	L	0.416	0.402
OF13	R	0.046	0.047
	L	0.044	0.047
OFF	R	0.385	0.392
	L	0.401	0.389

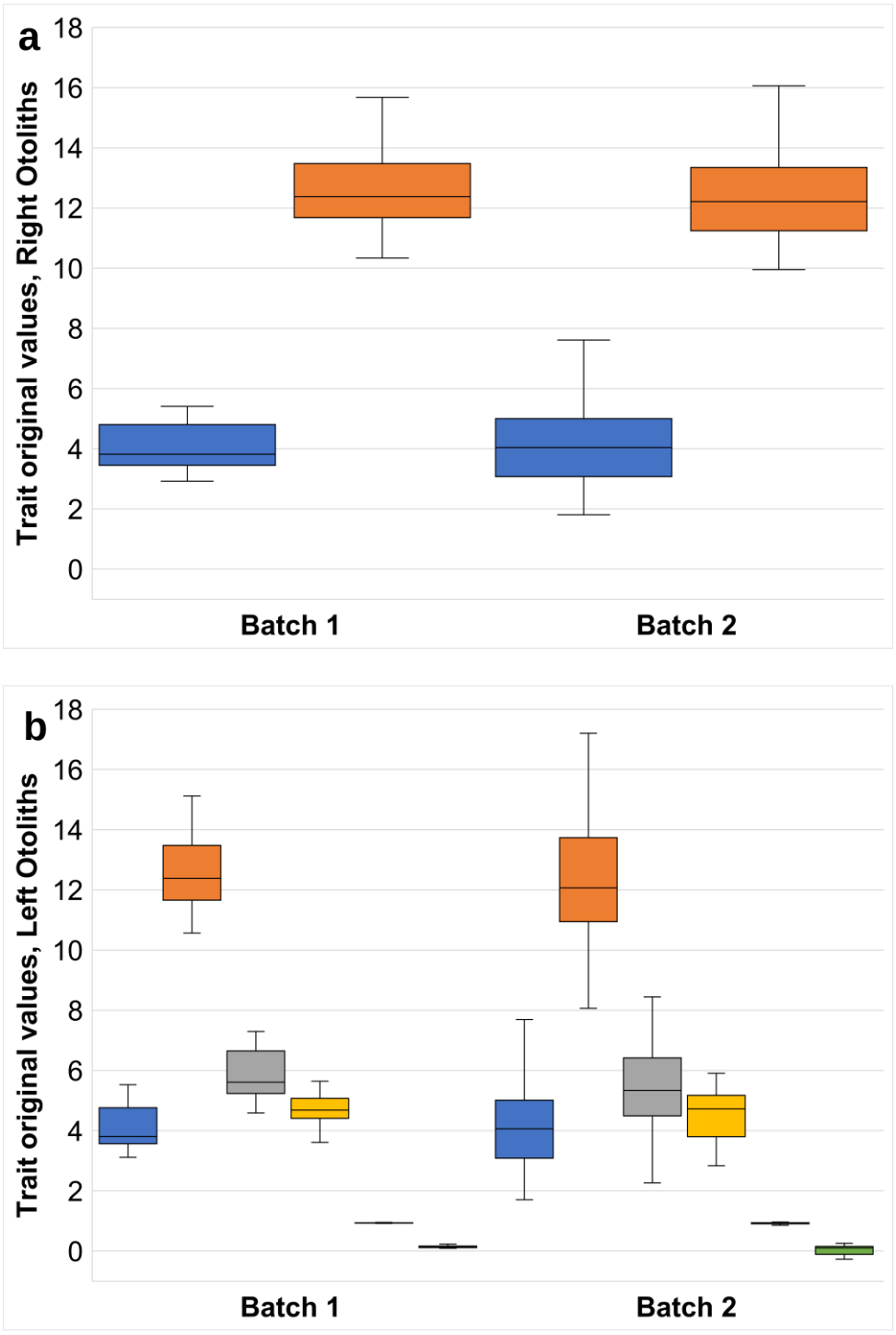


Figure V.IV.2. Data for the two presented batches, original values for the traits with significant statistical differences in the MANOVA. WO, OP, OA-L, OL-L, OE-L, and OF2-L. a) right, and b) left otoliths. Batch 1= wild, batch 2 = farmed.

ii. Discriminant Canonical Analysis

In the DCA output, WO (right and left) and OE (left) were selected (**Table V.IV.4**) as morphological variables to discriminate. They constituted a unique Canonical Discriminant Function (CDF), explaining the 100% of the total variance of the dataset. The CDF coefficients permit to assess the importance of the variables, with a higher value meaning higher importance. In this case, WO was the trait with higher discrimination importance for the otoliths from both sides. This is in accordance with the results obtained with the GLM test, where the WO was highlighted in both right and left otoliths, meanwhile OE was only present in left otoliths.

Table V.IV.4. Elemental based CDF outcoming from the DCA analysis information.

Side	CDF1 (coefficients)	Eigenvalues (%)	Lambda Wilks (sig)	Canonical correlation	% success (cross-validation)
Right	WO (1.000)	0.096 (100)	0.912 (0.003)	0.296	63.4
Left	WO (0.702) OE (0.580)	0.155 (100)	0.866 (0.001)	0.366	57.4

Afterwards, a cross-validation procedure was performed to assess the capacity of the selected variables to predict different provenance for the tested fish. In the right otoliths' DCA, the 63.4% of tunas were successfully classified, meanwhile in left otoliths this happened with the 57.4%. Nevertheless, regarding the classification accuracy by batch, tunas from the batch 2 were better classified, surpassing the 80% in both otoliths, and tunas from batch 1 were poorly classified with percentages lower than 20% (**Table V.IV.5**). This is an important fact, given that the farmed specimens could be better identified, being of highly interest for the economy and management of the species. This is in accordance with the previous mentioned conditions in farmed tunas, which have a more constant and plentiful diet, which would homogenize the otolith size results, giving better batch

discrimination. In addition, the ambient quality and chemistry conditions are much more controlled in aquaculture systems and it would permit more homogeneous otolith size results. In this study, the farmed tuna rearing was made in tanks with recirculating aquaculture systems, which allow better control on wastes (Blancheton et al., 1996). In addition, the wild tunas' ecosystem (Mazarron Bay, part of the west Mediterranean) could be exposed to more heterogeneous conditions, with water chemistry and quality shifts. Also, another main cause affecting the otolith size is environmental stress (Vinagre et al., 2014), which could be triggered specially by factors like marine pollution (i.e., agricultural wastes carried by the rivers, discharge of domestic and industrial wastes to the sea without treatment, petroleum-derived pollutants from sea accidents; Bat et al., 2018; Pokazeev et al., 2021).

Table V.IV.5. DCA percentages of classification accuracy (*) and missclasification by batch and side of ABFT otoliths. Batch 1= wild, batch 2 = farmed tunas.

Side	Batch	1	2
Right	**1**	19.4*	80.6
	2	12.3	87.7*
Left	**1**	13.9*	86.1
	2	18.5	81.5*

The graphical representation of these results (plotted as discriminant scores, formed by the addition of the DCA coefficient for each element, **Figure V.IV.3**) show that the batches are different enough to have very little spatial superposition, as batch 1 specimens' coefficients are mostly positive meanwhile batch 2 tunas have a wider sign range of the data.

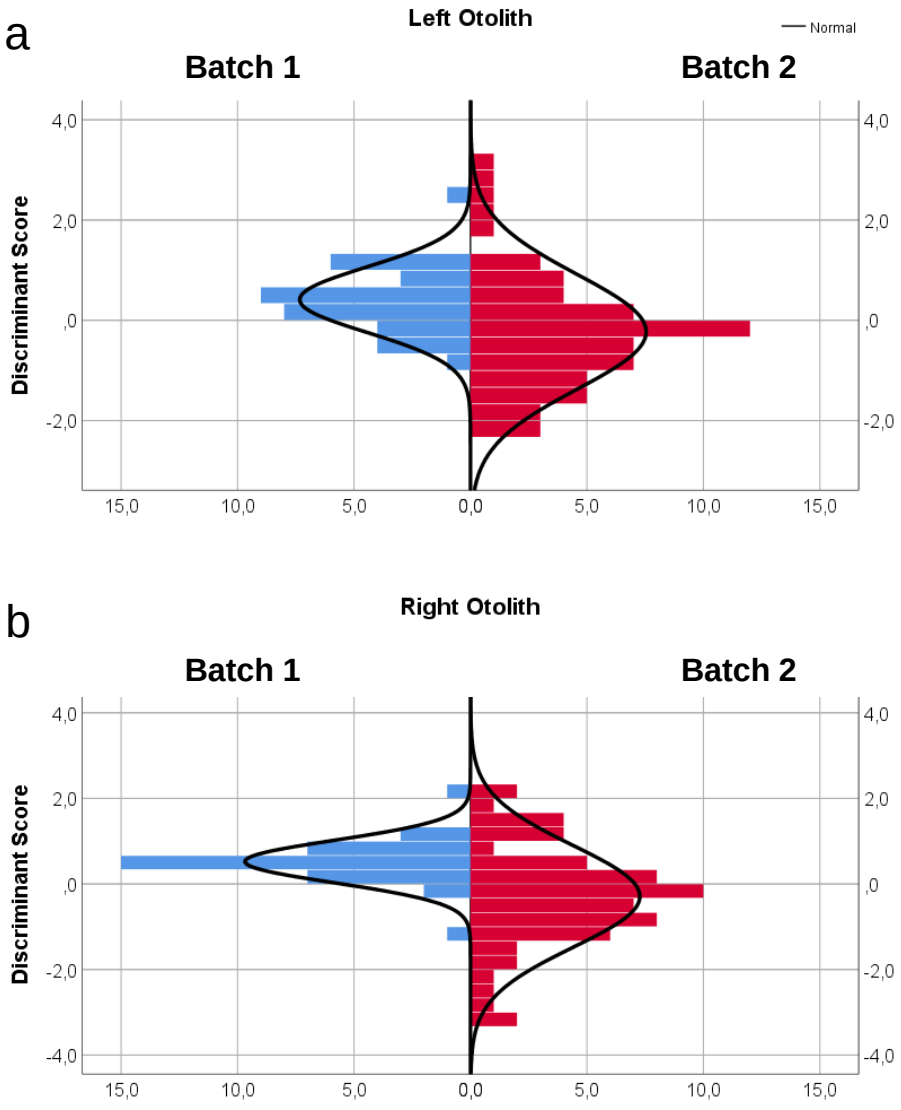


Figure V.IV.3. Graphic upcoming from the DCA with elements' concentrations. The axes represent the residuals values (x) and the number of them (y) for each batch.

Finally, the obtained formulae can be applied to unidentified tunas, given that when introducing the traits' values in the formula the result can tell us the probable tuna batch (**Table V.IV.6**). When substituting the trait value in each of the formula, the group with the higher number would be the probable batch of the fish, with a probability, in left otoliths, of 13.9% and 81.5% of being wild and farmed, respectively. Yet, we have to keep in mind that this results and specially the formulae presented can be only applied to the batches described in this study (wild and farmed tunas from the Mazarrón Bay). In this sense, further studies

including more batches and samples should be pursued to standardize these results and formulae.

Table V.IV.6. Formulae to identify the fish batch, where WO and OE= value for the given trait. Batch 1 = wild, batch 2 = farmed.

Side	Batch 1	Batch 2
Right	Z = [0.537 x (WO right)] + (-1.080)	Z = [(-0.571 x (WO right)] + (-0.495)
Left	Z = [0.319 x (WO left)] + [12.166 x (OE left)] + (-1.127)	Z = [(-0.632) x (WO left)] + [(-4.013) x (OE left)] + (-0.529)

Conclusion

The use of the otolith morphometry through shape indices and Moments of Region Boundaries, have shown their utility discriminating two batches of juvenile ABFT. Therefore, we recommend their use coupled with multivariate analysis tools like MANOVA and DCA. In this study, the DCA permitted to difference wild specimens with a high success rate. On the other hand, we recommend to abord these studies with otoliths from both sides, given that the side of the otolith seems to modify the batch differentiation results. The wild tunas presented left otoliths with higher area, length, eccentricity and F2, meanwhile their otoliths were heavier and had bigger perimeter in both sides. The environmental conditions and life regime in juveniles seem to be natural tracers in tuna otoliths, therefore, we recommend that future studies target adult tunas to see the evolution of these differences in the otoliths with time.

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CHAPTER V

Asymmetry study in otoliths from Atlantic bluefin tuna (*Thunnus thynnus)* form two different environments

Abstract

Captive-reared fish tend to present a higher level of asymmetry in bilateral structures. To test these predictions, the asymmetry in juvenile of Atlantic Bluefin tuna (ABFT, *Thunnus thynnus*) were studied as a proxy for group differentiation. More specifically, we addressed the following questions: (1) what types of asymmetry occur in these specimens? and (2) does the level of asymmetry vary among groups?. For this purpose, two ABFT batches were studied: captive-reared tunas (farmed) and extractive-fishing (wild) tunas. Eight otolith morphology landmarks were used to quantify different types of asymmetry: directional asymmetry, antisymmetry and fluctuating asymmetry, with two index, one standard and another developed through Principal Component Analysis and the Akaike Information Criterion. Only two types of asymmetry were found in both batches: antisymmetry and directional asymmetry, being antisymmetry more frequent. The asymmetric parameters in studied batches were the otolith weight, length, width, eccentricity and compactness, and differences were found specifically between batches for width and eccentricity, with higher asymmetry values in specimens from farmed tuna batch.

Keywords: asymmetry, Atlantic bluefin tuna, environment, juveniles, otolith

Introduction

The developmental stability (DS) refers to the capacity of an organism to withstand disturbances during its development (Leary et al., 1992; Somarakis et al., 1997a; Palmer & Strobeck, 2001; Băncilă et al., 2012). The core idea about DS is that both sides of an organism can be perceived as independent replicas of the same developing event. In a homogeneous ambiance, a bilateral symmetry will appear. Meanwhile, in a non-homogeneous environment, small random perturbations (developmental noise) can deviate the developmental pathway from the expected trajectory. As these processes act locally, their effect will accumulate on right and left side separately, leading to asymmetric phenotypes (Geladakis et al., 2020). The sensitivity to these random perturbations can be viewed as the tendency to produce structural changes in response, and is often called developmental instability (DI) (Klingenberg, 2002; Nijhout & Davidowitz, 2003). The phenotypic result of DI is called fluctuating asymmetry (FA), and its analysis is a common approach for assessing these DI and DS and therefore, acts as a biomarker for the individual's fitness and/or stress (Parsons, 1989; Dongen, 2006; Beasley et al., 2013; Sánchez-Chardi et al., 2013). In this sense, morphometric tools (i.e., measurable characters or length-based measures of specific body parts) have been proved to be more sensitive for the asymmetry detection, compared to studies that only use meristic tools (i.e., countable traits like the number of fins or branchial arcs on a fish; Beasley et al., 2013).

There are several types of asymmetry described (**Figure V.V.1**): i) the fluctuating asymmetry (FA), which refers to a pattern of bilateral variation in a sample of individuals where the mean of the side differences (R - L) is zero and the variation is normally distributed about that mean (**Figure V.V.1a)**; ii) directional asymmetry (DA), that occurs when there are right (R) and left (L) structural differences (Palmer & Strobeck, 1986, 1992; Palmer, 1994), and the side that is larger is generally the same in a sample (**Figure V.V.1b**), resulting in a non-zero mean distribution (i.e., R is always higher than L, or *vice versa*; Somarakis et al., 1997a; Loher et al., 2008; Kajajian et al., 2014); and iii) antisymmetry (AS) (**Figure V.V.1c**), that appears when the R-L structural differences varies randomly among individuals (i.e., resulting in a bimodal distribution). This last, can have a genetic

and/or a non-genetic basis and therefore can be undistinguishable from FA (Palmer, 1994), because for both types of asymmetry, some portions of the between-sides variation could be due to developmental noise, and therefore environmental causes (Palmer & Strobeck, 1992).

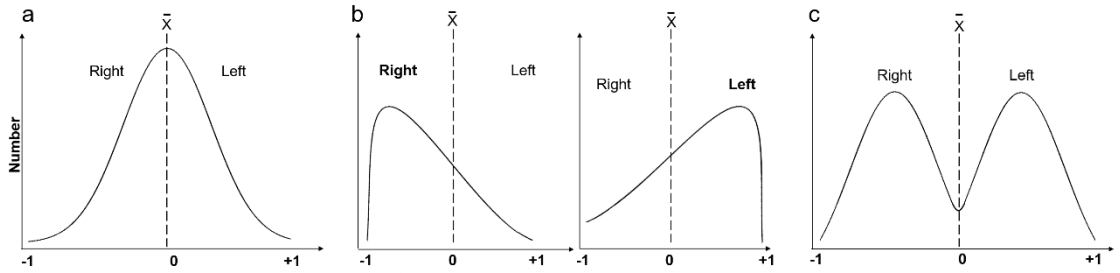


Figure V.V.1. Different types of asymmetries: a. Fluctuating asymmetry (FA), b. Directional asymmetry (DA), c. Antisymmetry (AS). Based on: Vallortigara & Rogers, 2005; Mahé et al., 2021.

In fish, some bilateral traits have been used to study DI, such as the eye diameter, the length of fin rays, dentition, form of pharyngeal arches, and otoliths' shape *inter alia* (Michaelsen et al., 2015; Leung et al., 2017). Concretely, the otolith shape (Mérigot et al., 2007; Mille et al., 2016; Vignon, 2018; Mahé et al., 2021), depend on a mixture of genetic and environmental factors and the separation of populations induces divergent otolith morphometries (Messieh, 1972; Lombarte & Lleonart, 1993), which could help to discriminate different populations or batches. This is because the otoliths have species-specific shapes and sizes (Fey et al., 2020). This structure is generally right-left symmetrical, except in flatfish and catfish (Panfili et al., 2002), reason why in the otolith, some asymmetries like the FA have been proposed to be useful indicators of fish growth, condition, fitness, or level of stress (Díaz-Gil et al., 2015). Some of the possible causes of DI and therefore asymmetry are water chemistry (Fey et al., 2022; Yedier et al., 2022a), diet (Browning et al., 2012; Johnsson et al., 2020; Jawad & Adams, 2021), diseases (Pasnik et al., 2007; Jawad & Adams, 2021) and environmental stressors (Manizadeh et al., 2018; Mahé et al., 2019; Yedier et al., 2022a; Yedier, 2022).

The FA has been the most studied asymmetry, and it has been measured using the number of pectoral fin rays, gill arks, fish body proportions, eye spot area, otolith size and shape in some species (*Heteropneustes fossilis* -Al-Hassan et al., 1990; *Mystus pelusius* - Al-Hassan & Hassan,1994; *Merluccius productus* - Escós et al.,1995; *Engraulis encrasicolus* - Somarakis et al., 1997a,b; *Tilapia zilli* - Jawad, 2001; four sparid fishes- Jawad, 2003; 2004; *Perca fluviatilis* - Øxnevad et al., 2002; *Salaria pavo* - Gonçalves et al., 2002). Nevertheless, in some species no data about its DI or otolith asymmetry has been reported. One of these species is the Atlantic bluefin tuna (ABFT, *Thunnus thynnus*), which biological cycle was fully disentangled in 2016 in the facilities of the Spanish Oceanography Institute (Ortega & De la Gándara, 2017). Therefore, specimens of this species from both aquaculture facilities (farmed), and extractive-fishing (wild) coexist in the market and methods to distinguish both baches should be found in a nearly future. Otoliths from juvenile ABFT were analyzed with the purpose of detecting the possible asymmetry presence and its types in ABFT. If present, the asymmetry was quantified to compare its level among wild and farmed tuna batches.

Material & Methods

i. Tuna sampling and otolith extraction

Otoliths were extracted from 101 ABFT juveniles weighing between 100 to 1500 grams. The specimens from batch 1 (wild, n=36) were caught by hook-and-line method (barbless hook) in October 2018 in Mazarrón Bay (Murcia, Spain) and sampled immediately after capture. The specimens from batch 2 (farmed, n=65) were cultured in land-based facilities of the Spanish Institute of Oceanography (IEO, Mazarrón, Spain). Fertilized eggs coming from spawning captive adults kept in sea cages belonging to Ricardo Fuentes Group, were reared in a 40 m3 tank. Hatched larvae were fed on rotifer, artemia, sea bream yolk-sac larvae and then weaned with an artificial diet (Magokoro, Marubeni Nissin Feed Co., Ltd., Tokyo, Japan). Overall temperature was 24.9°C and salinity 37.5 g L^{-1}. At 41 days post-hatching (dph), they were transferred to 900-m^3 tank in the Infraestructura

para el Cultivo del Atún Rojo (ICAR Cartagena, Spain) where they were fed with European anchovy, *Engraulis encrasicolus*, Round sardinella, *Sardinella aurita,* and Atlantic mackerel, *Scomber scombrus*. Fish dying due to traumatic events (collision against the tank walls) or for natural reasons were collected soon after death and sampled. In accordance with European legislation (Directive 2010/63/UE), the practices employed did not required animal experimentation permission.

The two otoliths were extracted carefully by opening the fish head from above. All the materials used for the otolith extraction were prepared carefully, using two phases to cleansing, consisting in the immersion in 96% ethanol, then in Milli-Q water. Finally, the otoliths were placed in tubes where they dried at room temperature previously to their storage.

ii. Morphometric analysis

Otoliths from each specimen were placed in a dark field, following the positioning recommendations from the Report of The ICCAT GBYP International Workshop on ABFT growth (Rodríguez-Marín et al., 2020). Then, as described in the previous Chapter ('**Otolith morphology in juveniles of Atlantic bluefin tuna (*Thunnus thynnus*)**'), each otolith pair was observed (x1 and x2 magnification) under a stereomicroscope (Leica, Greenough Stereo Microscopes S9 Series, www.leica-microsystems.com) connected to a computer. Acquisition, image processing and analysis were performed using image software analysis Otolab (Nava et al., 2018). Seven structure parameters were measured directly by the software in both right and left otolith from each specimen: measures of morphology (area – OA -, perimeter -OP-, length -OL -, width -OW-), and shape indices (circularity -OCI-, eccentricity -OE- and compactness -OCO-). Finally, the otoliths were weighted (weight of the otolith -WO-) to the nearest 0.001 mg using an electronic micro-balance Sartorius CP2P, balanced using a material reference tested following the normative (ISO 9001) with four decimals attached.

iii. Asymmetry analysis

A simple parameter (A_i) was calculated by specimen and trait. This parameter is obtained from the subtractions of the left value to the right value from a given trait R_i-L_i (Palmer, 1994), and shows the asymmetry of a particular trait for an individual (i), whose direction is shown just by regarding its positive (towards right side) or negative (towards left side) sign.

