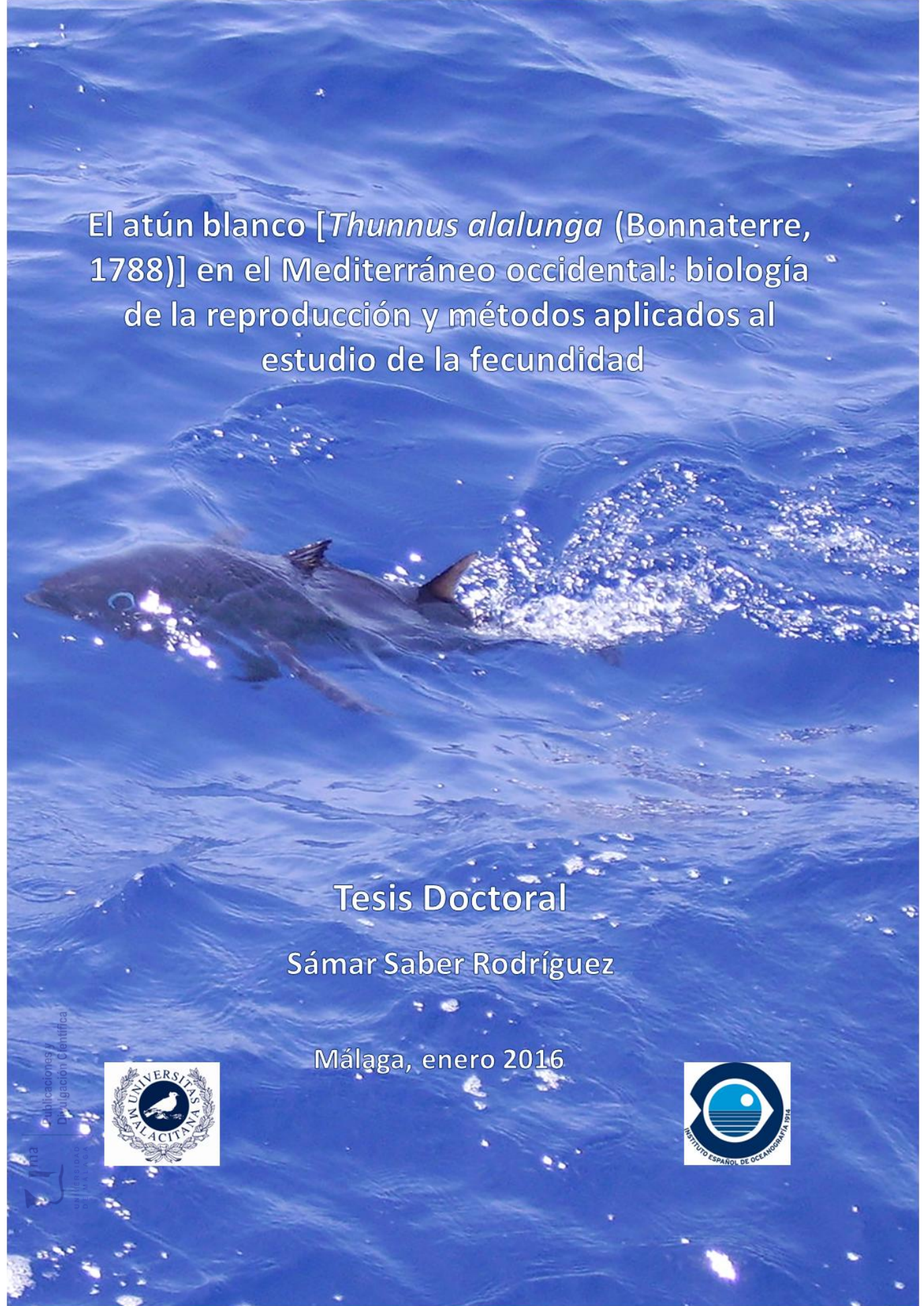

El atún blanco [*Thunnus alalunga* (Bonnaterre, 1788)] en el Mediterráneo occidental: biología de la reproducción y métodos aplicados al estudio de la fecundidad

Tesis Doctoral

Sámar Saber Rodríguez

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ACREDITAN

Que Dña. Samar Saber Rodríguez Licenciada en Biología, ha realizado en el Departamento de Biología Animal de la Facultad de Ciencias de la Universidad de Málaga las investigaciones contenidas en la presente memoria de Tesis Doctoral, titulada "El atún blanco [*Thunnus alalunga* (Bonnaterre, 1788)] en el Mediterráneo occidental: biología de la reproducción y métodos aplicados al estudio de la fecundidad".