To avoid possible differences associated to the weight of the tunas, the A_i data from the traits were weight-detrended using the residuals (Linear Regression analysis in the whole population, tuna weight used as a proxy for fish size) by subtraction of the common linear slope from the observed traits (unstandardized residuals):

$$Residual\ data\ =\ raw\ value - (a\ +\ b\ x\ tunaW),$$

where *a* is the constant and *b* is he slope for the tuna weight (*tunaW*).

To detect the presence of two types of AS (genetic and environmental), a one-sample Kolmogorov-Smirnov test was used for the A_i data (H_0: normal distribution, $p < 0.05$), for trait by batch. AS was considered present if a non-normal distribution was found (H_1). Second, to evaluate the DA presence, a Wilcoxon test was performed for the A_i data (H_0: median = 0, $p < 0.05$) for each trait by batch, and DA was considered present if the median was significantly different from zero (H_1).

To determinate FA, one standard index (FAI) was generated per individual and trait (Palmer & Strobeck, 2001). This index represents the mean of the absolute bilateral difference ($|A_i|$). To confirm the presence of FA, the normality of the FAI was tested (H_0: normal distribution, $p < 0.05$), being FA present if there was a normal distribution (Kolmogorov-Smirnov, $p< 0.05$).

iv. Statistical treatment

The statistical analyses were performed using the SPSS software (*Statistical Package for the Social Sciences*, *IBM 24.0*, New York). For the creation of the GIDI, Rstudio (Rstudio Team, 2020) was used. Due to the violation of normality (Kolmogorov-Smirnov Test, $p < 0.05$), non-parametric methods were used in this study.

The tuna weight was compared between batches (U-Mann-Whitney, $p< 0.05$). For the tuna weight, A_i and FAI (median, minimum and maximum) were obtained by batch (wild and farmed tuna). Differences for A_i between batches (U-Mann-Whitney, $p < 0.05$) were assessed.

On the other hand, an overall asymmetry index (Global Index of Developmental Instability, GIDI) was performed after standardizing the traits by dividing by the fish weight. It consists on a Principal Component Analysis (PCA) performed on all traits, for both left and right otoliths. With these analyses the most important traits were selected, and the Bartlett Score Factor (BSF) from Principal Component 1 (PC1) was extracted for each otolith (i.e., this factor proportions an unbiased estimate of true factor scores and is not correlated with other factors; DiStefano et al., 2009; STATS, 2009). Finally, right and left otolith's BFS from the same individual were subtracted to obtain the GIDI for each fish: GIDI = BFS Right - BFS Left. This index used the Akaike Information Criterion corrected for small samples (AICc) to compare the asymmetry results between batches:

$$AICc = AIC + \frac{2K(K+1)}{(n-K-1)}$$

where AIC is Akaike's information Criterion, *K* is the number of parameters and *n* is the number of observations (Burnham & Anderson, 2002).

AICc is used for model selection as it estimates the quality of each model, relative to other models. Consequently, it permits to determine if the general symmetry distribution in the samples have to be considered as one population or as two,

coinciding with our two batches. The AICc hypothesized two situations: the FA distribution is common, or separated in the batches. When the AICc for separated distributions is lower (in absolute value) than for the common distribution, the GIDI results can be treated as separated batches. In addition, as close is the Akaike Weight value to 1 (100% of probability) higher is the result reliability.

Results & Discussion

No differences in weight between batches were found (U-Mann-Whitney, $p < 0.05$; 328.2 and 417.0 grams for batch 1 and 2 specimens respectively, **Figure V.V.2**).

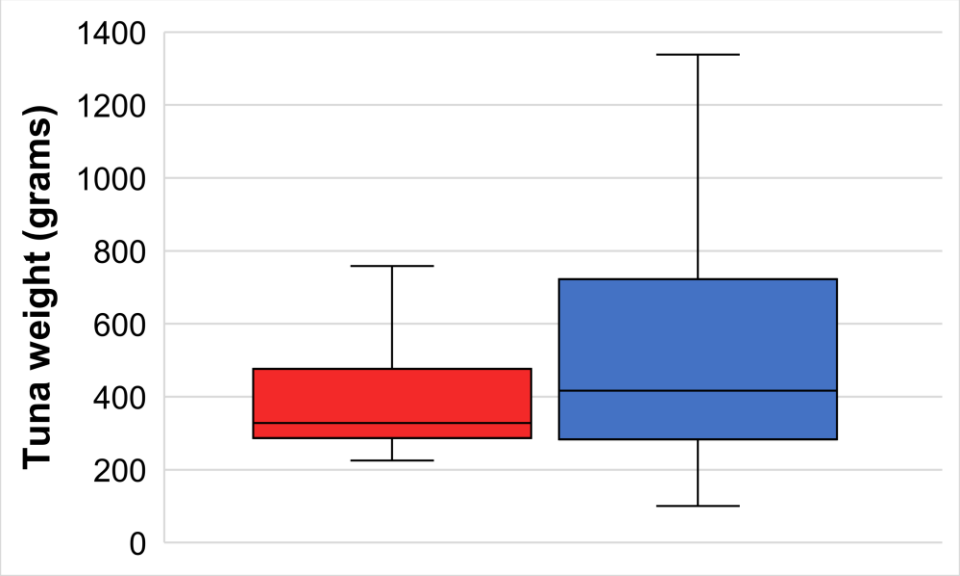


Figure V.V.2. Box -plots of tuna weight (median, 10th, 25th, 75th, 90th percentiles and outliers). Batch 1 (wild) and batch 2 (farmed).

This is the first study to analyze the asymmetry presence in juveniles ABFT otoliths. Results of the traits with AS, DA and FA are shown in **Table V.V.1**. Two types of asymmetry (DA and AS) were found when analyzing ABFT otolith traits, being more frequent AS. However, both AS and DA have been poorly documented in the literature by their own. In fact, a recent review (Mahé et al., 2019) signaled that less than 22% of the papers comparing groups through asymmetry have taken into account the AS measurement, being considered as a nuisance in the otolith asymmetry analysis and not being studied further. In fact, the AS is an asymmetry in which the structure differences appear randomly, and

according to Palmer (2005) it is the most common on fish. It depends on external aleatory stimuli (sometimes from the left and sometimes from the right) during ontogeny. According to some authors, this type of asymmetry could be consequence to adaptative advantages in relation to survival and reproduction (Nakajima et al., 2004; Palmer, 2005), and could eventually precede DA. On the other hand, the low DA frequency and the absence of FA in our study could be good indicator for the studied batches' fitness, because DA has been associated to unfavorable environmental conditions (Palmer, 1994), like shifts in the water temperature (Khedher et al., 2021) or the presence of pollutants (Yedier et al., 2022b); and FA has been associated to stressful factors like nutritional conditions (Grønkjær & Sand, 2003), a high eutrophization level (Almeida et al., 2008), or also chemical pollution (Sopinka et al., 2012).

Table V.V.1. Summary of the antisymmetry (AS), directional asymmetry (DA) and fluctuating asymmetry (FA) in the studied batches and traits; B1= batch 1, wild; B2= batch 2, farmed; nf= not found.

Trait	AS	DA	FA
WO	B1&B2	nf	nf
OA	B2	B1	nf
OL	B1&B2	nf	nf
OW	B1&B2	nf	nf
OE	B1&B2	B1&B2	nf
OP	nf	nf	nf
OCI	B1	B1	nf
OCO	B1&B2	nf	nf

In this study, AS appeared in both wild and farmed batches, however DA appeared in three traits (OA, OE and OCI) in wild and only in one (OE) in farmed (**Table V.V.1**). Regarding the literature, asymmetry factors have also been described on wild conditions, like genetic predisposition (Yedier et al., 2022a; Yedier, 2022), lower survival if abnormal otoliths present (David et al., 1994), or also environmental stress (Ma et al., 2008; Yedier et al., 2022a), nevertheless few studies of wilds' asymmetry have been developed to be conclusive (Yedier et al., 2022a). Thus, the A_i-residuals of the OE and OW were significatively higher in otoliths of specimens from batch 2 (**Table V.V.2, Figure V.V.3**), so the farmed specimens were more asymmetric, which agrees with the AICc of GIDI (higher AICc value for the separate distribution; the distribution was wider in specimens from batch 2, having more extreme values, **Figure V.V.4**), and with the literature findings. In this sense, many factors have been described as possible causes for asymmetry in farming conditions (**Figure V.V.5**), like the water chemistry (Fey et al., 2022; Yedier et al., 2022a), water quality (Vinagre et al., 2014; Yedier et al., 2022a), water temperature (Fey et al., 2022; Geladakis et al., 2022; Yedier et al., 2022a), diet (Browning et al., 2012; Jonhson et al., 2020; Jawad & Adams, 2021), environmental stress (Mahé et al., 2019; Yedier et al., 2022a; Yedier, 2022), diseases (Pasnik et al., 2007; Jawad & Adams, 2021), local physical or mechanical issues in the otoliths (Yedier et al., 2018; Mahé et al, 2019; Yedier & Bostanci, 2020; Yedier, 2022), and metabolic rate (Sweeting et al., 2004).

Table V.V.2. Descriptives (median, minimum-maximum) for A_i and FAI; * significant statistical differences between batches for A_i (comparing traits with asymmetry in both batches: WO, OL, OW, OE and OCO). Batch 1 = wild, batch 2 = farmed.

Trait	Batch	Ai	FAI
WO	**1**	0.211 (-0.598 - 0.231)	0.060 (0.001 – 0.598)
	2	-0.025 (-1.187 – 2.006)	0.144 (0.019 – 2.006)
OA	**1**	-0.138 (-0.690 – 0.233)	0.115 (0.006 – 0.648)
	2	-0.123 (-2.538 – 6.200)	0.249 (0.001 – 6.300)
OL	**1**	-0.035 (-0.570 – 1.264)	0.158 (0.11 – 1.191)

	2	-0.085 (-1.081 – 1.353)	0.388 (0.063 – 1.264)
OW	**1**	-0.008 (-0.228 – 0.069)	0.020 (0.002 – 0.228)
	2	0.024 (-1.123 – 0.773) *	0.053 (0.001 – 1.123)
OE	**1**	0.002 (-0.024 – 0.085)	0.005 (0.000 – 0.085)
	2	0.024 (-0.059 – 0.147) *	0.027 (0.002 – 0.147)
OP	**1**	-0.092 (-1.177 – 2.375)	0.402 (0.0129 – 2.375)
	2	0.031 (-6.888 – 2.779)	0.657 (0.002 – 6.888)
OCI	**1**	-0.013 (-0.200 – 0.046)	0.034 (0.003 – 0.200)
	2	-0.021 (-0.291 – 0.207)	0.062 (0.000 – 0.292)
OCO	**1**	-0.155 (-4.963 – 13.779)	1.424 (0.027 – 13.859)
	2	0.249 (-19.821 – 25.041)	3.086 (0.055 – 25.102)

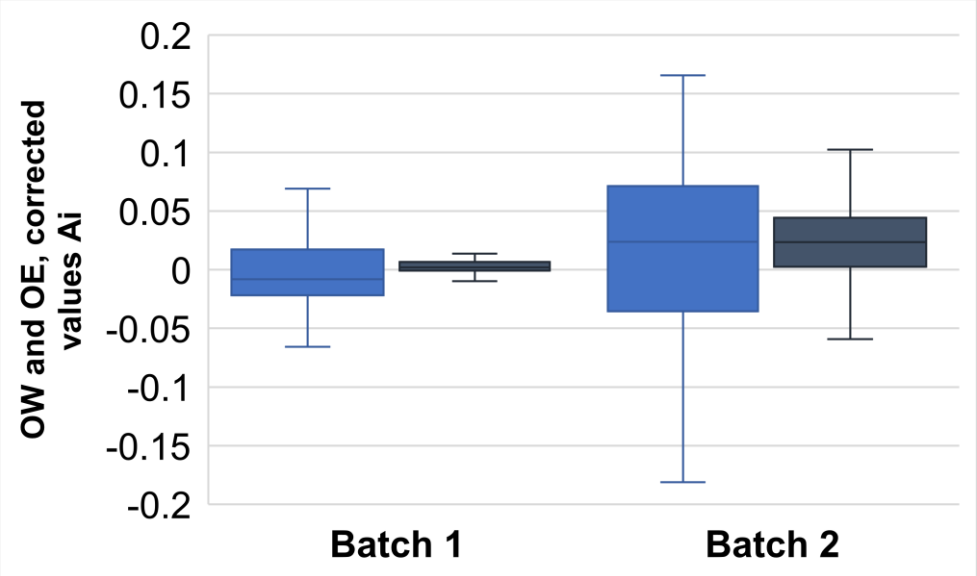


Figure V.V.3. Box-plots (median, 10th, 25th, 75th, 90th and percentiles) of the traits with significant statistical differences for A_i-residuals, OW and OE. Batch 1= wild, batch 2 = farmed.

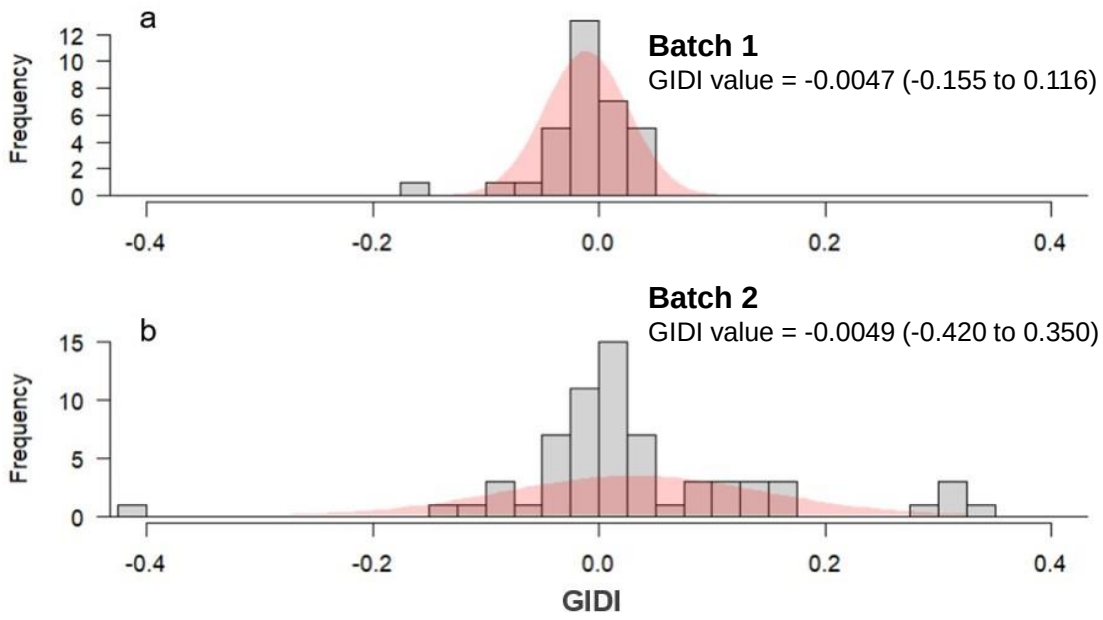
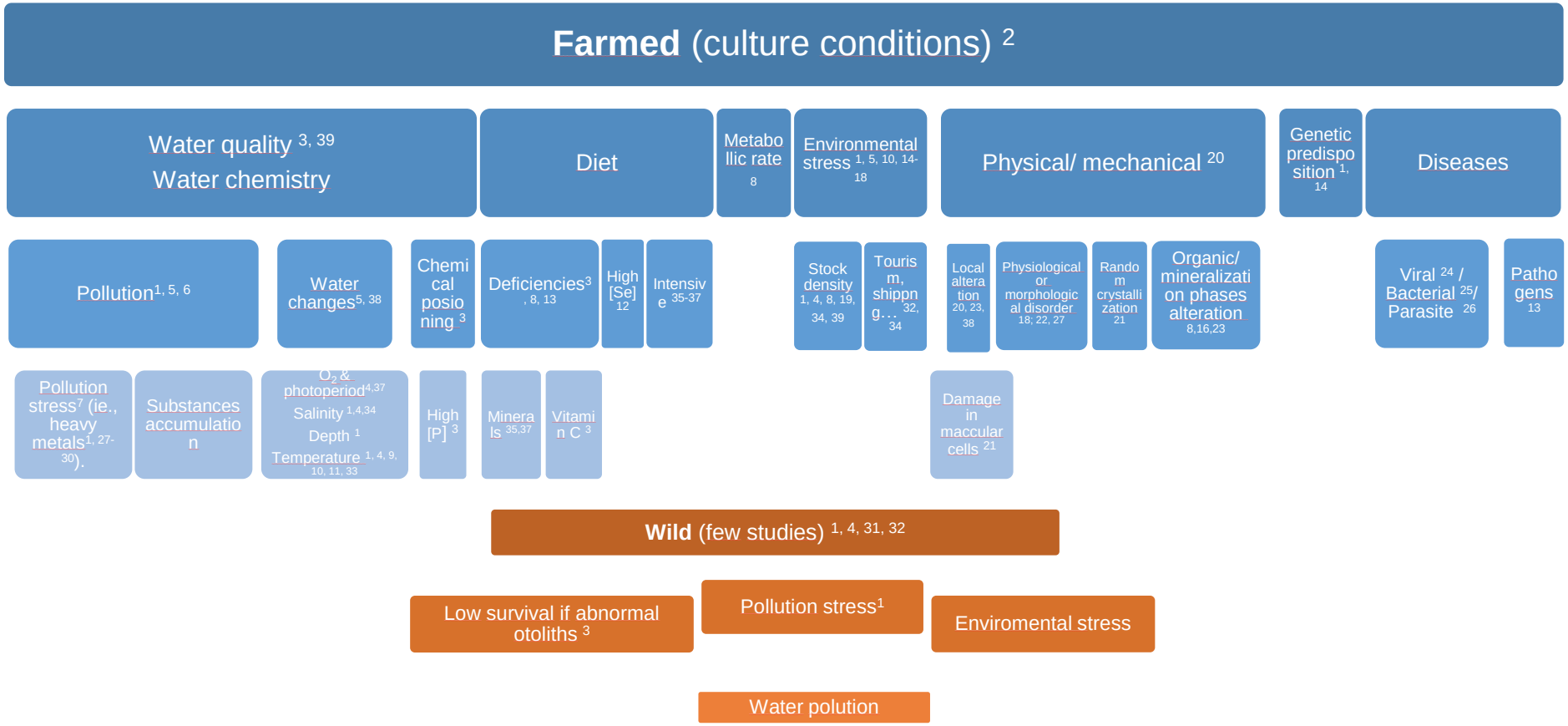


Figure V.V.4. Global Index of Developmental Instability (GIDI) distributions for the 2 batches. AICc= -170.92 for common asymmetry and AICc= -210.90 for separate asymmetry distribution. Akaike Weight = 1 (100% reliability). Batch 1= wild, batch 2 = farmed.

However, the most likely possible cause of asymmetry (**Figure V.V.6)** are the environmental problems (Khedher et al., 2021; De Carvalho Lapuch et al., 2022; Yedier et al., 2022b) and therefore, environmental stress for both type of conditions (farmed and wild), especially water pollution (Kessabi et al., 2013; Elie & Girard, 2014). Tunas from both batches came from a common background (Mediterranean Sea), in which they are exposed to stress from marine traffic, municipal sewers, tourism, agriculture and fish farms and illegal discharges (Öztürk et al., 2007; Telli-Keraklaç & Ediger, 2020); being known that pollutants like many pesticides, insecticides and heavy metals can cause fish anomalies (Kessabi et al., 2010; Yedier & Bostanci, 2019; Jawad & Ibrahim, 2021; Uzer & Karakulak, 2022).



References 1-39 **Figure V.V.5**. Diagram of the asymmetry causes depending on the fish origin. All the described causes in the discussion are referenced here.

[1]Yedier et al., 2022a; [2]Fey et al., 2022; [3]David et al., 1994; [4]Boglione et al., 2013; [5]Vinagre et al., 2014; [6]Sadighzadeh et al., 2011; [7]Yedier et al., 2018a; [8]Sweeting et al., 2004; [9]Gauldie, 1986; [10]Greszkiewicz & Fey, 2020; [11]Geladakis et al., 2022; [12]Bengtsson & Hindberg, 1985; [13]Jawad & Adams, 2021;

[14]Browning et al., 2012; [15]Manizadeh et al., 2018; [16]Ma et al., 2008; [17]Mahé et al., 2019; [18]Morales-Nin, 1987; [19]Casselman, 1990; [20]Morales-Nin, 1985; [21]Strong, 1986; [22]Yedier et al., 2018b; [23]Penttila & Dery, 1988; [24]LaPatra et al., 2001; [25]Pasnik et al., 2007; [26]Kent et al., 2004; [27]Yedier & Bostanci, 2019; [28]Jawad & Ibrahim, 2021 ; [29]Kessabi et al., 2010 ; [30]Uzer & Karakulak, 2022; [31]Fernández et al., 2008; [32]Koumoundouros, 2010; [33]Boglione et al., 2014; [34]Telli Karakoç & Ediger, 2020; [35]Cahu et al., 2003; [36]Graff et al., 2002; [37]Sullivan et al., 2007; [38]Budnik et al., 2020; [39]Portz et al., 2006.

Conclusions

In conclusion, only two types of asymmetry were found in both batches: antisymmetry and directional asymmetry, being antisymmetry more frequent. The asymmetric parameters were the otolith weight, length, width, eccentricity and compactness, and differences were found specifically between batches for the otolith width and eccentricity, with higher asymmetry values in farmed tuna specimens. However, this is not enough to signal the asymmetry as a reliable natural tracer to discriminate among batches, and future studies englobing more variables, like the pollution and pathogens exposition, or the genetics, could help to ameliorate the asymmetry analysis in the juveniles of this species.

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CHAPTER VI

Vaterite precipitation in Atlantic bluefin tuna (*Thunnus thynnus*) otoliths

Abstract

Fish otoliths are composed by calcium carbonate ($CaCO_3$) among other constituents. In biological systems, aragonite and vaterite are $CaCO_3$ isomorphs normally associated to normal and anomalous otoliths, respectively. In other studies, vateritic-otoliths occurs more frequently in farmed than wild fish. In this study, we examine 90 otoliths from wild and farmed juvenile Atlantic bluefin tuna. The otoliths were weighed and photographed to obtain their area, perimeter, length, width, eccentricity, circularity and compactness and their composition was analyzed by X-Ray diffraction. Vaterite otoliths were significantly more frequent in farmed than wild tuna. Morphological analyses show otoliths without vaterite were generally larger than vaterite ones. Abnormal morphologies appeared both in otoliths with and without vaterite. Further studies on the aragonitic-otoliths malformations in the future should be pursued, considering the apparition of morphology abnormalities in 'normal' otoliths (see aragonitic-otoliths).

Keywords: Atlantic bluefin tuna, otolith, vaterite, aragonite, discrimination

Introduction

Otoliths are sensory organs contributing to hearing, balance, gravity sensation and linear acceleration in fish (Popper & Lu, 2000). They are composed of calcium carbonate ($CaCO_3$), organic matter and inorganic constituents derived mostly from ambient water (Gauldie & Nelson, 1988). In biological systems, $CaCO_3$ has three iso-morphologies with identical chemical formulas but different crystalline structures: aragonite is orthorhombic, calcite is trigonal and vaterite is hexagonal (Carlström, 1963; Falini et al., 2005). These iso-morphs also differ in their chemical stability, being vaterite the most unstable, especially when entering in contact with water where it can dissolve (Irie, 1960; Kralj & Brečević, 1990; Kralj et al., 1994). $CaCO_3$ crystals are normally arranged as aragonite (Carlström, 1963). In *sagitta* otoliths, the largest otoliths of the three pairs, vaterite forms are anomalous and otoliths containing vaterite are sometimes referred as 'abnormals' (Strong et al., 1986).

Processes leading to crystallographic anomalies in $CaCO_3$ deposition are uncertain (Ma et al., 2008; Loeppky et al., 2019). A lower rate of aragonite deposition compared to vaterite in otoliths can occur when fish grow at fast rates and metabolism is high (Sweeting et al., 2004; Reimer et al., 2017), if there is a high concentration of phosphorus in the water or the fish has a poor feeding (David et al., 1994), if population densities are high (Sweeting et al., 2004), also with endolymph biochemical composition (Tomás et al., 2004) or pH (Holmberg et al., 2019) alterations. Otoliths with vaterite can be more translucent and granular than those with aragonite since vaterite replacement changes the frailness, density, size and shape of the otoliths (Sweeting et al., 2004). Vaterite otoliths can also be larger and lighter than their aragonite pairs probably due to the vaterite crystal structure (Tomás & Geffen, 2003).

Pacific bluefin tuna (*Thunnus orientalis*) aquaculture is developing rapidly and tunas cultured from eggs from broodstocks kept in captivity (Murashita et al.,

2021; Okada et al., 2021) are already available in some Japanese markets. Techniques to rear Atlantic bluefin tuna (ABFT, *Thunnus thynnus*) in captivity are also advancing fast (Ortega & De la Gándara, 2017), and in a next future these tunas will also be available in the market. As it is already stated in this Thesis, there is an increasing interest in developing methods that allow to discriminate aquaculture tuna from their wild counterparts, especially in the future scenario where both specimens will be commercialized for human consumption. In this sense, several studies have shown that abnormal otoliths' forms occur sporadically in wild fish being more frequent in hatchery-reared fish (i.e., up to 10 times more - Reimer et al., 2016; Gauldie 1986; David et al. 1994; Bowen et al. 1999; Sweeting et al. 2004). Therefore, the vaterite presence in otoliths has been suggested to be useful to distinguish unmarked hatchery fish from their wild counterparts (Bowen et al., 1999).

Vaterite deposition and/or prevalence in otoliths of both wild and farmed bluefin tuna species have never been investigated. In this study, we hypothesize that vaterite otoliths will be more frequently found in farmed than wild ABFT. Our objectives are therefore: i) being able to identify abnormal forms and/or vaterite in ABFT otoliths ii) if present, estimating the quantity of vaterite in both wild and cultured ABFT, and iii) describing and comparing the otolith morphometry depending on its composition, aragonitic or vateritic.

Material & Methods

i. Tuna sampling

Otoliths were extracted from 46 ABFT juveniles (474.7 ± 210.9 gr, average weight ± S.D.) both wild and farmed tunas. Farmed ABFT (n= 23) were cultured in land-based facilities of the Spanish Institute of Oceanography (IEO, Mazarrón, Spain). Fertilized eggs from spawning captive adults kept in sea cages belonging to Ricardo Fuentes Group were reared in a 40 m^3 tank. Hatched larvae were successively fed rotifer, artemia, sea bream yolk sac larvae and then weaned with an artificial diet (Magokoro, Marubeni Nissin Feed Co., Ltd., Tokyo, Japan) as described in Ortega (2015). Average temperature was 24.9°C and salinity 37.5

g L^{-1}. At 41 days post-hatching (dph), ABFT juveniles were transferred to a 900-m^3 tank at the Infrastructure for Atlantic bluefin tuna aquaculture (Cartagena, Spain) where they were fed European anchovy (*Engraulis encrasicolus)*, Round sardinella (*Sardinella aurita),* and Atlantic mackerel (*Scomber scombrus)*. Fish dying due to traumatic events (collision against the tank walls) were collected soon after death and sampled. Wild tunas (n=23) were caught by the hook-and-line method (barbless hook) in October 2018 in Mazarrón Bay (Murcia, Spain). In accordance with European legislation (Directive 2010/63/UE) the practices employed did not required animal experimentation permission.

ii. Otolith sampling

Every fish (n=46) was weighted to the nearest gram (0.01 gr) and measured to the nearest centimeter (0.1 cm) and the two *saggita* extracted (right, n=46 and left, n=44). Otoliths were extracted carefully by a frontal section of the head and extracting the brain, to localise the inner ear from above. All otoliths were cleaned through dry-elimination of the tissues. Right otoliths were also cleaned using purified water (Type 1 purified water, Milli-Q®) to test if the cleansing method modified the morphology and therefore the $CaCO_3$ disposition due to the instability of vaterite in contact with water.

iii. Morphometric and shape otolith analyses

After the dry-elimination of the tissues, otoliths were placed separately in a dark field, following the positioning recommendations from the Report of The ICCAT GBYP International Workshop on Atlantic Bluefin Tuna Growth (Rodríguez-Marín et al., 2020). Each single otolith was observed, using magnifications x1 and x2, under a stereomicroscope (Leica, Greenough Stereo Microscopes S9 Series, www.leica-microsystems.com) connected to a computer. Both otoliths were photographed after the dry-cleansing. Right otoliths were photographed also after wet-cleansing. We characterized the otolith morphology by measuring the otolith area – OA -, perimeter -OP-, length -OL -, width -OW- and the otolith shape by measuring the circularity -OCI-, eccentricity -OE-, and compactness -OCO- (the

otolith morphometry measurement is further explained in **Chapter IV**). All measurements were conducted using the image software Otolab (Nava et al., 2018). Both otoliths were also weighted (Weight of the otolith -WO) to the nearest 0.001 mg using an electronic micro-balance Sartorius CP2P, balanced using a material reference tested following the normative (ISO 9001) (www.sartorius.com) with 4 decimals attached. Finally, the otoliths were placed separately (right and left) in plastic tubes where they dried at room temperature and afterwards stored until further processing.

iv. Crystalline structure analyses

Crystalline structure and vaterite presence were identified by X-Ray diffraction. The $CaCO_3$ mineral phases were identified separately in right and left otoliths with a θ-θ mode diffractometer (Bruker D8 Advance, *Bruker Corporation*, Billerica, MA, USA), with CuKα radiation, 40 kV, 30 mA and a one-dimensional detector with a 3.5º window. The primary optics consisted of a 2º Soller slit, a 1 mm incidence slit, and an anti-air scatter screen. Secondary optics included an 8 mm antiscatter slit, a Nickel filter, and a 2.5º Soller slit. Each otolith sample, previously ground by hand in an agate mortar, was placed on a front-loading sample holder with a 0.1 mm cavity, and was analysed in the range 10–70º in 2θ, in steps of 0.05º, 3 s/step and without rotation to avoid sample loss. The powder diffraction file was evaluated with the equipment-linked program (DIFFRAC.EVA 5.0, Bruker AXS, 2019) and a crystalline powder database (PDF-4+ 2021, ICDD).

v. Statistical treatment

Normality (Kolmogorov-Smirnov) and homogeneity of variance (Levene's) of the morphometric data was tested. For the morphometric data medians, minimums and maximums were used, and for the vaterite percentages, the geometric means and the standard errors were obtained.

For the morphometric data, the data were compared using U-Mann-Whitney for: in the whole population between i) the otoliths wet-cleaned before and after this cleansing, and ii) the left and right otoliths (in the same conditions: dry-cleaned),

and finally; and in the farmed tuna batch between for the otoliths with and without vaterite.

For the presence (qualitative) and percentage (quantitative) of vaterite, it was used: i) a Chi-square test for the vaterite presence depending on the tuna batch, and ii) the U-Mann-Whitney test for the vaterite quantity in the farmed tuna otoliths depending on the side.

All analyses were conducted using the SPSS software (*Statistical Package for the Social Sciences*, *IBM 24.0*, New York). The significance levels for all tests were set at 0.05.

Results

First, the morphometry data from the whole population (left otoliths dry-cleaned and right otoliths dry-cleaned and after wet-cleansing), used for the pre and post purified water cleansing and right and left comparison are given in **Table V.VI.1**. Otoliths did not differ in morphology or size depending on the side or after being cleaned with purified water (**Figure V.VI.1**, U-Mann-Whitney). Secondly, the presence (qualitative) and quantity of vaterite data are given in **Table V.VI.3** by side and batch. Finally, **Table V.VI.4** shows the morphometry data of otoliths without and with vaterite.

Table V.VI.1. Descriptives (median, minimum-maximum) for left and right otoliths, and the pre and post wet-cleansing morphological comparison (U-Mann-Whitney) in the whole population.

Otolith	**Left**	**Right**	
Measurement	**Dry-cleaned**	**Dry-cleaned (Pre wet-cleansing)-**	**Wet-cleaned**
WO	3.83 (2.30 - 6.78)	3.57 (0.38 - 6.56)	3.73 (0.38 - 6.56)
OA	5.39 (3.42 - 7.70)	5. 46 (2.55 - 7.37)	5. 37 (2.73 - 7.15)
OL	4.56 (3.06 - 5.70)	4.68 (2.21- 5.63)	4.59 (2.20- 5.62)
OW	1.70 (1.46 - 1.86)	1.69 (0.91-2.15)	1.69 (0.91-2.20)

OP	0.94 (0.85 - 0.95)	0.94 (0.66 - 0.98)	0.94 (0.53 - 0.98)
OE	12.47 (10.31 - 14.98)	12.97 (7.48- 18.26)	13.07 (7.63- 22.96)
OCO	0.46 (0.26 - 0.58)	0.44 (0.26- 0.63)	0.44 (0.13- 0.63)
OCI	27.48 (21.54 - 48.65)	28.80 (20.08 - 49.16)	28.50 (19.94 - 96.29)

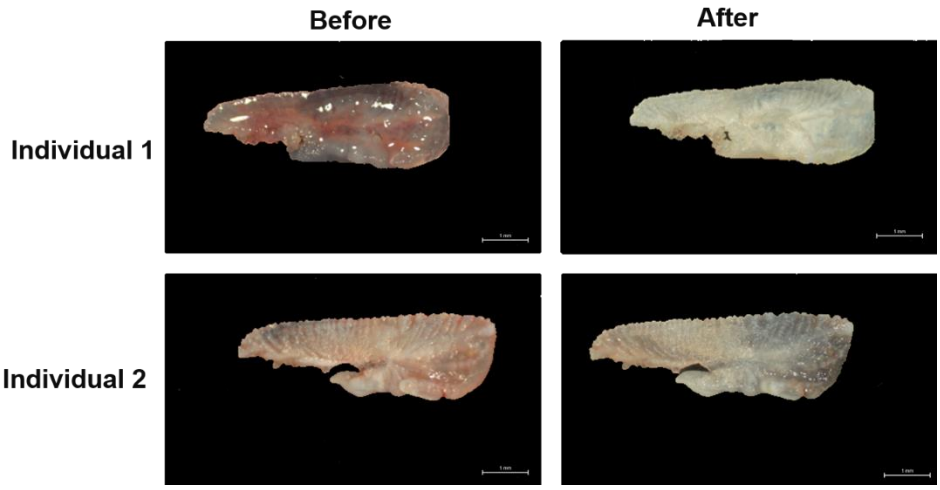

Figure V.VI.1. Example of two otoliths photographed before and after cleansing using purified water. Scalebar = 1mm

i. Vaterite presence and quantity

The X-Ray diffraction pointed out the presence of some otoliths with vaterite depositions (vateritic-otoliths from now on, **Figure V.VI.2**). Firstly, we are going to analyse the vaterite presence (qualitatively), where 12 out of 45 of the otoliths from farmed tunas had vaterite (26.67%), meanwhile 2 out of 44 wild tunas' otoliths (4.55%). The Chi-square analysis determined that this difference between origins was statistically significant ($\chi^2(1) = 8.998$, $p<0.05$). In farmed tunas the vaterite presence was bilateral and appeared in both otoliths from the same individuals. In contrast the presence of vaterite in wild tunas was unilateral.

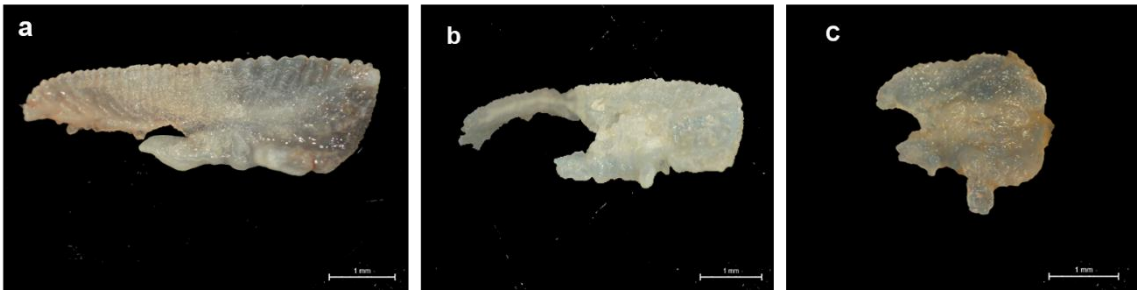

Figure V.VI.2. Otoliths (a) without (aragonitic-otoliths) and (b and c) with vaterite (vateritic-otoliths) in ABFT. Scalebar = 1mm.

Secondly, considering vateritic-otoliths, the quantities of vaterite (vaterite rate) by otolith are showed in **Table V.VI.2**. The quantity of vaterite in farmed tunas' otoliths was higher: 85.3 ± 4.3% in right otoliths and 91.0 ± 1.8% in left (geometric mean ± standard error), than in wild tunas' otoliths: 13% in right and 11% in left otoliths (**Figure V.VI.3**). However, differences in vaterite quantity between right and left otoliths were not statistically significant (U-Mann-Whitney, $p < 0.05$, in this comparison an outlier was not considered -individual 4 in **Table V.VI.2**-).

Table V.VI.2. Vaterite (%) in vateritic-otoliths

Individual	Origin	Percentage of Vaterite (%)	
		Right Otoliths	Left Otoliths
1	Wild	13	0
2	Wild	0	11
3	Farmed	87	93
4	Farmed	91	47
5	Farmed	87	89
6	Farmed	91	92
7	Farmed	81	93
8	Farmed	81	90

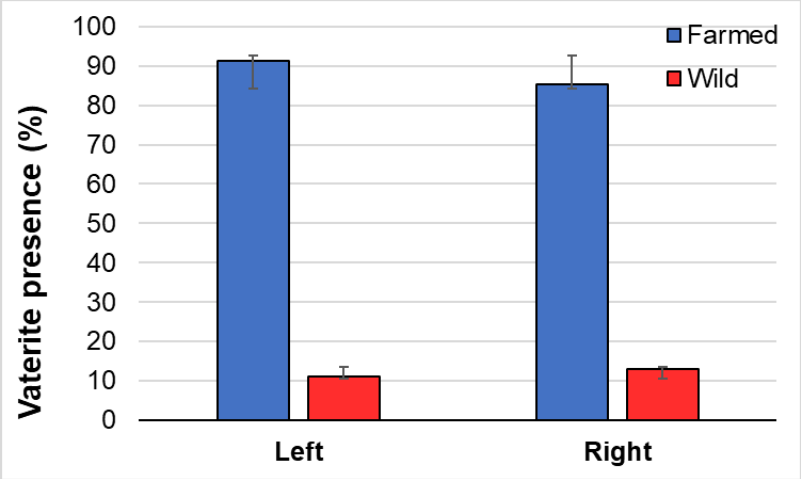

Figure V.VI.3. Quantitative study: vaterite quantity (presented in %) in those otoliths identified as vateritic-otoliths, by side and group. Farmed Wild.

ii. Morphometry comparisons

Morphometric differences between the two otoliths of the same fish (**Table V.VI.2**), were no significantly different (U-Mann-Whitney, $p<0.05$). Using the morphometry data of aragonitic and vateritic-otoliths among the farmed tunas (**Table V.VI.3**), aragonitic-otoliths had greater otolith area, length and eccentricity than vateritic ones while vateritic-otolith had greater width (U-Mann-Whitney, $p< 0.05$, **Figures V.VI.4-6**).

Table V.VI.3. Morphological descriptors (median, minimum-maximum) and *statistical significant differences for aragonitic and vateritic-otoliths from farmed tunas and both sides (no significant statistical differences, U-Mann-Whitney, were found between sides). The morphological traits were compared within the farmed batch due to the scarce vateritic-otoliths found in the wild group.

	Aragonitic	Vateritic
WO	3.45 (2.34 – 6.78)	3.11 (1.84 – 4.05)
OA *	5.02 (3.25 – 7.70)	4.67 (2.77 – 6.95)
OL *	4.50 (3.87 – 5.70)	3.81 (2.21 – 5.15)
OW *	1.57 (1.07 – 1.93)	1.70 (1.58 – 2.15)
OE *	0.94 (0.90 – 0.98)	0.89 (0.66 – 0.94)
OP	12.24 (10.31- 14.56)	11.91 (7.48 – 18.26)
OCI	0.46 (0.26 – 0.56)	0.39 (0.26 – 0.63)
OCO	27.44 (22.63 – 48.64)	32.59 (20.08 – 49.16)

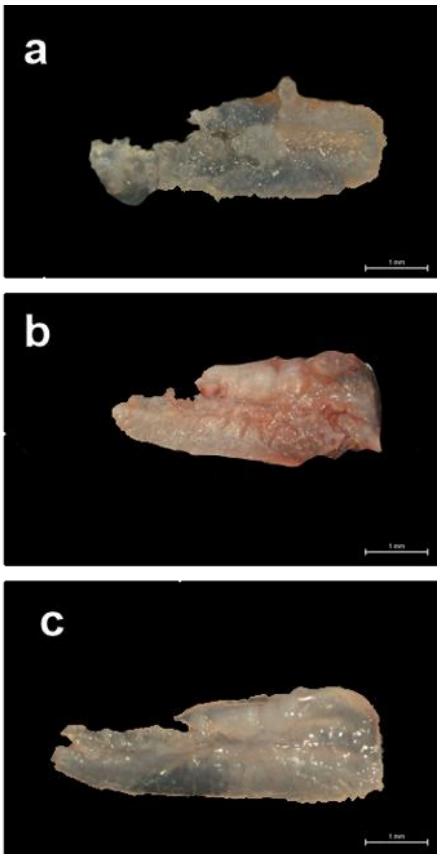

Figure V.VI.4. Farmed individuals a) vateritic-otoliths, and b) and c) aragonitic-otoliths. Scalebar= 1mm.

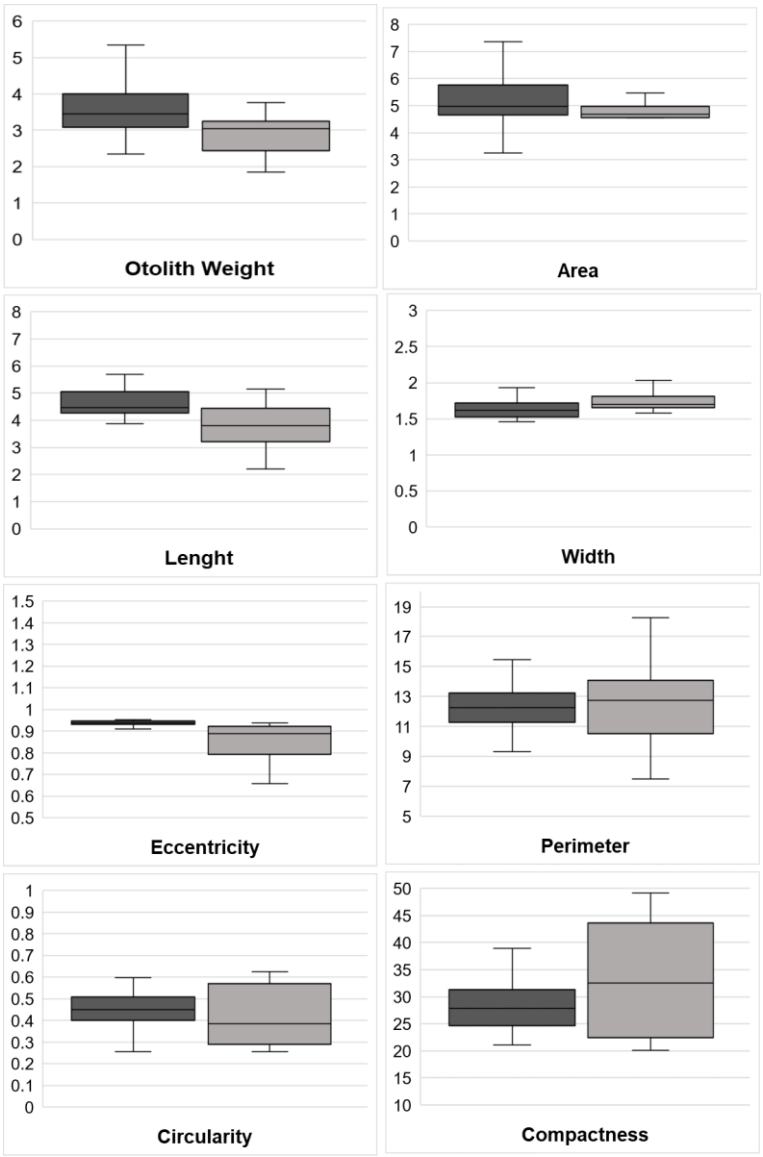

Figure V.VI.5. Box-plots (showing median, 10th, 25th, 75th, 90th percentiles and outliers), in otoliths from farmed tunas, ■ without and □ with vaterite.

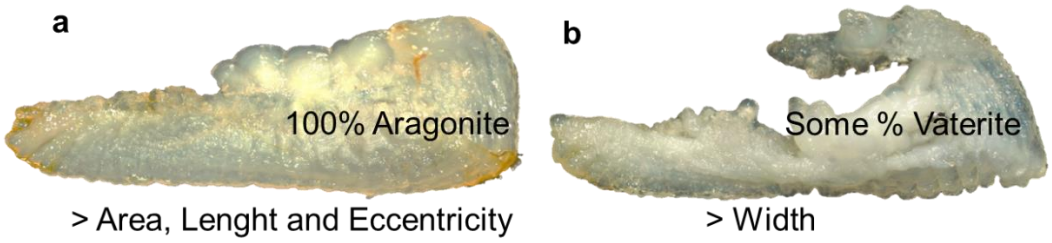

Figure V.VI.6. Resume of the morphometry comparison results between otoliths a) without vaterite (aragonitic-otoliths), and b) with some percentage of vaterite (vateritic-otoliths) in farmed tunas.

Discussion

Vateritic-otoliths occur in ABFT, having farmed individuals higher incidence than wild. Regarding their morphometry, there are differences among 'normal' and 'abnormal' otoliths, being aragonitic-otoliths larger than vateritic ones. After this study, we can conclude that morphometry abnormalities in ABFT otoliths are presented in both aragonitic and vateritic-otoliths. In addition, the standardized protocol for otolith decontamination is based on a series of purified water baths (Super-Q or Milli-Q® water), which could result in a modification of the otolith morphometry, given that vaterite could eventually dissolve in contact with water, as it is the least stable of the three CaCO3 anhydrous polymorphs (Kralj & Brečević, 1990; Kralj et al., 1994). However, in our study no morphological changes have been associated to the use of purified water on the otolith cleansing.

In our study, higher proportion of vaterite otoliths in the farmed tunas compared to wild ones was found. This agrees with other authors (drafted in **Table V.VI.4**) who described higher vaterite prevalence in aquaculture than in wild populations for different species. This assumption is important, given that tuna batches with higher prevalence of vateritic-otoliths would likely be farmed. However, in this and other studies (**Table V.VI.4**) vateritic-otoliths have been found to occur sporadically in wild fish. Therefore, the vaterite presence should be regarded from a quantitative point of view instead of qualitative (Casselman, 1986, 1990; Bowen et al., 1999) considering the individuals with a high rate of vaterite as farmed fish. Besides, it should be considered that in our study most of the sampled farmed tunas were dying due to collisions, which could be related to the vaterite presence in otoliths (impared balance and/or hearing), and therefore the vateritic-otoliths' incidence in farmed tunas could be overrated. In addition, the quantitative study of vaterite rate in vateritic-otoliths can offer valuable information. In our study, while vateritic-otoliths coming from farmed tunas contained between 87 and 93% (only an outlier was under 50%) this rate was much lower (12%) in the vateritic-otoliths coming from wild tunas. We also found that when one otolith had vaterite its contralateral tended to develop vaterite as well. This is consistent with Reimer

et al. (2016) results, who described bilateral vaterite in salmon. For them, once one otolith has begun the vaterite deposition, the other otolith is more likely to deposit it. Our results suggest that high rates of vaterite and vaterite bilaterality are only found in farmed tunas. But as the number of vateritic-otoliths found in wild tuna was low, further research with a greater number of wild tunas should be carried out.

The causes behind the vaterite deposition are still unknown. Some studies point out to the rapid fish growth which is characteristic of cultured fish (Reimer et al., 2016; Loeppky et al., 2019). One of the mechanisms that could explain the disruption of aragonite in the otoliths is the composition of the organic matrix and its influence in the crystal polymorphism (Mann, 2001; Falini et al., 2005). The presence of the macromolecule-64 (OMM-64) in combination with Otolin-1 in the otolith matrix favours the formation of aragonite whereas the presence of OMM-64 alone favours the vaterite deposition (Tohse et al., 2009). Other mechanism is the inorganic carbon HCO_3^- transport to the endolymph by energy dependent mechanisms (Cl^-/HCO_3^- and HCO_3^-—ATPase) across the saccular membrane (Tohse & Mugiya, 2001). Presumably, low $[Ca^{2+}]/[CO_3^{2-}]$ ratios due to a greater transport of HCO_3^- relative to Ca^{2+} promotes vaterite formation over other calcium carbonate polymorphs (Chen & Xiang, 2009; Reimer et al., 2017). But apart from this, the otolith formation regulation is controlled by several genetic and neuroendocrine factors, and the perturbation of one or more of these factors may cause the shift to vateritic-otolith formation or just an abnormal aragonite deposition (Tomás & Geffen, 2003).

The consequences for the fish having vateritic-otoliths have been partially investigated. They have a direct negative impact in the inner-ear fish functions. In farmed fish, vateritic-otoliths may impair hearing sensitivity (Oxman et al., 2007; Reimer et al., 2016) hampering prey perception (Reimer et al., 2017; Vignon & Aymes, 2020). These hearing problems could be one of the causes of the higher mortality found in ABFT from aquaculture when compared with wild tunas (Ortega et al., 2019). But as it has been stated before, vaterite rate in

farmed tunas could be overrated, and further studies with active sampling are highly recommended. Also, vaterite formation may impair hearing directionality due to mass asymmetry, although serious problems in directionality may only occur where mass asymmetry between otoliths exceeds a threshold (Lychakov & Rebane, 2005). This directional impairment may also avert the expression of normal behavior, which is especially relevant in some farmed species (Reimer et al., 2016).

Comparing the morphometry of aragonitic and vateritic-otoliths: weight, area, length, perimeter and eccentricity were greater in aragonitic-otoliths. This is not similar to Reimer and colleagues (2016) findings, in which vateritic-otoliths were 17% larger and 8% lighter on average than their aragonite counterparts. In that study, the target species was the Atlantic salmon. Therefore, differences with our results could be explained by either the ontogeny (the otolith morphometry varies between species), the environmental conditions or the fish size. Parallelly abnormal otoliths with aberrant forms but no vaterite were found, implying that the vaterite presence is not the main actor in these abnormal shapes. Deeper studies to discover the causes of this strange otoliths' forms, as well as if they are functional or have any external or physiological consequences should be done. In fact, in other chapters of this thesis we discuss the otolith morphometry and asymmetry in the same tuna batches studied in this chapter.

Table V.VI.4. Other studies comparing vaterite otolith incidence between batches in means of different parameters.

Study	Species	Sample	Results
Peck, 1970	Coho salmon, *Oncorhynchus kisutch*	> 50	Wild fish: 1.4%, hatchery: 55.9% vaterite.
Mugiya, 1972	Rainbow trout, *Oncorhynchus mykiss* Alaska pollock, *Theragra chalcogramma* Common sole, *Solea solea*	5	Not comparing
Gauldie, 1986	Chinook salmon, *Oncorhynchus tshawytscha*	368	Wild fish: 0.4 - 14% vaterite.
Strong et al., 1986	Saithe, *Pollachius virens*	10851	Larger otoliths have more vaterite. Wild fish: 2.7-3.1% vaterite.
David et al., 1994	Red croaker, *Sciaenops ocellatus*	2863	Wild fish: 0%, hatchery: 0.8 - 4.8% vaterite.
Bowen et al., 1999	Lake trout, *Salvelinus namaycush*	486	Wild fish: 7-15%, hatchery: 53-84% vaterite.
Tomás & Geffen, 2003	Herring, *Clupea* spp.	601	Wild fish: 5.5%, hatchery: 7.8 – 13.9% vaterite.
Sweeting et al., 2003	Coho salmon, *Oncorhynchus kisutch*	300	Wild fish: 4.5% and 5.7%, hatchery: 33.5% and 38.3% vaterite.
Sweeting et al., 2004	Coho salmon, *Oncorhynchus kisutch*	2000	Wild fish: 12%, hatchery: 46-56% vaterite (3.5 times more).
Tzeng, 2007	*Anguilla Anguilla*, European eel	108	Not comparing
Ma et al., 2008	Ayu, *Plecoglossus altivelis*	31	Not comparing

Brown et al., 2013	Steelhead (*Oncorhynchus mykiss*)	82	Wild fish: 5%, hatchery: 50% vaterite.
Reimer et al., 2016	Atlantic salmon, *Salmo salar*	210	Wild fish: 8.6%, hatchery: 48.7% vaterite (3.7 times more).
Reimer et al., 2017	Atlantic salmon, *Salmo salar*	270	Slow-growing: 29%, fast-growing: 90% vaterite.
Loeppky et al., 2019	Lake sturgeon, *Acipenser fulvescens*	23	Not comparing
Yedier & Bostanci, 2019	Blackbellied angler, *Lophius budegassa*	100	Not comparing
Yedier & Bostanci, 2020	Mediterranean horse mackerel, Trachurus *mediterraneus* Spanish seabream*, Pagellus acarne* Sheephead bream, *Diplodus puntazzo* Merling, *Merlangius merlangus*	703 (294, 80, 104, 125)	Not comparing
Vignon & Aymes, 2020	Brown trout, *Salmo trutta*	60	Not comparing
Austad et al., 2021	Atlantic salmon, *Salmo salar*	1568	Not comparing
Long et al., 2021	Goldeye, *Hiodon alosoides*	3	Not comparing

Conclusion

Otoliths containing vaterite were identified in bluefin tuna. These otoliths were more frequent and their vaterite quantity was higher in farmed tunas than wild. In addition, morphometry differences were found between otoliths with and without vaterite in farmed tunas.

Moreover, abnormal morphologies (like missing otoliths parts), were not related with the vaterite deposition in ABFT otoliths, and they were not due to the purified water cleansing. A future study of these malformations in aragonitic-otoliths should be pursued in order to discover their origin.

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THIRD SECTION,

artificial marking of the otoliths

CHAPTER VII

Is oxytetracycline useful for marking otoliths of juvenile Atlantic bluefin tuna?

Abstract

The increasing importance of the Atlantic bluefin tuna aquaculture has created a need to differentiate wild and captive-reared fish. Otoliths have, for several decades, been used as marking tools in fish. This study investigates the applicability of mass marking of otoliths with oxytetracycline chlorhydrate (OTC) in juvenile Atlantic bluefin tuna. Two different concentrations of OTC (100 and 200 ppm) administered via intramuscular injection, were tested. Fish were sampled between one day and three months after the OTC injection. Using ultraviolet-light microscopy, a fluorescent OTC band could be detected in 100% of the marked individuals. The intensity of the marks in the otoliths was compared between concentrations and no statistical differences were detected. The time elapsed since the injection was also studied and no decrease in the signal intensity in the otoliths was found. Owing to its good mark retention and detectability, the injection of OTC resulted a reliable mass marking method in Atlantic bluefin tuna.

Keywords: bluefin tuna, otolith, marking, oxytetracycline, fluorescent

Introduction

The use of chemical compounds in aquaculture as a method for mass marking is a widely employed procedure. Testing the applicability of this approach in Atlantic bluefin tuna (ABFT, *Thunnus thynnus*) is necessary due to the need for product tracking when fish products from both wild and aquaculture origin are mixed in the fish market. Their quick and easy administration make chemical compounds a popular tool in large-scale stock evaluations of small fish species (Simon et al., 2009). Fish are exposed to a chemical compound (i.e., a trace element, stable isotope or pigment) by ingestion (Weber & Ridgway, 1967; Stańczak et al., 2015), immersion (Beckman & Schulz, 1996; Liu et al., 2009; Honeyfield et al., 2011; Caraguel et al., 2015) or injection (Weber & Ridgway, 1962; Wexler et al., 2003) resulting in the physiological incorporation of the compound into the fish tissue (Guy et al., 1996; Warren-Myers et al., 2018). The presence of these compounds can then be examined at a future date to identify the fish exposed to them (Uglem et al., 2020).

Chemical compounds are often used as fluorescent pigments, the most common being tetracycline, alizarin red and calcein (Mohler, 1997; Williamson et al., 2009; Smith et al., 2010; Wells et al. 2013; Warren-Myers et al., 2018). The tetracycline is an antibiotic that is incorporated into calcified structures within hours (Nagięć et al., 1995; Lagardère et al., 2000) and shows up as an identifiable fluorescent mark in fish bony parts such as scales, fin rays, vertebrae, bones and otoliths under ultraviolet (UV) light. The viewing of these signals requires no great expertise or expensive equipment (just a UV light microscope). Oxytetracycline chlorhydrate (OTC), the most commonly used form of tetracycline, has two maximum absorption peaks in its UV range, at 270 mμ and 360 mμ in the yellow-green fluorescent spectra (Weber & Ridgway, 1967; Brooks et al., 1994; Wells et al., 2013). OTC marking can be performed by immersing fish in this antibiotic, combining it with feed or by injecting it as a solution (Warren-Myers et al., 2018). Marks are permanent, which makes them suitable for long term studies, and each exposure mark in otoliths is represented by a separate ring. Nevertheless, these

marks are susceptible to photodecomposition (caused by light exposure) and the otoliths should be protected from light (Doi & Stoskopf, 2000).

In large fish like tuna, immersion may not be appropriate due to the huge tank volumes required and the body mass of the fish. In fact, the survival rates of OTC-injected yellowfin tuna (*Thunnus albacores*) kept in captivity for over three years has been shown to be high, which suggests OTC marking to be a successful technique for this tuna species (Wexler et al., 2003). In addition, fish may benefit from the antibiotic properties of this compound (Ahmed & Tan, 1992). To the best of our knowledge, no data on ABFT marked with fluorescent chemical compounds have previously been reported. In this study, OTC marking was therefore applied to this species to test its validity as a marking technique for the first time.

Material & Methods

Juvenile ABFT were obtained from eggs naturally spawned in sea cages (San Pedro del Pinatar, Murcia, Spain) in July 2019. Fertilized eggs were moved to Infraestructure for Atlantic Bluefin Tuna Aquaculture, a Research centre belonging to the Spanish Institute of Oceanography (Spanish National Research Council) and placed in Mazarrón (Murcia, SE Spain). Hatched larvae were fed on rotifer, artemia, YSL and then weaned on an artificial diet (Magokoro S-3, *Marubeni Nissin Feed Co.*, Ltd., Tokyo, Japan) provided *ad libitum* several times per day. At 42 days post-hatching (dph) some tunas were moved to another tank (55 m^3) where they were cultured for a month in a flow through system with a continuous supply of oxygen and under natural photoperiod. Mean dissolved oxygen was always above 100%, salinity 37.5 g L-1 and temperature ranged between 24 and 27. Tuna juveniles were fed with bait fish *(*round sardinella -*Sardinella aurita*-, pilchard -*Sardina pilchardus*- and Atlantic mackerel -*Scomber scombrus-).*

After a month, when the juveniles had an average weight of 100 g (98.47± 28.45 g), the experiment began. 15 tunas were injected with OTC (Oxytetracycline Chlorhydrate, *Acofarma*, Spain) and tagged with a PIT TAG (Trovan Co.). Injections were performed in the dorsal muscles, between the dorsal fin and the lateral line, and between the first and the sixth fin ray. The tags were inserted into the muscle in front of the first dorsal fin (**Figure V.VII.1**). The OTC was administered at two different doses: 100 ppm (100 mg OTC/ kg of tuna) and 200 ppm (200 mg OTC/kg of tuna). As OTC is a dry powder, for the administration it was diluted in a sodium chloride solution (sterile NaCl single dose, *Visclean,* Spain) at a rate of 100 mg of OTC per ml of solution. Then, 8 specimens were injected with 100 ppm (0.1 ml of the prepared OTC solution) and 7 with 200 ppm (0.2 ml of the prepared OTC solution).

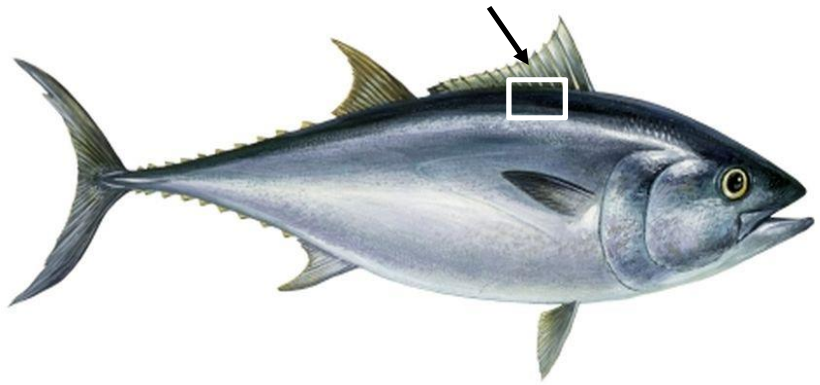

Figure V.VII.1. Injection place.

After treatment, juveniles were kept in a separated tank for three days. Then, six specimens (three each of the high and low OTC doses) were euthanized using an overdose of the anaesthetic MS-222 (300 ppm). The remaining fish were moved and placed in a greater tank where a hundred tuna juveniles were being ongrowed. Mortality of all the tunas was recorded during the following 40 days to notice the effect of treatment on survival. When a tuna marked with OTC died, it was dissected to study the long-term persistence and visibility of the OTC mark, not only in this period but until the last OTC treated juvenile died. Following

euthanasia, the OTC-treated tunas were weighed (to the nearest gram), their total lengths measured (to the nearest centimetre) and their otoliths extracted to examine the effects of the OTC as a mark. Otoliths were prepared using two-stage cleansing consisting of immersion in 96% ethanol and then in Type 1 purified water (Milli-Q®). Then, the otoliths were placed in Eppendorf tubes where they were dried at room temperature before being stored in a light-proof box. The extracted otoliths were polished before being studied to check for marks: they were fixed with a mounting adhesive (*Crystalbond ™ 509, Aremco*) on microscope slides and then ground and polished using sandpaper with a grain size of 1200–3000 (3M™ and *MicroCut*).

Afterwards, the presence of OTC marks was assessed under UV light (Excitation Filter 355-425, in x2.0 magnification) of a Leica DM LS microscope (Leica, leica-mycrosystems.com) at the Otolith Research Laboratory at DTU Aqua (Denmark). The presence of marks was assessed using the *ImageJ* (image analysis free software: *Fiji package*, Schindelin et al., 2012; Rueden et al., 2017). Firstly, the marks were registered by a visual qualitative score, being described as: 1) Low (slightly visible), 2) Good (clearly visible) and 3) Excellent (bright mark). Secondly, images were processed using *ImageJ* and the signal intensity measured; specifically, the *Macro* tool was employed for this intensity quantification. A *Macro* called *Otc intensity* was generated with help of the professors from DTU Aqua (Kongens Lyngby, Denmark) and the technicians from the Department of Image Analysis (University of Murcia, Spain), this Macro measures the average grey value within the region of interest of an image. For each specimen, 10 measurements were performed in the marked zone (region of interest) selecting 10 different zones following the mark line (**Figure V.VII.S1**). The statistical analyses were performed using the statistical programme SPSS (*IBM, SPSS 24.0*), in which the comparisons of the response to the two concentrations were tested using non-parametric methods (Mann–Whitney U-test); p values of less than 0.05 were considered to be statistically significant.

Results & Discussion

In the three days following tagging and the OTC treatment, 2 from 15 tunas died. This mortality (13.3%) is higher than the mortality observed in tagged or injected juveniles separately (unpublished data) but in this study juveniles were tagged earlier (100 gr instead of 250-300 gr) and it was the first time that both treatments (tagging and injection) were applied at the same time. Then, after the transfer of the treated tunas to the untreated tunas tank, the mortality rate (during the following 40 days) was similar: 57% in treated tunas against 50% in untreated fish. As treated tunas were handled to be transferred unlikely untreated tunas, this handling could explain the small difference in mortality rates observed in the two groups.

Concerning the marks, all the marked otoliths examined under the UV light microscope had a visible green ring (**Figure V.VII.2**). The qualitative visibility score of the marks in fish marked with low concentrations (100 ppm) was good in 62.5% and excellent in 37.5%, whereas in the high concentrations (200 ppm), visibility was low in 28.6%, good in 42.8% and excellent in 28.6%. Thus, in both concentrations, all the marked otoliths could be noted and the visibility of marks was mostly good, even when the low dose was used. However, when the signal intensity was quantified by the image software analysis, results were quite different (**Figure V.II.3)**. The means of the mark intensities of each examined specimen are given in **Table V.VII.1**, meanwhile complete measurements of each specimen are provided in Supplementary Material (**Table V.VII.S1).** There was no relationship between the visual qualitative scores and the mark intensity measures and some otoliths with marks visually classified as excellent had lower intensities. Therefore, the visual score was imprecise, being the image analysis much more reliable to sort out the mark intensity. The mean intensity for the 100 ppm was 102.23 ± 30.99 and 116.81 ± 47.45 for the 200 ppm. There were no statistical differences in OTC mark intensity between concentrations (Mann-Whitney U-test, $p < 0.05$).

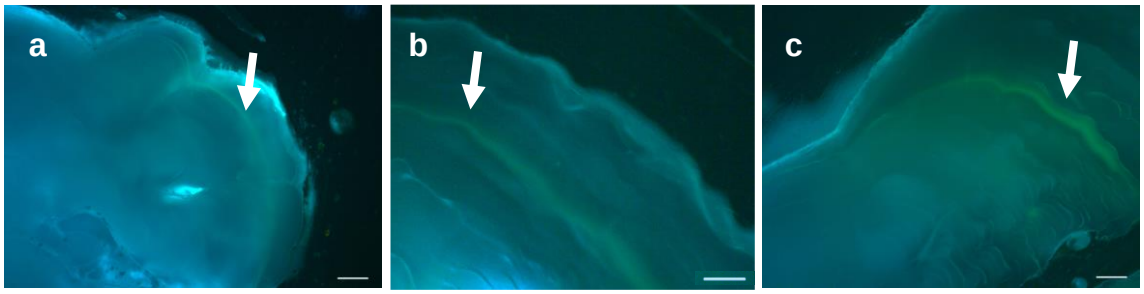

Figure V.VII.2. Oxytetracycline chlorhydrate marks in juvenile ABFT viewed under UV light stereomicroscope. Scalebar = 100 µm. a) 85 dph, 104.4 grs, low intensity (1); b) 80 dph, 109.3 grs, good (2); c) 104 dph, 383.6 grs, excellent (3).

Table V.VII.1. Overview of juvenile ABFT injected with oxytetracycline chlorhydrate, including fish size, injection concentration, time since injection and mark intensity.

Concentration (ppm)	Tuna weight (g)	Time from injection (days)	Mean mark intensity	Visibility qualitative mark
100	89.9	1	172.87	2
200	84.20	1	80.88	1
200	109.30	3	90.70	2
100	161.57	3	94.01	2
200	100.00	3	77.99	2
100	99.78	3	82.54	3
200	53.58	3	188.28	3
100	89.45	3	102.73	2
200	104.40	8	166.41	2
100	171.40	17	72.18	3
100	253.60	27	101.44	2
100	383.60	27	83.85	3
200	361.80	38	140.31	1
100	630.00	73	108.19	2
200	945.00	98	73.09	3

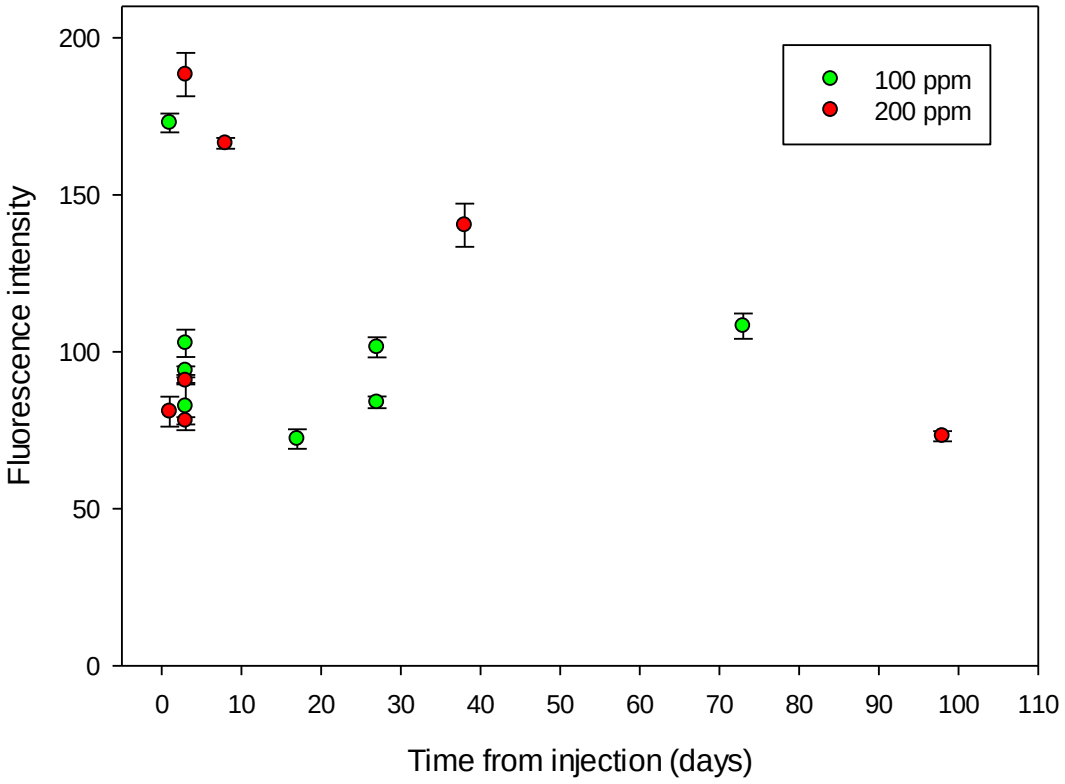

Figure V.VII.3. Fluorescence intensity of the oxytetracycline chlorhydrate mark in otoliths of juvenile ABFT in relation to days post treatment (data in mean ± S.D).

OTC marking has been demonstrated to be a useful technique for short-term otolith marking in juvenile ABFT since it generates a mark that identifies the marked fish. This technique has advantages over other mass-marking methods such as otolith thermal marking, which takes from several days to weeks to be visible. In addition, OTC seems to be a useful compound for recognition of the mark over time as fish still had visible marks three months after marking. Nevertheless, it seems that marks in the high dose treatments tends to loss intensity over time, and as the experiment only was conducted for three months and with a low number of tunas, further experiments should be carried out to survey this loss of intensity in time and how the dose affect the mark intensity in the long term. This agrees with other authors that signal that OTC otolith marking can be used due to the high mark retention and good detectability in the short term (Warren-Myers et al., 2018), and even that it is the only chemical method

whose marks can appear within 24 hours (i.e., Walt & Faragher, 2003; Crook et al., 2009; Wickström & Sjöberg, 2014; Caraguel et al., 2015; Warren-Myers et al., 2015a, b). In our study, two tunas showed fluorescence marks in their otoliths after less than 24 hours, even after being exposed to low concentrations of the compound. In addition, the marking success of the two tested concentrations was 100%, so the use of the lower concentration (100 ppm) could be recommended; and we would advocate that the use of even lower concentrations should be studied, as they are less invasive for fish (Lü et al., 2019). Once it has been demonstrated that oxytetracycline is useful for marking juvenile ABFT, further studies should be done dealing with another administration routes or marking ages like: immersion (possibly in larval stages) or feeding, mixing OTC with feed (in juveniles), because injections are less cost effective and have some issues with welfare that could be overcome with these alternatives.

Regarding the possible side effects of this marking method, there is little information in the literature on whether or not OTC influences the survival and growth of marked fish (Simon et al., 2009). Simon and Dörner (2005) showed that, as we have found in this study for ABFT, potential marking-related mortality in a species as sensitive as the European eel (*Anguilla anguilla*) was negligible over a three-week period. Most literatures' conclusions state that fluorochromes dyes like OTC have no persistent adverse effect on the marked organisms (Lang & Buxton, 1993; Taylor et al. 2005; Liu et al. 2016). However, minor health issues from OTC marking have occurred in some fish species (i.e., toxicity in striped bass (*Morone saxatilis)* immersed in OTC at 500mg 6 hours, Bumguardner & King (1996); and spinal fractures in Atlantic salmon (*Salmo salar*) fed with OTC at 2%, Toften & Jobling (1996). Side-effects always differ among species, life stages, compounds, application methods, dosage and concentration (Lü et al., 2019). Concretely in tunids, only Wexler and colleagues (2003) examined the short-term (2-3 weeks) and long term (3 years) effects of OTC injection on survival. The dosage of 100 mg/mL used by Wexler et al. (2003) did not affect the short term or long-term survival of captive yellowfin (*Thunnus albacares*), as all fish survived throughout the experimental period and beyond.

Other important points to consider are the environmental and legislative aspects. Although the method is approved in the United States, in Europe there is greater concern for the environment and the use of antibiotics for marking purposes could generate problems. Therefore, finding alternatives for the otolith dyeing would be of interest in the near future.

Conclusion

OTC is a useful technique for short-term otolith marking in ABFT juveniles and it does not affect survival rate. However, further studies should be done on long term duration of marking as well as other routes of administration.

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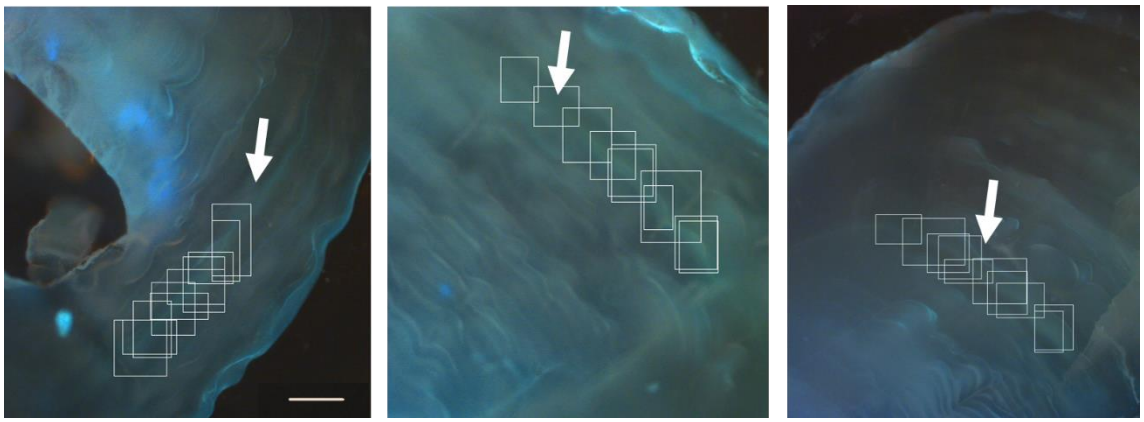
Supplementary material

Table V.VII.S1. Fluorescence intensity by individual (10 measurements per specimen).

Concentration (ppm)	1	2	3	4	5	6	7	8	9	10
100	93.44	92.50	94.65	95.79	92.66	94.35	95.75	92.66	92.73	95.60
100	82.84	97.91	80.32	75.53	86.65	74.96	82.29	91.41	75.84	77.70
100	100.01	99.03	107.07	109.73	98.76	98.62	107.42	99.02	106.24	101.42
100	170.12	170.31	172.92	169.01	174.02	173.11	179.48	172.24	172.17	175.37
100	73.54	69.25	74.78	71.61	67.50	75.88	74.80	67.73	72.02	74.70
100	107.82	113.25	102.41	106.20	106.81	105.83	114.56	110.10	111.41	103.52
100	103.04	100.73	109.08	100.58	100.60	99.52	103.36	97.41	98.79	101.33
100	84.41	85.24	81.97	86.73	81.07	85.05	85.86	82.83	83.26	82.09
100	75.91	85.41	73.64	81.49	86.20	78.26	76.70	79.40	86.48	85.36
200	90.89	89.54	91.28	90.64	91.72	92.44	90.56	91.18	90.23	88.50
200	78.88	77.03	78.25	78.77	75.69	78.50	78.64	78.35	76.60	79.15
200	189.45	198.28	180.50	186.57	187.23	189.55	187.48	199.99	177.32	186.38
200	166.72	166.48	166.58	164.10	167.37	168.45	164.48	164.22	169.21	166.46
200	70.90	73.70	75.48	74.24	74.57	72.72	71.37	74.52	71.35	72.03
200	131.94	132.51	146.44	139.18	140.69	145.50	151.81	133.98	135.22	145.88

Figure V.VII.S1. Measure of the fluorescence intensity in the otoliths' specimens (10 measures per specimen), scalebar = 100 µm (first caption). The arrow points the mark line.



CHAPTER VIII

Is Alizarin red S useful for marking otoliths of Atlantic bluefin tuna eggs?

Abstract

In fish aquaculture, mass-marking methods using chemical compounds like fluorochromes are often used. Among the fluorochromes, alizarin red S (ARS) is the most popular, and the direct immersion method is widely used in fish larvae marking. However, only a few studies have been pursued in marine fish larvae in relation to ARS marking. Therefore, we aimed to determine if ARS immersion is a feasible marking method for Atlantic bluefin tuna eggs.

Two trials were made with ABFT eggs: First, 50 or 100 ppm of ARS immersion were tested during 3h or 6h. Second, the best time-concentration combination was selected to mark new eggs. These eggs were hatched, and the larvae sampled: their otoliths were extracted for the visualization of the mark and the analysis of its intensity on an UV light microscope. As a result, all the otoliths were marked regardless the treatment duration or concentration, and the mark could be easily identified. In the first trial, 100% marking was achieved with the lower concentration and shorter time immersion in ARS. However, there were no statistically significant differences in the hatching rates between groups. In the second trial, still there were no statistically significant differences in hatching rates between the control and the treated eggs (at 50 ppm and 3h of immersion). In general, the intensity remained constant through the growth of the larvae (from 0 to 22 dph), and there were no Intensity differences caused by the preservation time. In conclusion, ARS proved to be a high efficiency, reliable and not harmful method in ABFT egg marking, and allowed us to distinguish the marked larvae and fingerlings in the short term.

Keywords: bluefin tuna, alizarin, otolith, mass-marking

Introduction

In fish aquaculture, mass-marking methods using chemical compounds like fluorochromes are often used (Baras et al., 2000; Simon, 2007). A fluorochrome produces detectable pigmented or fluorescent marks in bony structures, thanks to the formation of complexes with calcium that are deposited in calcified structures with the fish growth (Eckmann, 2003). Therefore, fish exposed to these chemical compounds will incorporate them, being later detectable under specialized equipment (Guy et al., 1996; Warren-Myers et al., 2018; Uglem et al., 2020).

Among the fluorochromes, alizarin red (ARS) is the most popular (Williamson et al., 2009; Smith et al., 2010; Wells et al., 2013; Warren-Myers et al., 2018). This compound is a feasible alternative to other compounds like oxytetracycline (OTC) and calcein (Bashey, 2004; Simon et al., 2009), especially because they are destined to be used as fluorescent dyes and described as harmless (Warren-Myers et al., 2018). In Spain, OTC mass-marking at a commercial scale is not viable as it can only be used experimentally, and calcein is a fluorescent dye which had toxic effects on fish in some studies and is described as irritant and possible carcinogen in Europe (Moran, 2000; ECHA, 2022a, b) with exclusive use in experimentation (MedChem, 2022). In addition, alizarin marking is a more cost-effective method compared to OTC or calcein marking. ARS fluorescent signals range from violet-red to yellow, depending on the light source (Beckman & Schulz, 1996; Lagardère et al., 2000; Liu et al., 2009).

The application methods for visible internal chemical marking in general, and fluorescent marking in particular, are: feeding, immersion (direct or by osmotic induction), and injection. For fish larval mass-marking due to their small size, only the two first approaches are of interest. In general, the feeding method requires more time in both preparation and administration, but it avoids the fish larvae handling, which is a huge advantage. Meanwhile, the immersion method is faster, a high number of individuals can be marked in a short time with a low handling

stress (Liu et al., 2009; Lü et al., 2016, 2019), and success rates of 100% have been achieved with either little or no effect on mortality in juvenile fish (using optimal time-concentration combinations; revisited in Warren-Myers et al., 2018). In this method, the direct approach consists in placing the desired eggs or fish in pre-prepared fluorochrome dye solution for a certain time, normally a few hours (i.e., Beckman & Schulz, 1996; Eckmann, 2003; Liu et al., 2009), meanwhile in the osmotic induction, fish are exposed to highly saline water for a short period before the fluorochrome dye. This osmotic shock increases the later rate of dye uptake, reducing the immersion time needed to a few minutes and generating brighter marks than in the direct immersion (Mohler, 2003; Negus & Tureson, 2004). Nevertheless, the drawback of the osmotic induction is the physiological stress to which fish are submitted, for example, in the study from Crook and colleagues (2007) fingerlings from golden perch (*Macquaria ambigua*) required several minutes to regain a normal swimming behaviour. Therefore, this technique can affect the growth and survival of the treated larvae and it requires detailed investigation. In whole, this makes the direct immersion method to be the most used, especially for small fish.

Regarding the marine fish larvae ARS marking, there are few studies (Sánchez-La Madrid, 1997). Therefore, we aimed to determine if ARS immersion is a feasible method for mass- marking Atlantic bluefin tuna (ABFT, *Thunnus thynnus)* eggs. A preliminary test to determine when to perform the treatment was carried out with sea bream eggs (unpublished results). This trial showed that survival increased if the treatment was performed in the gastrula stage, so the treatment described below was also conducted with eggs in this stage. The experiment was carried out in two steps: a first trial to assay two different concentrations and two times of exposure, to notice if the mark treatment was successful, and analyze its effect on hatching rate; and a second trial to notice if the marks were permanent.

Material & Methods

Naturally spawned ABFT eggs were collected from captive adults located in sea cages in San Pedro del Pinatar (Murcia, Spain), and transported to the facilities of the Spanish Institute of Oceanography in Mazarrón (IEO-CSIC, Spain).

i. Trial I

In the first trial, two concentrations (50 and 100 ppm) and two immersion times (3h and 6h) were tested. Fertilized eggs were cleaned, counted and placed in 18 tanks (10 L Volume), at a rate density of 100 eggs/L with sea water flow through, aeration and natural photoperiod. When the eggs reached the gastrula stage, the water flow was closed and the experiment began. The ARS was previously diluted by mixing Alizarin red S (*Scharlab* S.L., Spain) with sea water at 25ºC and homogenized during 3h in an automatic shaker. When the water flow was closed the ARS dilution was added to the incubation tanks (two different doses, 6 tanks per dose, and 6 control tanks). After 3 hours or 6 hours, according to the immersion time tested on each tank, the treatment finished and the water flow was reopened to achieve complete removal of ARS in less than two hours. The temperature values ranged between 25.1ºC and 25.3ºC during this incubation period.

When the treatment finished, a sample from each tank was taken and kept in smaller tanks to determine hatching rate and to visualize the marks in the otoliths as it is detailed in the section below (*Otolith extraction and visualization*), see a design of the process in **Figure V.VIII.1**.

ii. Trial II

In the second trial, 50 000 eggs/tank in the gastrula stage were immersed in two 150 L aerated incubators, one with an ARS concentration of 50 ppm, the other tank without ARS, kept as control. During 3 hours, both tanks had no water renewal, and 3 hours after it was reopened to 150L/h. Temperature ranged between 25.3ºC and 25.8ºC and eggs hatched after 35-40 hours. Two days after

hatching, 15.000 larvae from the ARS treated incubator were extracted and cultured in three 1500 L tanks (5000 larvae per tank) during 22 days. Larvae were fed on rotifer (5-10 rotifer/mL, twice per day) until 15 dph, on artemia from 12 to 22 dph (0.3 artemia/mL, 2 times per day), and on sea bream yolk sack larvae from 18-22 dph (supplied *ad libitum*, 2 times per day). During larval rearing, the temperature ranged between 22.3 - 25.6°C, salinity was 37.5 g L^{-1} and the photoperiod was 12L:12D

In order to control survival rate during embryonic development and hatching rates, four samples of 50 eggs from the control and alizarin treatments were placed in 250 mL boxes. 12 hours after treatment (prehatching stage) all the eggs in the boxes were counted, dead eggs extracted and survival rate ($number\ of\ live\ eggs\ x\ 100\ /number\ of\ initial\ eggs$) was calculated. After hatching, the hatching rate ($number\ hatched\ larvae\ x\ 100\ /\ number\ of\ initial\ eggs$) was also determined.

In order to notice the intensity of the mark, a representative sample of larvae from the three tanks were sampled at 1, 2, 8 and 22 dph, see a design of the process in **Figure V.VIII.1**.

iii. Otolith extraction and visualization

Sampled larvae were measured and the otoliths were observed under an specialized microscope and the fluorescence intensity was measured (quantitatively) in each of the ages, and re-measured (quantitatively) in different times. For this purpose, we used a UV light microscope (Leica DMi8 Thunder Imager) using the filter DFTC-TRITC (Maximum excitation: 550 nm, maximum emission: 573 nm, ARS signals in this equipment shined from red to orange), and a *Macro* tool in the *ImageJ* software of image analysis (*ImageJ2, Fiji package*, Schindelin et al., 2012; Rueden et al., 2017). This *Macro* was generated by the Department of Image Analysis (University of Murcia, Spain) and once selected the zone of interest (the marked area) it measured the fluorescence intensity.

After extraction, otoliths mounted in a microscope slide and protected by a coverslip were preserved at -80ºC. In order to notice if the time of preservation affected the intensity of the measurement, all the otoliths were visualized 24 hours, 15 days and 30 days after their extraction.

iv. Statistical analyses

All the statistical analyses from the fluorescent signal and mortality results were performed using the statistical programme SPSS (*IBM,* SPSS 25.0). In the first trial, A Chi-square Test was made to test the differences in mortality between treatments. In the second trial, for comparing the survival or hatching rates between treatments, parametric tests were used: T-test for two groups and ANOVA for more than two groups. For the comparison between the mean fluorescence intensity non-parametric methods were used (Mann–Whitney U-test) due to violation of normality (Levene's Test). In all the tests, p values of less than 0.05 were considered to be statistically significant.

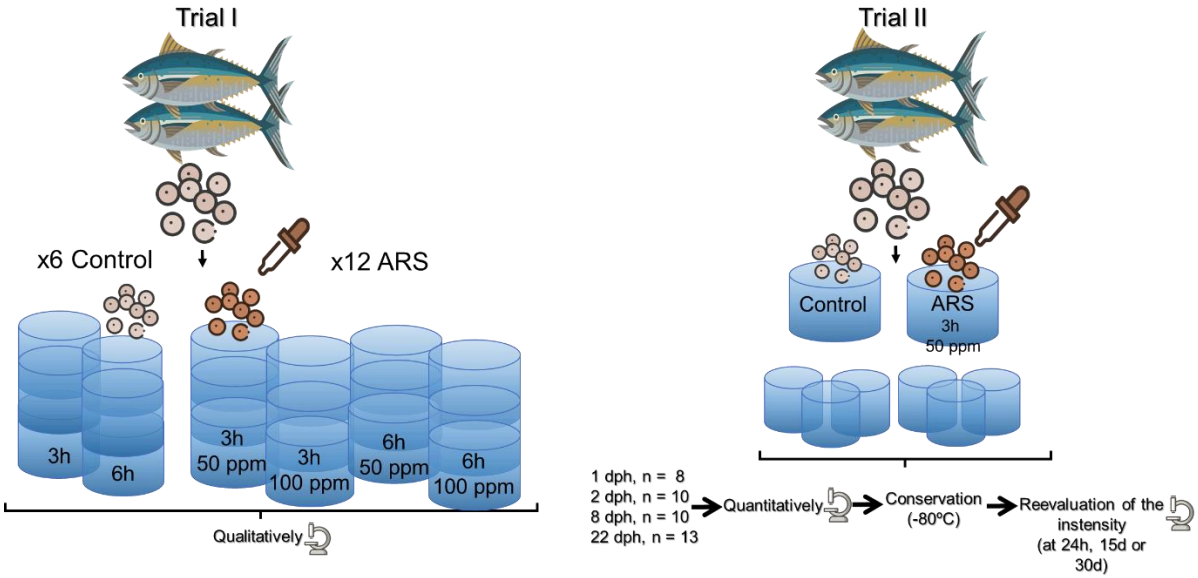


Figure V.VIII.1. Trials development and organization. ABFT eggs were marked by direct immersion in ARS and their fluorescence intensity was measured (qualitatively in the Trial I and quantitatively in the Trial II). In the Trial I representative samples were taken and in the Trial II larvae were sampled four times (the samplings showed are per tank, x3 control and x3 ARS). The otoliths were conserved by simple frozen, and their fluorescence intensity was re-measured (quantitatively) in 3 different periods (24 hours15 days and 30 days).

Results

i. Trial I

Results obtained in the first trial are showed in **Table V.VIII.1** Regardless treatment duration and concentration, all the otoliths were marked. Concerning the hatching rate, best results were achieved with the lowest concentration and the shortest time (**Table V.VIII.2**). However, there was no statistically significant difference between groups for the Hatching rate (One-way ANOVA $F = 0.446$, $p = 0.774$).

Table V.VIII.1. Hatching rates (mean ± S.D.) in Trial I.

Treatment	C 3h	C 6h	3h 50	3h 100	6h 50	6h 100
Hatching rate (%)	80.7 ± 10.7	84.9 ± 13.1	88.0 ± 9.4	77.5 ± 7.4	76.6 ± 14.4	46.6 ± 46.3

ii. Trial II

For the second trial, survival rates and hatching rates are shown in **Table V.VIII.2**. There were not statistically significant differences between groups nor for mortality rates 12 h after treatment (T-student test, $t = -0.666$, $p < 0.05$: 6.42% in control eggs and 9.48% in ARS treated eggs) nor for hatching rates (T-student test, $t = 1.108$, $p < 0.05$: 84.98 % in control and 80.91% in ARS treated).

Table V.VIII.2. Survival rates (12 h. after treatment) and Hatching rates (mean ± S.D.) in Trial II.

Treatment	Control	ARS
Survival rate (%)	93.58 ± 2.79	90.52 ± 2.75
Hatching rate (%)	84.98 ± 8.75	80.91 ± 8.33

iii. Otolith visualization

The mark in the otoliths could be visualized easily (**Figure V.VIII.2**), and the results of the analyses of mark intensity are showed in **Figure V.VIII.3**.

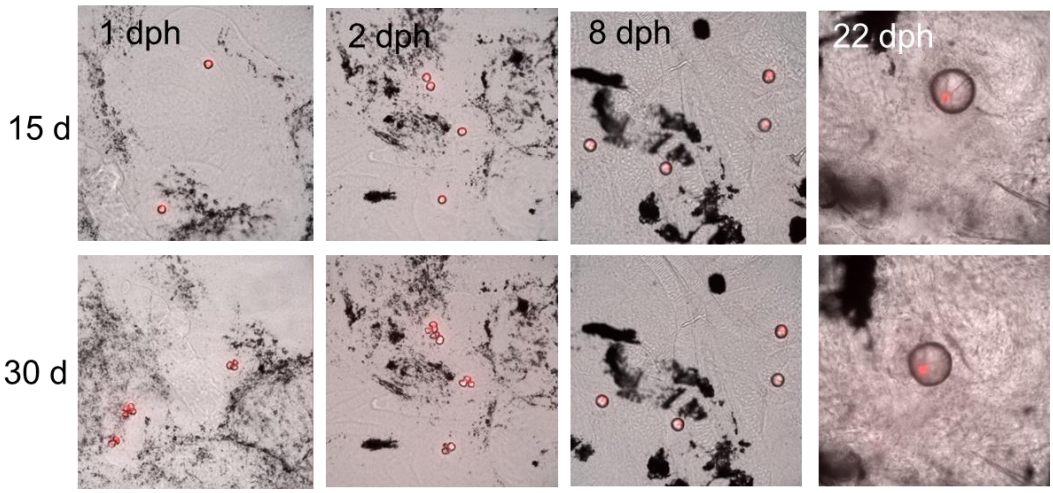


Figure V.VIII.2. Marked otolith images (UV-fluorescence pictures are superposed to the light microscope image) in tunas sampled with different ages (1, 2, 8 and 22 dph), at the same time after their conservation (15 and 30 days after freezing).

Comparison tests between the fluorescent results were made (U-Mann-Whitney, $p< 0.05$). In general, the intensity remained constant except for the older larvae which presented higher intensity. With regards to the preservation time, results did not show significant differences, even when the otoliths from larvae 8 dph had a slightly higher intensity visualized 30 days after freezing.

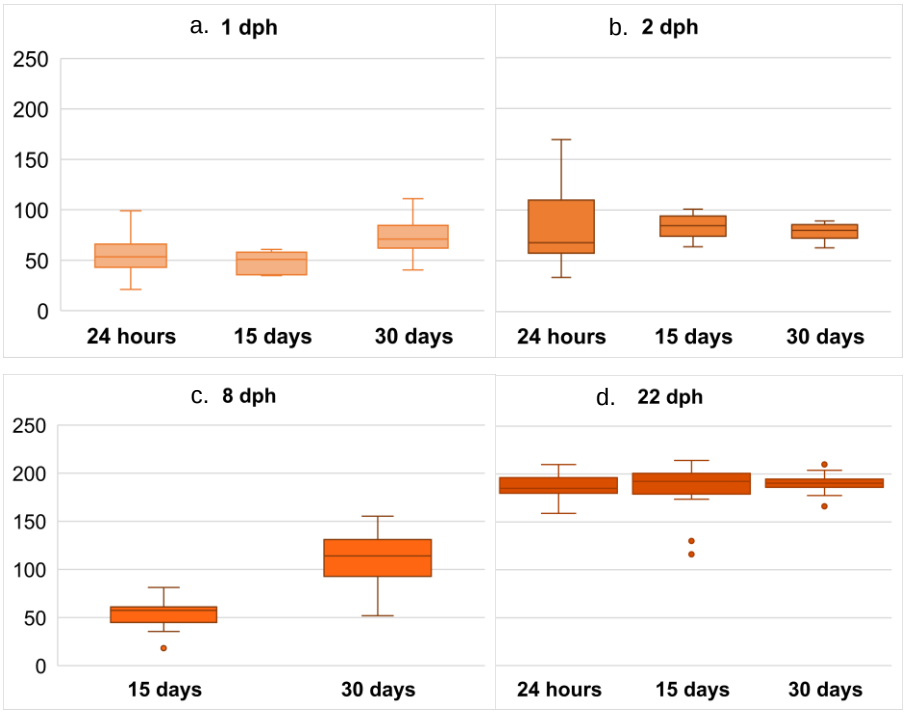


Figure V.VIII.3. Mean fluorescence intensity: in each plot the intensity of the marked otoliths at different sampling ages after 24 h, 15 days and 30 days of preservation is showed.

Discussion

The employed technique for marking the otoliths of ABFT eggs with ARS was successful. When exposing a bony structure like the otoliths to ARS, all the surface was dyed. This has been observed in other studies, where they refer the Alizarin red S as a calcium-chelator, and thus, it has the ability to form complexes with calcium ions that are already embedded in skeletal structures like the otoliths (Campana, 2001; Stańczak et al., 2015). Consequently, regarding the otoliths from the marked individuals long-time after the immersion, we could easily identify the after-marking deposited material: the after-marking region was not fluorescent meanwhile the before-marking region remains really visible and differentiated (**Figure V.VIII.4**).

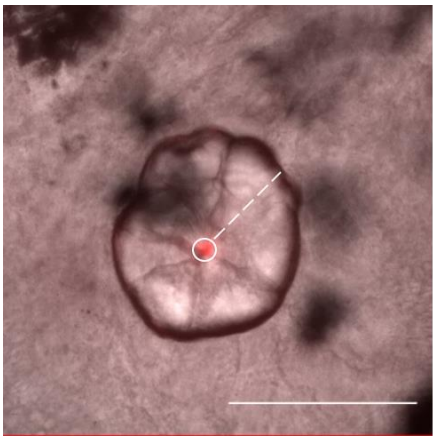


Figure V.VIII.4. Growth before and during the bath (○) and after (----) the marking in ABFT of 22 dph (fresh), x20 magnification. Scalebar = 100 µm.

As it can be observed in the figure, the dye marks the whole otolith during the bath, and the new material deposited in the otolith after the bath is not marked. The ARS mark remained in the otoliths over time (from 0 to 22 dph). We also found in another marking experience (**Chapter VII of this Thesis**) that OTC can be a successful marking method in ABFT juveniles. OTC could also be used as a dye for eggs, but the Spanish legislation only permit its use with experimental purposes and the amount of OTC used and, consequently, released to environment is much higher in an immersion treatment.

At the tested concentrations and time, there were no effect of the survival and hatching rates, so we can state that the ARS immersion marking is a reliable mass-marking method, because it permits to mark individuals with low time and concentration obtaining 100% of signal success. In comparison with other application methods, fluorescent marking by direct immersion could take much less time to apply (Hettler, 1984; Eckmann, 2003; Mohler, 2003; Negus & Tureson, 2004; Logsdon & Pittman, 2012). Regarding the long-term effects of chemical marking in fish, most studies have concluded that it has no persistent effect on the marked organism (i.e., Blom et al., 1994; Tsukamoto, 1988; Tsukamoto et al., 1989; Baumann et al., 2005; Liu et al., 2009; Hansen et al., 2015). However, Meyer et al. (2012) found sub-lethal effects like reduced growth rates, hatching and first feeding success, when marking Atlantic cod larvae and

eggs with alizarin, possibly because the larvae had ingested the compound. In contrast, Blom and colleagues (1994) did not find comparable effects when marking similar stages of Atlantic cod. Moreover, the ultimate conclusions drawn from most studies were that fluorochrome dyes (i.e., CAL, ARS, ALC, TC, OTC) have no persistent adverse effects on the marked organisms (i.e., Lang & Buxton, 1993; Mohler, 2003; Taylor et al., 2005; Stańczak et al., 2015; Lü et al., 2016) by immersion (Bashey, 2004; Taylor et al., 2005; Lü et al., 2014a, b; Hansen et al., 2015) even double (i.e., Tsukamoto, 1988).

Marked otoliths keep their fluorescence intensity for several weeks after being extracted. Therefore, the simple freezing is a reliable conservation method to follow. Finally, regarding the sampling age, even though the specimens were marked all in the same stage (before hatching), the older specimens had generally more signal than the younger.

Conclusion

The ARS proved to be a high efficiency, reliable and not harmful method in ABFT eggs that allowed us to distinguish the marked larvae and fingerlings through their otoliths. In this study, the 100% of the tunas were marked, the mark visualization was easy, and no effect on hatching rate was observed. This chemical method could be useful in ABFT mass-marking, but further studies on the long-term prevalence of this marks should be carried out.

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VI. General Discussion

The study of the ABFT and possible techniques that facilitate its identification is a fascinating field of research due to its novelty and the few references in the literature of specimens bred entirely in aquaculture. Specially, the identification of ABFT juvenile specimens whose hatch and rearing take place in onshore tanks has never been described, since the information usually found refers to juvenile wild specimens and/or adults kept in fattening cages, which are usually bigger than 50 kg. Due to the various types of tracers and markings for the identification of ABFT that have been described throughout the 3 Sections and 8 Chapters of this thesis, we want to integrate the findings in a General Discussion that allows us to display the advantages and disadvantages of each method, as well as the most useful one(s). Throughout this General Discussion, the information obtained in each chapter will be highlighted by Sections, in order to integrate it and compare the different types of natural chemical tracers first, natural morphometrical tracers later, and artificial markings at last. Finally, for the First and Second Sections, and for the three Sections altogether, the traceability systems proposed in this Thesis will be compared.

VI.I. First Section: Natural chemical tracers

Natural tracers have been broadly used to distinguish individuals (i.e., Wilson et al., 2006; Gillanders, 2009), and have brought good results when discriminating groups of fish (i.e., Sogut & Percin, 2011). However, in Scombridae species and especially in ABFT they are poorly developed, except for a few studies in tissular chemical composition (Percin et al., 2011; Sogut & Percin, 2011). In this Thesis, natural tracers in ABFT juveniles have been useful, as differences in group characteristics (possibly due to diet and environmental conditions) permitted to discriminate batches using several hard and soft tissues.

From the 32 detected elements by ICP-OES, only 7 (Ca, Fe, Mg, Na, P, S and Zn) were detected in 100% of the samples: liver, kidney, muscle, brain, gill, bone

and otolith. Others, like K, Cu and Mn, appeared in all the samples in soft tissues, gill and bone; also, Sr appeared in all the samples of gill, bone and otoliths, and Al, Rb and Ti only in the otolith samples. Therefore, *a priori* these first 7 elements would be the ones to stablish differences among batches. However, the contrast of the obtained results requires to observe the data closely.

One of the most important aspects found in this study was the non-coincident results obtained in the means (or medians) comparison tests and the discriminant analysis (DCA). Thus, regarding the 7 elements found in all the analyzed tissue samples, the Ca and Na concentrations did not serve to discriminate among juvenile batches trough the DCA, and there were no statistical differences in Ca concentrations between batches (**Table VI.I.1**). However, there were statistical differences in Na concentration of the otoliths. These two elements fulfill important functions in the organism. Regarding the Ca, it is physiologically regulated (osmoregulation) (Lall & Kaushik, 2021), which could justify the absence of differences among batches. In the case of Na, it is an abundant element in water, also present in food, so its deficiencies are rare (Lall, 2002). Therefore, despite both elements did not provide information in the discriminant analysis, their use in future discrimination or traceability studies should not be ruled out.

The rest of the 5 elements have utility on the batches' discrimination in the DCA, depending on the tissues. For example, phosphorus and S served to discriminate batches in 4 of the 7 analyzed tissues. The P is an important element in the composition of hard tissues, like the bone and the otolith, and plays an important role in the cells of the organism (Lall & Kaushik, 2021). Its entry is mainly from the diet (Coloso et al., 2003), which could justify its different tissue concentration among batches and therefore its role as discriminating element. In the case of S, even though it is the fourth most important marine element, having an important role in protein composition, very few simple sulfated compounds have been identified in marine vertebrates (Kornprobst et al., 1998). In both elements, we did not find a complete coincidence (element with statistically significant

differences between batches *vs.* discriminant element), however we found some coincidences in both soft and hard tissues (**Table VI.I.1**). Therefore, the study of ABFT tissular levels with different statistical tests could be useful in the search for tools to determine their traceability, although it is necessary to carry out new studies in which the stable isotope analysis could be considered ($^{34}S/^{32}S$).

The resting elements (Fe, Mg and Zn) were discriminant in 3 tissues (**Table VI.I.1**). For these elements, only in bone they signaled both statistically significant differences among batches and discrimination in the DCA. Therefore, these three elements could be relevant in traceability studies in bone (**Table VI.I.1**). For its part, Fe had both statistically significant differences in concentration among batches and was discriminant in several tissues: kidney, muscle (soft tissues), and bone (hard tissue). Fe is found in all the cells of the organism (National Research Council, 2011; Lall, 2021), mainly in hemoproteins, hemoglobin and myoglobin (Lall & Kaushik, 2021), which could justify this coincidence. Meanwhile Mg only coincided having statistically significant differences in concentration among batches and being discriminant in brain (soft tissues) and bone, and Zn in bone. Magnesium is an important macroelement present in soft tissues such as muscle (Knox et al., 1981), and together with Zn they participate in many physiological functions, biochemical processes and metabolism, and are crucial as components or co-factors in different enzymatic systems (Lall, 2002; National Research Council, 2011; Lall, 2021). However, the absence of a common pattern prevents selecting an element, tissue or statistical test exclusively, so the combined study of all of them could provide more complete and relevant information in traceability studies from these specimens.

On the other hand, even though not being present in the 100% of the otolith samples, elements like Mn and Cu are also important at a cellular level (Lall & Kaushik, 2021). In our studies, Mn served to discriminate among batches in the DCA from 4 tissues, and Cu in 3. Both elements were discriminant for brain and gill, with concentration differences among batches (**Table VI.I.1**). In the organism, Mn plays a key role in multiple physiologically functions, including the bone mineralization (Aschner & Aschner, 2005), so it could be an important element in

hard tissues for discrimination. Then, Cu was the only element with statistically significant differences between groups for all tissues (soft and hard, but not the otolith). This element needs to be incorporated within the diet (National Research Council, 2011), and its requirements are compromised by other factors, such as the physiological state, its water concentrations and the presence of potentially antagonistic elements (National Research Council, 2005). Therefore, future studies with older specimens and in which these factors are controlled could provide new information on the discriminating power of this element. Also, Sr and K served to discriminate batches even though not being present in the 100% of the samples, but only in 2 tissues. In the case of Sr, these tissues were the bone and the otolith (**Table VI.I.1.**), tissues in which Sr has key biological functions including increasing bone mineral density (Siccardi et al., 2010). The tissues in which K served for discrimination were liver and kidney, not being useful in the gills, probable due to its osmoregulatory functions (Borgatti et al., 1992). Finally, Al, Rb and Ti, appeared only in 100% of the otolith's samples. These are elements without known physiological function and did not serve to discriminate batches except for Rb, which despite being an element with little biological importance could be taken into account in future studies, especially since it is an analogue of K with which it competes (Peters et al., 1999; Tipsmark & Madsen, 2001).

In light of the above, it seems interesting to consider mainly the following elements in future studies: P, S, Mn, Cu, Fe, Mg and Sr, without ruling out the possible interest of the rest of the elements investigated in this thesis. Among these elements, it has to be mentioned that S was the only element that appeared repeatedly in the tissues with higher discrimination success (≥80%).

Table VI.I.1. Summary of the information obtained in the First Section of this Thesis. *Elements in 100% of the samples in all the tissues. Nc = not considered (not in 100% of the samples), ^{d} statistically significant differences in the mean comparison ($p<0.05$), $^{(d)}$ marginally statistically significant differences in the mean comparison, ^{s} selected by the DCA and therefore for discriminating among batches.

Elements	LIVER	KIDNEY	MUSCLE	BRAIN	GILL	BONE	OTOLITH	DCA selected times
Ca*	-	-	-	-	-	-	-	0
Cu	*d*	*d*	*d, s*	*d, s*	*d, s*	*d*	*nc*	3
Fe*	-	*d, s*	*d, s*	-	-	*d, s*	-	3
K	*d, s*	*d, s*	*d*	-	-	-	*nc*	2
Mg*	-	*(d)*	*d*	*d, s*	*s*	*d, s*	*d*	3
Mn	-	-	*s*	*d, s*	*d, s*	*s*	*nc*	4
Na*	-	*(d)*	-	-	-	-	*d*	0
P*	*(d)*	*(d), s*	-	*d, s*	-	*s*	*d, s*	4
S*	*(d)*	*d, s*	-	*s*	*d, s*	*s*	-	4
Zn*	-	-	*s*	-	*s*	*d, s*	-	3
Sr	*nc*	*nc*	*nc*	*nc*	*(d)*	*s*	*d, s*	2
Al	*nc*	*nc*	*nc*	*nc*	*nc*	*nc*	-	0
Rb	*nc*	*nc*	*nc*	*nc*	*nc*	*nc*	*d*	0
Ti	*nc*	*nc*	*nc*	*nc*	*nc*	*nc*	-	0
DCA discrimination sucess	60.8	88.6	79.5	82.2	80.0	77.0	78.4	-

The analyzed tissues with higher chemical discrimination in ABFT were the kidney, brain and gill (≥80% of discrimination success), followed by muscle, otolith and bone (>75%), being the liver the tissue with the worst discrimination success (60.8%). So, soft tissues (in general) seem to be more useful than hard tissues as discriminating tools, and had been previously used in some studies with this purpose (Percin et al., 2011; Sogut et al., 2011). In the literature, we have not found arguments referring to the soft tissues' capacity to better highlight differentiate among tuna batches. But perhaps one of the causes of this characteristic (even if it is mere speculation) could lie in physiology, given that the rate of cellular renewal of soft tissues is higher and that they have greater irrigation. Hence, future studies in this line, including older specimens, may shed light. Apart from what is mentioned above, from a practical point of view each group of tissues (soft *vs*. hard) have its advantages and disadvantages, and each tissue its own characteristics. Firstly, regarding soft tissues, the kidney is a by-product, and its sampling avoids losses or cuts that could depreciate the product, however its sampling is complicated, especially in small specimens. For its part, the brain is a small organ in tunas, sensitive to conservation and slippery, so it is not an easy tissue to handle *a priori*. Then, the muscle is a soft tissue easy to obtain and the most common commercialized part of fish, usually used for public health, food and ecotoxicology studies (i.e., Di Bella et al., 2015). Finally, the liver is another by-product, easy to sample, but had the lowest discrimination success in the DCA. Secondly, regarding hard tissues, gill is an interesting tissue for discrimination or traceability purposes, being a vital route for mineral uptake (Evans & Clairbone, 2009; Blust, 2012; Hogstrand, 2012; Grosell, 2012; Lall et al., 2021). For its part, the bone is also a by-product and easy to sample, but had lower discrimination success through the DCA. Then, the otolith could be considered as a useful organ in discrimination, because it is versatile (i.e., many methods can be applied on them with this purpose), has a continuous growth (during the fish's life), and its mineral part remains unaltered after deposition (Campana & Thorrold, 2001). However, it was the fifth tissue on the discriminating success ranking, and its usefulness for this purpose should be taken with caution. As it can be seen, it is not easy to determine the best tissue for discrimination or traceability purposes, although *a priori* the muscle could be the best choice.

In addition, this study has permitted to known the tissue of higher success in discrimination for each batch, and therefore, of higher utility for each group (**Table VI.I.2**): in wild tunas, the muscle had the greatest success on discrimination (96.3%), meanwhile in onshore tanks it was the otolith (87.3%), and in sea cages the brain (93.3%). On the other hand, this study it allowed us to know the best discriminated batch by tissue. In this sense, wild tunas were best discriminated in muscle, gill and bone; onshore tanks in liver and otoliths; and sea cages in kidney and brain. We have not found technical and/or scientific studies in which such data are discussed, but except for liver, it could be affirmed that the chemical characteristics of wild and farmed tuna tissues allow to plan batch discrimination studies based on their chemical fingerprint, so any further research in this field could yield interesting results in the search for traceability markers in these tunas.

Table VI.I.2. Success percentage in the DCA for each batch and tissue.

Batch	Liver	Kidney	Muscle	Brain	Gill	Bone	Otolith
Wild	67.9	87.5	96.3	82.2	82.8	89.3	63.6
Farmed	73.0	86.7	70.8	83.3	79.2	75.0	87.3
Sea cages	36.4	91.7	68.2	93.3	77.3	63.6	-

Among batches, wild tunas had higher concentrations of some of the studied elements in soft tissues and bone (**Table V.I.1.** and **Table V.II.1, Chapter I** and **II**, respectively)**.** A possible explanation for this could be the weight, the diet or the differing environmental conditions, as related by Percin and colleagues (2011). Wild individuals were migrating through the sea, changing their location constantly until they were fished in the Mazarrón Bay, and therefore they had a higher variety of preys (ABFT juveniles have an opportunistic diet, Karakulak et al., 2009; Van Beveren et al., 2016) including the presence of shrimps, cephalopods and crustaceans (Uotani et al., 1990; Sarà & Sarà, 2007; Sinopoli et al., 2004). This could explain the higher presence of microelements in soft tissues (with high turnover rate), the biomagnification of ultra-trace elements

through the trophic chain (i.e., Rb, Campbell et al., 2005), and the possible exposition to trace elements in the end of their lives (i.e., the western Mediterranean Sea has element-rich waters with high presence of Mg and K, Minas & Minas, 1993; Guerzoni et al., 1999; Dafner et al., 2001; Talley et al., 2011). In contrast, the sea cages individuals are also found in the sea but in a constant location with lower dimensions, lower water renewal (due to the cages' nets), and a controlled diet based on defrosted bait (like in onshore tank tunas). Finally, the onshore tanks individuals are found in fix tanks with controlled, homogeneous conditions and a water recirculation system. However, in gills and otoliths, higher elemental concentrations were found in onshore tank tunas (only otoliths from onshore tanks and wild tunas were analyzed in **Chapter III**) than wild tunas. Thus, an exhaustive study following an experimental protocol that controls all the other possible variables that could bias the discrimination due to water conditions and feeding regimes should be pursued.

In resume, we consider that the study of the chemical profile in juvenile ABFT tissues could be a useful tool for the discrimination of batches with distinct origin. The tunas' coming from differing environments (wild *vs.* captivity) have chemical signatures that can differ given that their ambient conditions and diets are different. Regarding the tissues, we confirm the muscle and otoliths' usefulness for this traceability purposes, something already reported in different studies (see **Table V.VI.4, Chapter VI**). Nevertheless, the best batch discrimination results (through DCA) were given by the kidney, which is considered a trace-elements' storehouse in the fish body (Sogut & Percin, 2011), but is poorly mentioned in the literature and it has a difficult sampling. In this sense, the muscle is the most common commercialized part of the fish and is usually used for risk assessment studies (i.e., Percin et al., 2011), which would facilitate the sampling. On the other hand, the otoliths can constitute an area-specific 'fingerprint' (Walther & Limburg, 2012) which make them as well very interesting for traceability studies, but as with the kidney, their difficult obtention combined with the lack of farmed juvenile specimens could have limited their use in ABFT specimens. Future studies including adult ABFT could corroborate the utility of these tools for traceability purposes.

VI.II. Second Section: Natural morphometrical tracers in ABFT, the otoliths. If you find them, you win.

Knowing the special characteristics of the otoliths and their countless use possibilities for differentiation purposes, we decided to explore their use in juvenile ABFT group discrimination. Morphology, shape, asymmetry and/or vaterite composition have been successfully used in many species with differentiation purposes, including some members of the Scombridae family (i.e., Itoh et al., 2000; Megalofonou, 2006; Brophy et al., 2016, **Supplementary Material, Table VI.II.S1**), but none in ABFT, possibly due to the lack of interest and the difficult sampling of the otoliths. Our study found that the morphometrical parameters of the otoliths might help to discriminate groups, subtracting additional information about the individuals' fitness trough asymmetry and vaterite analyses (see **Table VI.II.1** for a summary). Currently, the measurement equipment and developed software permit to obtain many data to perform comparisons, and in the **Second Section**, diverse statistical analyses were envisaged, being DCA one more time used in **Chapter IV** to summarize the most useful morphometrical traits.

Table VI.II.1. Summary of the information obtained in the Second Section of this Thesis. *Analyzed in the three chapters of this Second Section. Nc = not considered, d = statistically significant differences in the mean comparison, (d) = marginally statistically significant differences; s = DCA selected; y = parameters with asymmetry in both batches

Study/ Trait	Morphometry[1] Right	Morphometry[1] Left	Asymmetry[2]	Vaterite[3]
WO*	*d, s*	*d, s*	*y*	-
OA*	-	*d*	-	*d*
OL*	-	*d*	*y*	*d*
OW*	-	-	*d, y*	*d*

OE*	-	*d, s*	*d, y*	*d*
OP*	*(d)*	*(d)*	-	-
OCI*	-	-	-	-
OCO*	-	-	*y*	-
OF1	-	-	*nc*	*nc*
OF2	-	-	*nc*	*nc*
OF3	-	-	*nc*	*nc*
OF13	-	-	*nc*	*nc*
OFF	-	-	*nc*	*nc*
Traits in DCA	1	2	x	x
DCA discrimination I	63.4	57.4	x	x

[1] Analyzed in the size-corrected traits between batches. [2] Analyzed in the size-corrected Ai between batches, no DCA was conducted. [3] Analyzed in the raw traits between aragonitic and vateritic-otoliths, no DCA was conducted.

The otolith morphometry discriminating scores from both sides (**Chapter IV**, 57.4 and 63.4% for right and left, respectively) were poorer than the obtained from their chemical profile (**Chapter III**, 78.4%). Even though the chemical profile of the otoliths gave better results in the DCA, with the morphometry of the otolith we could obtain further information, including the fish fitness. The combined use of the otolith morphometry and chemistry have already been signaled as potential tools to identify nursery areas of different commercially important species (Rooker et al., 2001; Gillanders et al., 2003; Tanner et al., 2013; Tournois et al., 2013; Avigliano et al., 2015; Bailey et al., 2015; Bouchard et al., 2015; Avigliano & Volpedo, 2016), but until our knowledge no references to the higher success of one over another have been described in the literature, which is of great importance in batch discrimination or traceability studies.

Regarding the side, right otoliths discriminated better with a smaller number of variables (only the weight of the otolith -OW- gave statistical differences in the mean comparison and was selected by the DCA) meanwhile using left otoliths, more traits gave differences among batches (4 traits gave differences among

batches in the mean comparison test, and in the DCA were the weight of the otolith -WO- and the otolith eccentricity -OE- were selected). These right and left side differences have been previously described and could be explained by the existence of some pathologies (i.e., calcification abnormalities, asymmetry, etc.) that result in larger otoliths on one side (Tomás & Geffen, 2003; Reimer et al., 2016). In any case, it is difficult to establish a selection criterion extolling a side for studies of this type. Thus, a combination of data from both otoliths in one through expressions like A_i (R_i – L_i) were used in **Chapter V**. This expression is described as the mean of the side differences and has been previously used to illustrate the possible types of asymmetry on an organism (Palmer & Strobeck, 1986, 1992; Palmer, 1994; Somarakis et al., 1997; Loher et al., 2008; Kajajian et al., 2014), and we considered that in the future it could be the most useful way to obtain discriminating information for the batches.

On the other hand, in the asymmetry and vaterite studies the DCA was not used (**Chapters V and VI**, respectively), given the complexity of the data interpretation. However, the used analyses permitted to identify some questions: First, two types of asymmetry were found (antisymmetry -AS- and directional asymmetry -DA-) in both wild and farmed tunas, having farmed higher asymmetry (**Chapter V,** '**Asymmetry study in otoliths from Atlantic bluefin tuna (*Thunnus thynnus)* from two different environments'**). This is in accordance with the literature, where causes for asymmetry in both open waters (i.e., genetic predisposition, environmental stress, etc., Yedier et al., 2022a) and rearing conditions (water conditions, diet, diseases, physical or mechanical problems in the otolith sacculus, environmental stress, etc., Jawad & Adams, 2021; Fey et al., 2022; Yedier & Bostanci, 2020; Yedier, 2022; Yedier et al., 2022a) have been described, but higher asymmetry have been associated to hatchery-reared fish (Gauldie, 1986; David et al., 1994; Bowen et al., 1999; Sweeting et al., 2004) even up to 10 times more than wild fish (Reimer et al., 2016). Second, the vaterite presence was identified in both wild and farmed tunas (**Chapter VI,** '**Vaterite precipitation in Atlantic bluefin tuna (*Thunnus thynnus*) otoliths**'), having farmed higher prevalence of vaterite, and having vateritic-otoliths lower area, length, eccentricity and higher width than their aragonitic counterparts. These results were partly in

accordance with the literature findings, where hatchery-reared individuals have higher vaterite prevalence (see the **Table V.VI.4 in Chapter VI** for a review of these studies and their results). However, the morphometry results differ with some of these studies. For example, Tomás and Geffen (2003) found that vateritic-otoliths had higher area, length and perimeter but lower width than aragonitic otoliths in juvenile herring (*Clupea harengus*). In conclusion, for this species and age group, both models can be considered useful to discriminate batches aiming a quantitative point of view instead of qualitative, given that both batches had either side asymmetry and vateritic-otoliths; consequently, these models can be considered useful to obtain information about the fish fitness, having more asymmetric individuals and individuals with higher quantity of vaterite in their otoliths lower performance and welfare (Tomás & Geffen, 2003; Reimer et al., 2016; Yedier et al., 2022a).

In this context is difficult to perform a quantitative comparison among the obtained data in morphometry, asymmetry and vaterite analyses, and not such comparison haven been found in the literature. Only similar studies to the **Chapter VI** of this Thesis ('**Vaterite precipitation in Atlantic bluefin tuna (*Thunnus thynnus*) otoliths**') combining morphometry and vaterite analysis to compare the morphometry from aragonitic and vateritic-otoliths have been performed to our knowledge (i.e., Tomás & Geffen, 2003; Geladakis et al., 2020; Long et al., 2021). In resume, in this Thesis it seems that the morphometry study (**Chapter IV**) gives more information to discriminate among batches given the nature of the data, seeming the best and clearer discriminating tool of this **Second Section**. Meanwhile the asymmetry and vaterite studies permit to compare batches in a quantitative but no qualitative point of view and we consider that the combination of all the data (morphometry, asymmetry and vaterite) can give more complete and interesting information in relation to the fish welfare and fitness; something already mentioned by other authors (Palmer, 1994), because asymmetry and vaterite can be related to stressful conditions *inter alia* (Vinagre et al., 2014, see **Figure V.V.5** in **Chapter V**).

Regarding the batches, the farmed ABFT group was the best discriminated using the morphometry of the otolith in both sides (87.7% and 81.5% *vs.* 19.4% and

13.9% for right and left otoliths, respectively in farmed and wild tunas), which coincides with the fact that farmed were also the best discriminated group using the otolith chemical profile. This shows that the otoliths are good natural tracers for groups reared in homogeneous conditions like farmed tunas, that can stand clearly different from more heterogeneous groups like wild tunas. In relation to this, the study of Couillard and colleagues (2022) could discriminate the more homogeneous Atlantic herring (*Clupea harengus*) group and signaled that these individuals are those which have less connectivity with other areas. In this Thesis, this was the case of farmed tunas, especially the onshore tanks tunas, which were hatched, weaned and raised in tanks during their entire life.

On the other hand, the rearing conditions can modify the farmed tunas' fitness, triggering a more 'abnormal' otolith development (Bowen et al., 1999; Sweeting et al., 2004; Reimer et al., 2016). In fact, in this Thesis farmed tunas had more asymmetry and vaterite in their otoliths (**Figure V.V.4** and **Table V.VI.3**, in **Chapter V** and **Chapter VI** respectively). The possible causes of the found differences in otoliths among batches of this Thesis are summarized in **Table VI.II.2**. In resume, these differences can be mostly caused by discrepancies in diet (Browning et al., 2012; Jonhson et al., 2020; Jawad & Adams, 2021) and ambient water chemistry conditions (Vinagre et al., 2014; Fey et al., 2022; Geladakis et al., 2022; Yedier et al., 2022a). However, concretely for the asymmetry and vaterite development, much more possible causes have been described (see **Figure V.V.5** in **Chapter V** for the asymmetry possible causes). In conclusion, we have discovered that the information given by the otoliths are numerous, being a tissue with high efficiency and possibilities, something searched in discrimination and traceability studies. Between the asymmetry and vaterite analyses seen, it is difficult to pick up a method, given that both conditions appear in the two batches studied and farmed specimens display higher quantities of both conditions. However, we can state some discrepancies: firstly, the nature of the quantification of both conditions is different, meanwhile for asymmetry we take into account the whole population statistics, the vaterite information is by individual, so the preference of one or another depends on the number of samples or the type of study that is to be done; Secondly, the

asymmetry analysis gives the opportunity of conserve the samples, meanwhile with the vaterite (X-Ray diffraction) analysis they are destroyed, being the first method of election if the obtention of more data from the samples is needed. In contrast, this discrepancy could be an advantage, permitting to perform both analyses one after another (1st asymmetry, 2nd X-Ray diffraction vaterite). Thirdly, the interpretation of the vaterite is easier, at least following the protocol stablished in this Thesis, being this method more helpful in studies with many samples or tight deadlines.

Table VI.II.2. Summary of the possible causes driving the differences in the otoliths of wild and farmed tunas for both natural chemical and natural morphometrical tracers (Chapters III-VI). Mg= Magnesium, Na = Sodium, P = Phosphorous, Sr= Strontium, Rb= Rubidium. WO= Weight of the Otolith; OA= Otolith Area; OL= Otolith Length; OP= Otolith Perimeter; OE= Otolith Eccentricity; OF2= Otolith F2.

Study	Differences	Causes
Composition **(Chapter III)**	Mg, Na, P, Sr and Rb	Sr uptake is probably related to surrounding water concentrations (Secor & Rooker, 2000), and the recirculation system used for the rearing of farmed specimens. Mg, Na and P, have been suggested to be physiologically regulated in fish (Dorval et al., 2007; Hamer & Jenkins, 2007; Thresher et al., 1994; Proctor et al., 1995). These differences in the studied batches could be mostly explained by the divergent diets: farmed specimens were fed on defrosted bait *ad libitum* composed of small pelagic fish, which are rich, oily and highly nutritive preys. Finally, Rb also seems to be related to diet, given it is transferred from prey to predator (Johnson & Reeves, 1995; Nyholm & Tyler, 2000), biomagnifying throughout the trophic chain (Campbell et al., 2005).
Morphometry **(Chapter IV)**	WO, OA, OL, OP, OE, OF2 Higher values in wild	Greater irregularities in wild could be related to stressful environmental conditions in open waters (i.e., abrupt shifts in water composition, temperature and/or salinity, Vinagre et al., 2014). Then, farmed individuals can also experience environmental stressors (i.e., high fish densities, and frequent human presence, or unknown stressor due to the artificial rearing, Sweeting et al., 2004; Reimer et al., 2016; Loeppky et al., 2019). Stressful environmental conditions and alterations in its homeostasis may generate side differences or differing

		forms of crystals. Farmed individuals gave more homogeneous results probably due to the more constant and controlled conditions.
Asymmetry (Chapter V)	OW and OE Higher asymmetry in farmed	Many factors have been described as possible causes of asymmetry in rearing conditions: water temperature (Geladakis et al., 2022), chemistry (Fey et al., 2022), quality (Vinagre et al., 2014), diet (Johnsson et al., 2020), metabolic rate (Sweeting et al., 2004), diseases (Jawad & Adams, 2021), physiological and mechanical issues on the otolith sacculus (Mahé et al, 2019; Yedier & Bostanci, 2020) and environmental stress (Yedier, 2022).
Vaterite (Chapter VI)	OA, OL, OW and OE Higher vaterite presence and quantity in farmed	Aragonite disruption can be caused by shifts in the otoliths' organic matrix composition (Mann, 2001; Falini et al., 2005; Tohse et al. 2009) and/or energetic mismatches in the otolith sacculus membrane pumps (Tohse & Mugiya, 2001). In addition, the otolith formation regulation and/or mineralization is controlled by several genetic and neuroendocrine factors, and the perturbation of one or more of these factors may cause the shift from aragonite to vaterite during the otolith formation (Tomás & Geffen, 2003).

For future studies, the otoliths constant growing nature makes that they could experience important shape changes from juvenile to adult (Itoh, 2000). In addition, with the age of the fish, both the asymmetry and the vaterite of the otoliths could increase due to the exposition to environmental conditions (Jawad et al., 2001). Consequently, new studies should be raised with the aim of standardizing the otolith morphology and shape of ABFT in different phases of the fish development, for a better knowledge of its growth and its best use as a tool to discriminate between groups, including wild and farmed counterparts.

Seeing the information from **First and Second Section**s altogether, the otoliths stand as the best natural tracers, especially for groups with different life regimes. They were especially good at highlighting the more homogeneous group, and could give hints about the fitness differences among batches. Therefore, they are not only good natural tracers for group discrimination but also a key tool in animal welfare and production. This is why, if possible, we recommend the use of both otolith morphometry and chemistry, given that both analyses can be done in the same samples in the given order. Nowadays, there are several multivariate analyses that permit to combine all the obtained information of a sample, for example its chemical profile, vaterite, morphometry and asymmetry analysis, opening many possibilities to discriminate among groups, and permitting to obtain the advantages of each type of analysis. However, if the budget of the study is limited, the morphometry would be of choice given its lower costs, equipment needs and sample process, and its wider possibilities (**Figure VI.II.1**).

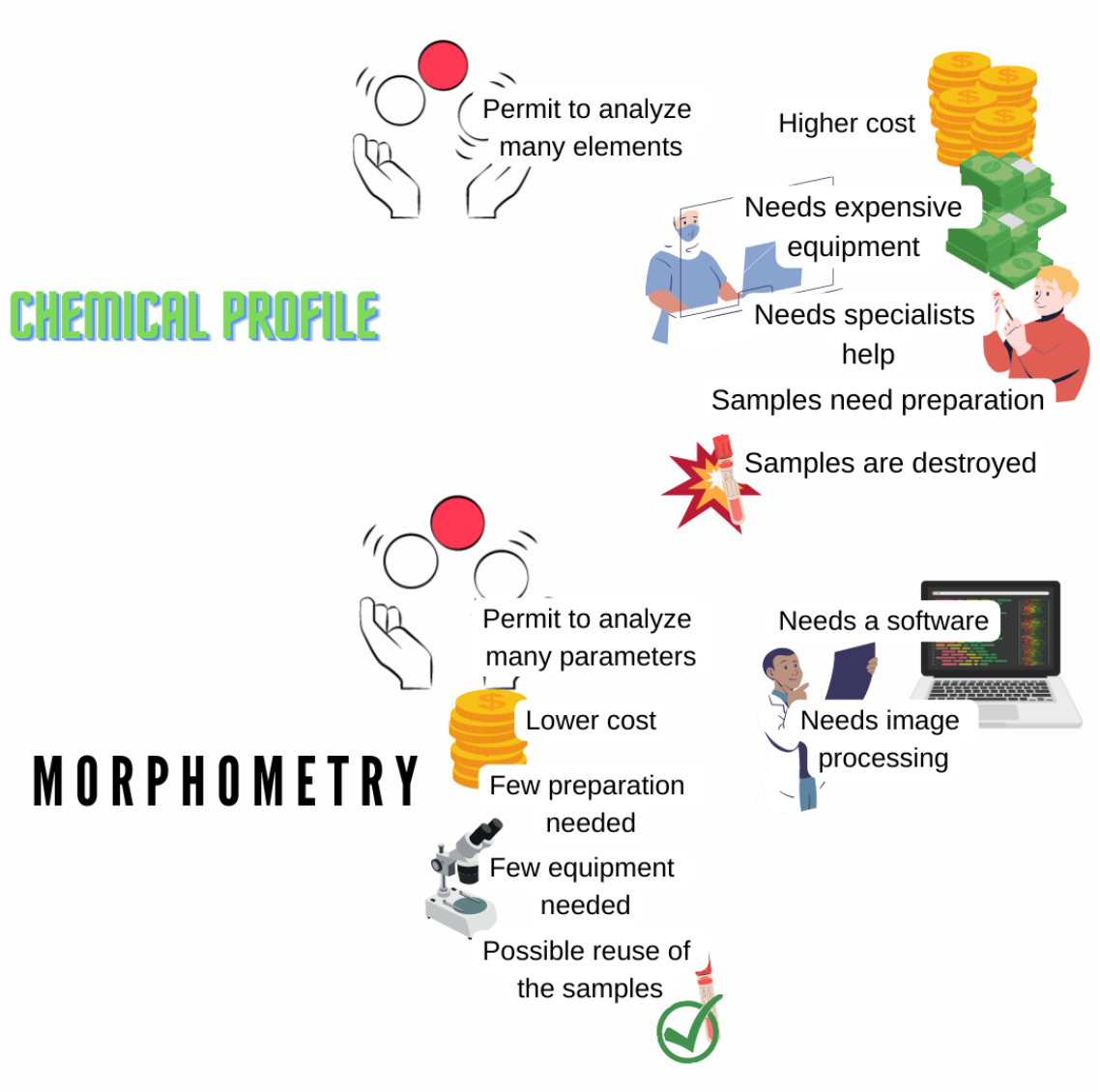

Figure VI.II.1. Advantages and disadvantages of the analysis pursued in the otolith of this Thesis (Chapters III-VI).

VI.III. Third Section: Artificial marking, can you see it? Is the artificial mass-marking the best option?

The artificial markings used in this Thesis (chemical fluorochrome markers) have been used to label and track groups of various fish species through hard structures like the otoliths (Gillanders, 2009). The main point about otolith mass-marking is that the marks are considered permanent, due to their non-resorbable nature, and that their structure is commonly used for chemical marks retention due to their concentric growth which permit to measure age and daily growth increments (Barker & McKaye, 2004). Therefore, the use of chemical markers could be an excellent alternative to conventional marking techniques (Barker & McKaye, 2004). There are many otolith artificial mass-marking techniques (i.e., thermal marking in salmonids -Warren-Myers et al., 2018-, chemical markers like tetracyclines and calcein in pikes -Brooks et al., 1994-, bass - Bumguardner & King, 1996-, salmonids -Mohler, 1997, 2003- or trouts -Negus & Tureson, 2004-), because *sagittae* in general have advantages for marking retention over other structures: they are one of the first calcified tissues in a fish (McElman & Balon, 1979) allowing marking during early life stages, they are easily collected and they exhibit daily growth rings (Brown et al., 2002). Apart from otolith marking, there are a lot of marking methods used on fish such as fin amputation, coded wire tags, pop-up satellite tags… which are often used for tracking (migration, localization and wild stock differentiation, i.e., ABFT - Cermeño et al., 2015-) in juveniles and adults, but not for mass marking in early life stages.

However, the most widely method used for otolith mass-marking in fish larvae and therefore in most farm aquaculture hatcheries are fluorochromes (Brothers, 1990; Baras et al., 2000; Simon, 2007). In this Thesis, none of the two markings used had been previously tested in Scombridae. Even though the possible toxicity or side-effects from the chemical markings tested (**Table VI.III.1**) are still being

investigated, they were efficient and successful, giving 100% of marking and a simple visualization of the marks. Among both methods, the use of ARS immersion in eggs was the most successful, given that it was easy to apply, did not require direct handling, it was cost effective for mass marking, the marks were clearly visible in the short-term, and no mortality effects were found on the treated eggs. However, it is possible that the same vehiculation of the OTC would give similar results, permitting to mark ABFT eggs instead of juveniles, dyeing the whole otolith, and without greater handling of the fish (in this Thesis OTC was injected in juvenile tunas). For example, OTC immersion has been successfully proved in species like: larval and juveniles walleyes (*Stizosteton vitreum,* Brooks et al., 1994) with 500 mg/L during 6h; yellow perch fingerlings (*Perca flavescens*) with 500 mg/L during 6h (Unkenholz et al., 1997) and yellow perch juveniles in 700mg/L during 4, 6 and 8h (Brown et al., 2002); Palmetto bass (*Morone spp.)* juveniles in 500 and 700 mg/L during 6h (Mauk, 2008); and small European eel (*Anguilla anguilla)* (Simon & Dörner, 2005). Most of the studies did not find effects on survival, only Brooks and colleagues (1994), encountered some affectation related more to the water temperature. Moreover, Tsukamoto (1985) observed a positive effect of tetracycline marking on survival in long-term (3-6 months) using 200-300mg/L during 24-48h and 3-24h in eggs and larvae of ayu (*Plecoglossus altivelis)* respectively, which could be related with their antibiotic properties. Therefore, to test the use of OTC immersion in early life stages of ABFT, as it was done with ARS in this Thesis, would be of interest.

Table VI.III.1. Summary of the properties of OTC and ARS used for marking purposes referenced by other authors and experienced in this Thesis.

Marking	OXYTETRACYCLINE injection in juveniles	ALIZARIN RED S Immersion in eggs
Advantages	100% of marking success in different studies and ours	Specifically used for marking purposes[11]
	Incorporated into calcified structures within hours[12]	No mortality related proved, described as harmless[3]
	Good for short marking	More cost-effective than other markings[13]
	May be beneficial due its antibiotic properties[14]	
	Not mortality related observed (including tunas)[15]	
	No need for sanding until 60 days post treatments[16]	
Disadvantages	There are important restrictions on the use of antibiotics in UE	Little information about potential toxicity

[11] Warren-Myers et al., 2018; Moran, 2000; ECHA, 2022a,b; MedChem, 2022

[12] Nagięć et al., 1995; Lagardère et al., 2000; Walt & Faragher, 2003; Crook et al., 2009; Wickström & Sjöberg, 2014; Caraguel et al., 2015; Warren-Myers et al., 2015a, b

[13] Warren-Myers et al., 2018

[14] Ahmed & Tan, 1992

[15] Brooks et al., 1994; Brown et al., 2002; Wexler et al., 2003; Simon & Dörner, 2005; Mauk, 2008; Barker & McKaye, 2011

[16] Brooks et al., 1994

	Only tested by injection in this study (in juveniles)	Potential species-specific side effects[17]
	Not correspondence between marks' intensities and dose/time	
	OTC will degrade in natural light[18]	
	Otolith autofluorescence interference can give mark identification problems[19]	
Conclusion	Medium efficiency. Other method than injection should be tested.	High efficiency, the mark visualization was easy, and no larval mortality related was observed

[17] Toften & Jobling, 1996; Bumguardner & King, 1996

[18] Muth & Bestgen, 1991; Doi & Stoskopf, 2000

[19] Jenkins et al., 2002

Thus, it is recommended to continue this mass-marking evaluation in the long-term, to test the evolution of the fish's marks and to assess their persistence over time and the effect of the storage conditions (i.e., some fluorescent marks are photosensible - Doi & Stoskopf, 2000-). There is also interesting literature about fluorochrome marking through feeding, with the possibility of direct fluorochrome feeding, or the use of preys as vectors (Stańczak et al., 2015) because fish larvae commonly accept live food willingly (Brett, 1971; Wolnicki et al., 2009). The use of live food as a vector was first tested by Nagięć & Nagięć (1983), and Nagięć and colleagues (1983), and this could be a new field to study given the highly voracious piscivorous behaviour in ABFT from early ages (Hunter & Kimbrell, 1980; Young & Davis, 1990; Sabate et al., 2010; Catalán et al., 2011).

To resume the three Sections of this Thesis (**Table VI.III.2**), the otolith would be the tissue of election because it permits the artificial marking in egg or larvae stages, but also the analysis of natural tracers (chemical or morphometrical) in bigger individuals. So, if the combination of both artificial marking and natural tracers is possible, we will recommend it. In the context of a hatchery for supplying fish to human consumers there is the possibility of mass-marking fish since egg or larval stage, and then control the specimens until slaughter. For the comparison of wild against different farmed groups in the future context of several ABFT hatcheries, it will be necessary to develop a standardized method, probably including natural tracers, artificial marking and genetic marks to be able to distinguish the origin of the different batches.

Table VI.III.2. Summary of the three Sections of the Thesis.

	First Section	Second Section	Third Section
Best result	Kidney discriminating success	Morphometry gives the most detailed information	ARS eggs mass-marking was successful
Main conclusion	Otolith give good results and wider applications.	Asymmetry and vaterite analyses also gives information about animal welfare and fitness. The otolith chemical profile, morphometry and asymmetry analyses can be done in the same samples, also vaterite if we use one side for chemistry and the other for vaterite (right and left differences should be previously tested).	ARS immersion in eggs was the most successful: it was easy to apply, did not require direct handling, it was cost effective, the marks were clear in short-term, and no mortality effects were found on the eggs.

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Supplementary material

Table VI.II.S1. Articles reviewed in this thesis for the Second Section**:**

Study	Species	Aim	Chapter/Use
Morales-Nin, 1987	Three demersal fish in Namibia: hakes (*Merluccius capensis and M. paradoxus)*, and kinglip (*Genypterus capensis*)	Relate otolith microstructure and features to environmental factors	Morphometry
Bölles & Begg, 2000	Silver hake (*Merluccius bilinearis*) in Northwest Atlantic	Stock distinction based on the whole otolith morphometrics	Morphometry
Itoh et al., 2000	ABFT (*Thunnus thynnus)*	Examine the periodicity of the daily increments' formation	Morphometry
Tuset et al., 2003	Comber (*Serranus cabrilla)* from the Atlantic and Mediterranean.	Examine the performance of shape for discriminate regions using Fourier Series	Morphometry
Megalofonou, 2006	ABFT (*Thunnus thynnus)*	Discriminate age ABFT groups	Morphometry
Mérigot et al., 2007	Common sole (*Solea solea*) in Northewestern Mediterranean	Characterize local populations using otolith morphometrics (morphology and shape)	Morphometry

Reichenbacher et al., 2009	Arabian pupfish *(Aphanius dispar)* in the Arabian Peninsula	Examine if allopatric divergence (genetic diversification) can be detected in isolated populations.	Morphometry
Vignon & Morat, 2010	Coral reef snapper (*Lutjanus kasmira*) in the Hawaiian Islands	Investigate if genetics and environment regulate the otolith shape	Morphometry
Joh et al., 2015	Cresthead flounder (*Pseudopleuronectes schrenki*) laboratory reared	Enable analysis of the otolith microstructure of farmed specimens	Morphometry
Mille et al., 2015	Various marine species, four roundfishes Whiting (*Merlangius merlangus),* haddock (*Melanogrammus aeglefinus),* herring (*Clupea harengus*), and red mullet (*Mullus barbatus)* and four flatfishes European plaice (*Pleuronectes platessa)*, common dab (*Limanda limanda),* common sole *Solea*	Investigate morphogenesis patterns using the otolith shape with Fourier Descriptors	Morphometry

	solea, and megrim (*Lepidorhombus whiffiagonis)* within the Gulf of Lions and Bay of Biscay		
Brophy et al., 2016	ABFT (*Thunnus thynnus)*	Discriminate wild ABFT stocks	Morphometry
Wang et al., 2019	Many species (otolith use symposium)	Use of the otolith shape variation to identify species	Morphometry
Mahé et al., 2019	Bogue (*Boops boops)* in the Mediterranean	Explore the directional asymmetry effects in the otolith morphology on stock discrimination using otolith shape analysis and the bogue stock structure in the Mediterranean.	Morphometry and Asymmetry
Yedier et al., 2019	Big-scale sand smelt (*Atherina boyeri*) in Lake Eğirdir and İznik, and Hirfanlı Dam Lake in Turkey	Examine the morphometry and otolith contour, and intraspecific differences in sagittal otoliths' patterns.	Morphometry
Yedier & Bostanci, 2020	Four species: Axillary seabream (*Pagellus acarne*), Mediterranean horse mackerel (*Trachurus mediterraneus*),	Describe the morphology of normal and polymorph crystalline otoliths and compare	Morphometry and vaterite

	Sharpsnout seabream (*Diplodus puntazzo*), Whiting (*Merlangius merlangus)* from Aegean Sea, Black Sea and Sea of Marmara	the morphology of aragonite and vaterite otoliths	
Geladakis et al., 2021	Gilthead seabream (*Sparus aurata)* wild and farmed	Examine differences in shape between wild and farmed sea bream	Morphometry
Mahé et al., 2021	Common sole (*Solea solea)*, Bogue (*Boops boops)* in the Mediterranean Sea	Investigate if the spatial variation of directional asymmetry could be used as stock discrimination tool.	Morphometry.
Yedier, 2021	European barracuda (*Sphyraena sphyraena)* in the Mediterranean Sea	Evaluate the otolith shape and estimate fish length and otolith dimension relationships.	Morphometry
De Carvalho Lapuch et al., 2022	Gulf toad fish (*Opsanus beta)* in Paraguaná Estuarine Complex (PEC) in Brazil.	Determine the otolith ontogenic variation between native and PEC populations	Morphometry and asymmetry
Geladakis et al., 2022	Juveniles Gilthead seabream (*Sparus aurata)* in laboratory	Effect of developmental temperature in the otolith shape and asymmetry of the species	Morphometry and asymmetry

Vøllestad & Hindar, 1997	Atlantic salmon juveniles (*Salmo salar*) in Norway	Test the fluctuating asymmetry correlation with heterozygosity and environmental stress within and between groups (three localities, wild and hatchery origin)	Asymmetry
Casselman, 1990	Northern pike (*Esox Lucius),* Lake trout (*Salvelinus namaycush),* Muskellunge (*Esox masquinongy).*	Examine the effect of growth on calcified structures relative size (like otoliths) using tetracycline label to measure distances	Asymmetry
Iguchi et al., 2005	Ayu (*Plecoglossus altivelis)* from Japan	Test the suitability of fluctuating asymmetry as indirect measure of genetic diversity	Asymmetry
Fey & Hare, 2008	Atlantic menhaden (*Brevoortia tyrannus*) larvae	Evaluate the possibility of using FA of sagittal otoliths as condition indicator related to DI	Asymmetry
Fernández et al., 2008	Gilthead seabream (*Sparus aurata)* larvae	Examine the performance and skeletal deformities in fish exposed to different vitamin A diets	Asymmetry

Koumoudouros, 2010	Mediterranean marine finfish aquaculture	Inform of morpho-anatomical abnormalities	Asymmetry
Sadighzadeh et al., 2011	Klunzingeri's mullet (*Lizta klunzingeri)* in the Persian Gulf	Study the fluctuating asymmetry in some otolith parameters (Otolith lenght, Otolith width, Otolith thickness) of this species	Asymmetry
Browning et al., 2012	Juvenile red drum (*Sciaenops ocellatus)*	Study the relation between otolith asymmetry, abnormalities on fish and cortisol response (behaviour differences)	Asymmetry
Jawad et al., 2012	Bengal snapper (*Lutjanus bengalensis*) larvae in the Sea of Oman	Quantify and asses the variability of asymmetry in this species.	Asymmetry
Jawad, 2012	Bengal snapper (*Lutjanus bengalensis*) larvae in the Sea of Oman	Provide information related to the detection of suitable settlement habitat to this species using the otolith length and width asymmetry.	Asymmetry

Jawad et al., 2016	Guinean tilapia (*Coptodon guineensis*) in Lake Ahémé and Porto Novo Lagoon Bénin, West Africa	Reveal the asymmetry level in the otolith lenght and width of the species	Asymmetry
Manizadeh et al., 2018	83 species randomly sampled in the Persian Gulf and Gulf of Oman	Report abnormal otoliths in these species	Asymmetry
Yedier at al., 2018	Mediterranean horse mackerel (*Trachurus mediterraneus*) in the Black Sea	Give information about the otolith asymmetry of this species in this region	Asymmetry.
Yedier & Bostanci, 2019	Blackbellied angler (*Lophius budegassa*) in the Sea of Marmara	Analyze aberrant sagittal otolith morphology	Asymmetry and vaterite
Greszkiewicz & Fey, 2020	Pike (*Esox lucius*) larvae	Determine the water temperature effect on the age and size of cannibal predators	Asymmetry
Jawad et al., 2020	Guinean tilapia (*Coptodon guineensis*) in Lake Ahémé and Porto Novo Lagoon Bénin, West Africa	Examine the asymmetry in the otolith mass and size.	Asymmetry

Jawad & Adams, 2021	Anchovies (*Engraulis australis*), the food of Australasian gannet *(Morus serrator*) in New Zealand	Determine the amount of bilateral dissimilarity in the otoliths' size	Asymmetry
Jawad et al., 2021	Tigertooth croaker (*Otolithes ruber*) of Iraq marine waters	Asymmetry in the otoliths' length and width of this species.	Asymmetry
Fey et al., 2022	Northern pike fry (Esox lucius*)*	Determine if skeletal deformities on fish under laboratory conditions are due to stress and reflected by fluctuating asymmetry	Asymmetry
Yedier, 2022	Greater weever (*Trachinus draco)* in Black Sea	Analyze the abnormal status in the sagitta morphology	Asymmetry
Yedier et al., 2022a	4 flatfish species: Common sole (*Solea solea*), San sole (*Pegusa lascaris*), Four-spot megrim (*Lepidorhombus boscii)*, European flounder (*Platichthys flesus*) in the Aegean Sea, Black Sea and Mediterranean	Determine the morphometry characteristics of normal and abnormal otoliths in these species	Asymmetry

Yedier et al., 2022b	4 Alburnus species: Danube bleak (*Alburnus chalcoides),* Pearl mullet *(A. tarichi),* Sakarya bleak *(A. escherichii), and* Mossul bleak *(A.mossulensis)* in Turkish inland waters	Inform about the otolith asymmetry of the species	Asymmetry
Mugiya, 1972	Alaska pollock (*Theragra chalcogramma),* Sohachi *(Cleisthenes pinetorum),* and Rainbow trout *(Oncorhynchus mykiss)*	Identify and characterize vaterite in teleosts	Vaterite
Gauldie, 1986	Chinook salmon (*Oncorhynchus tshawytscha)*	Examine the vaterite structure specially related to daily increments.	Vaterite
Strong et al., 1986	Pollock (*Pollachius virens)* in the Scotian Shelf, USA	Determine the crystalline composition of normal and aberrant otoliths	Vaterite
David et al., 1994	Juvenile reared red drum *(Sciaenops ocellatus)*	Report the occurrence of anomalous sagitta in hatchery-reared juvenile fish	Vaterite

Bowen et al., 1999	Lake trout (*Salvelinus namaycush)* in the Laurentian Great Lakes, Canada and USA	Identify the vateritic-otoliths, their prevalence in wild and farmed, and their consequences	Vaterite
Tomás & Geffen, 2003	Juvenile herring (*Clupea harengus)* laboratory reared	Study the prevalence of vaterite and its relation with abnormal fish	Morphometry, composition and vaterite
Sweeting et al., 2003	Coho salmon (*Oncorhynchus kisutch)* in the Strait of Georgia	Study the replacement of wild by aquaculture	Morphometry and vaterite
Sweeting et al., 2004	Coho salmon (*Oncorhynchus kisutch)* in the Strait of Georgia	Prevalence of vaterite and comparison between wild and farmed salmon	Vaterite
Tzeng, 2007	European eel (*Anguilla Anguilla)* in the Curonian Lagoon and Baltic Sea	Examines the existence of vaterite in eels' otoliths	Vaterite
Ma et al., 2008	Ayu (*Plecoglossus altivelis*) in the Japan Sea and Western North Pacific	Examine the otolith abnormality and compare the composition of normal and abnormal otoliths	Vaterite, composition and morphometry.
Wells et al., 2012	Yellowfin (*Thunnus albacares*) in the Hawaiian Islands	Use of the otolith isotopes (composition) to determine the nursery origin	Composition

Vinagre et al., 2014	Common sole (*Solea solea) & Senegal sole (*S. senegalensis*)* in Tagus and Douro estuaries	Describe the types and incidence of otolith anomalies in wild and farmed sole juveniles	Vaterite
Reimer et al., 2016	Atlantic salmon (*Salmo salar)* wild and farmed in Norway	Identify the differences between aragonite and vaterite, and study the vaterite prevalence and consequences	Vaterite
Rooker et al., 2016	Bigeye tuna (*Thunnus obesus*) and yellowfin tuna (*Thunnus albacares) in the Pacific Ocean*	Use chemical markers (isotops and trace elements) to examine the origin and movement of young fish (1 to 2+)	Composition
Reimer et al., 2017	Atlantic salmon (*Salmo salar)* laboratory reared	Determine the vaterite causes and its possible control	Vaterite
Kitchens et al., 2018	Yellowfin (*Thunnus albacares*)	Differences nursery areas	Composition
Loeppky et al., 2019	Lake Sturgeon (*Acipenser fulvescens)* laboratory reared	Analyse the early formation of vaterite, search the best quantifying method	Vaterite
Budnik et al., 2020	Rainbow trout /steelhead (*Oncorhynchus mykiss)* in Lake Erie (Canada-USA)	Use aragonite and vaterite otolith composition to assign the fish origin, to determine if vaterite otoliths are useful	Composition

Austad et al., 2021	Atlantic salmon (*Salmo salar)* laboratory reared	Quantify the prevalence of vateritic otoliths of hatchery reared Atlantic salmon smolt and compare with adult salmon of the same cohort returning to the river	Vaterite
Long et al., 2021	Goldeye (*Hiodon alosoides)* in Lake Texoma, USA	Detailed assessment of the otolith morphology and composition of Hiodon, using Goldeye as the representative for the genus	Vaterite and morphometry
Vignon & Aymes, 2020	Brown trout (*Salmo trutta)* laboratory reared	Test if individuals with vateritic otoliths have altered kinematic behavior	Vaterite

VII. Conclusions

The specific conclusions of this Thesis are presented in each of its chapters. Here we summarize those conclusions and present the general conclusions of the whole tesis.

VII.I. Specific of the Chapters

Study	Conclusion	Recommendation
Chapter I	The essential elemental composition in soft tissues in ABFT could be used to discriminate different tuna batches. Kidney and muscle appear to be the best tissue to be used with multivariate analysis tools (PCA and DCA). The elements selected for analysis are the essential, present in high enough concentrations to guarantee good analytical results.	Future research into the elemental composition of tuna diet and different fish origins.
Chapter II	The chemical profile has demonstrated its utility for comparing batches in bones and gills from ABFT juveniles. Of the elemental concentrations, mostly Cu, but also Fe, Mg, Mn and S were found to be the most useful elements The gills were the best tissue for comparing batches.	
Chapter III	Ambient natural tracers within the otoliths like Sr, and some presumably physiologically controlled elements (Mg, Na, P and Rb) could be considered of interest discriminating two batches of juvenile ABFT.	
Chapter IV	The morphometry of the otolith has been proved to be a useful group biomarker. We found important differences between batches, being left	

	otoliths, and their weight and eccentricity of choice for a group differentiation analysis.	
Chapter V	Two types of asymmetry were present in ABFT batches, being the antisymmetry the most common. Within the otoliths, the width and eccentricity were the traits with the best utility to discriminate between batches using their asymmetry. Higher asymmetry values were found in farmed individuals rather than wild.	A deeper study of AS in ABFT populations is highly recommended.
Chapter VI	In this study, vaterite otoliths were identified in ABFT with a higher percentage in farmed than wild individuals. In addition, morphometry differences were found between otoliths with and without vaterite. Abnormal morphologies were found in ABFT otoliths, but were not related with the vaterite deposition.	A future study of malformations in aragonitic-otoliths should be pursued in order to discover their origin.
Chapter VII	OTC was a useful technique for both short- and long-term otolith marking.	Controlled laboratory experiments on ABFT growth after OTC marking should be conducted
Chapter VIII	The ARS proved to be a high efficiency, reliable and not harmful method in ABFT larvae until 22 dph.	We would recommend this chemical marking method to be further developed in future ABFT mass-marking studies.

VI.II. General of the Thesis

1. For the natural chemical tracers, kidney was the best discriminating tissue through its chemical profile among ABFT batches, and even though the muscle and the otolith had lower discrimination, it was also good and both are the most used tissues for fish discrimination in the literature. The best discriminating (Mn, S and Sr) elements are recommended to be included in these tissues analysis, but also other elements with good signal (Cu, Fe, Mg, P and Zn) and present in all the ABFT samples. In the future, adult specimens and wider samplings could be included to follow the progression of the natural chemical traces with time.
2. For the natural morphometrical tracers, the otolith morphometry gave the best and clearer discriminating results, but just by combining the morphometry information is possible to learn also about the fish fitness through asymmetry and vaterite.
3. In resume, the otoliths stand as the best natural tracers, especially for groups with totally different life regimes. They were especially good at highlighting the more homogeneous group (farmed tunas in this Thesis), and could give hints about the fitness differences among groups. Therefore, it is not only a good natural tracer for groups discrimination but also a key tool in animal welfare and production. This is why, if possible, we encourage the use of both otolith morphometry, and chemistry given that both analyses can be done in the same samples.
4. Among the artificial marking methods tested, the use of ARS immersion in eggs was the most successful. It did not require direct handling (the eggs were not manipulated by hand), the otoliths' marks were visible without any processing of the otoliths (just specific UV-light equipment), and no mortality effects were found on the treated eggs.
5. Globally, the otolith would be the tissue of election to discriminate among ABFT batches because it permits the artificial marking in egg or larvae stage, but also the analysis of natural tracers (chemical or morphometrical) in older individuals.