Como director y tutora de la misma consideramos que la presente memoria reúne todos los requisitos para ser sometida a juicio de la Comisión correspondiente, por lo que autorizamos su exposición y defensa para la obtención del Grado de Doctora en Biología.

Y para que así conste, en cumplimiento de las disposiciones vigentes, firmamos la presente acreditación en Málaga a 10 de noviembre de 2015.

Profa. Dra. Mª Eugenia Manjón-Cabeza Clouté Dr. Ángel David Macías López

FACULTAD DE CIENCIAS

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Memoria presentada por Dña. Samar Saber Rodríguez para optar al grado de Doctora en Biología por la Universidad de Málaga

La doctoranda:

Fdo.: Samar Saber Rodríguez

El presente trabajo de investigación ha sido desarrollado principalmente en el Centro Oceanográfico de Málaga-Fuengirola del Instituto Español de Oceanografía, gracias a la ayuda Predoctoral de Formación de Personal Investigador del Ministerio de Ciencia e Innovación asociada al proyecto de investigación GPM-4 del Instituto Español de Oceanografía y parcialmente financiado por:

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Parte de la información contenida en esta tesis ha sido publicada en los siguientes artículos científicos:

- Saber, S., Ortiz de Urbina, J., Gómez-Vives, M.J., Macías, D., 2015. Some aspects of the reproductive biology of albacore *Thunnus alalunga* from the western Mediterranean Sea.
  Journal of the Marine Biological Association of the United Kingdom 95 (8), 1705–1715.

- Saber, S., Macías, D., Ortiz de Urbina, J., Kjesbu, O.S., 2015. Stereological comparison of oocyte recruitment and batch fecundity estimates from paraffin and resin sections using spawning albacore (*Thunnus alalunga*) ovaries as a case study.
  Journal of Sea Research 95, 226–238.

- Saber, S., Macías, D., Ortiz de Urbina, J., Kjesbu, O.S., 2016. Contrasting batch fecundity estimates of albacore (*Thunnus alalunga*), an indeterminate spawner, by different laboratory techniques.
  Fisheries Research 176, 76–85.

ÍNDICE / INDEX

Introducción general

Clasificación taxonómica

El atún blanco, también denominado bonito del norte o albacora, *Thunnus alalunga* (Bonnaterre, 1788) (Fig. 1) se clasifica sistemáticamente según Nelson (2006) como sigue:

Filo Chordata
Subfilo Craniata
Superclase Gnathostomata
Clase Actinopterygii
Subclase Neopterygii
División Teleostei
Subdivisión Euteleostei
Superorden Acanthopterygii
Series Percomorpha
Orden Perciformes
Suborden Scombroidei
Familia Scombridae
Subfamila Scombrinae
Tribu Thunnini
Género *Thunnus*
Especie *Thunnus alalunga* (Bonnaterre, 1788)

Quince especies de túnidos componen la tribu Thunnini repartidas en cinco géneros: ocho especies corresponden al género *Thunnus*, una especie al género *Katsuwonus*, tres especies al género *Euthynnus*, dos especies al género *Auxis*, y una especie al género *Allothunnus* (Collette *et al*., 2001).

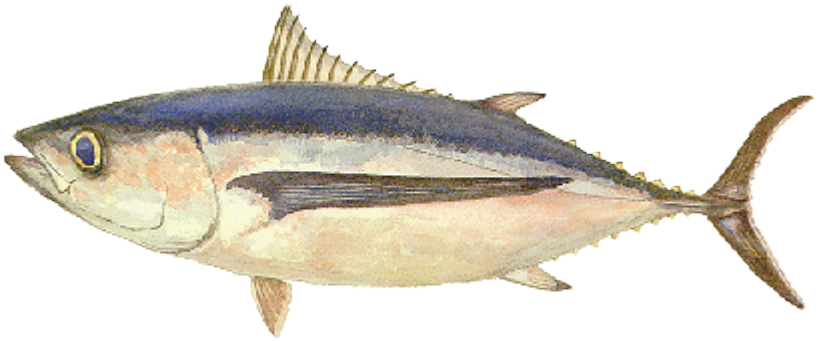

Figura 1. Atún blanco *Thunnus alalunga* (fuente: http://iccat.es/en/ICCATManual.asp?mId=5).

Características descriptivas y distribución geográfica

El atún blanco tiene el cuerpo alargado y fusiforme, cubierto de pequeñas escamas cicloideas. La parte dorsal es de un color azul oscuro metálico, los flancos grisáceos más o menos plateados y la parte ventral blanca plateada. Las aletas pectorales son largas, hasta el 30% o más de la longitud a la horquilla (en inglés *fork length*, FL). En individuos inferiores a 50 cm FL las aletas son proporcionalmente más pequeñas, similares a las del patudo (*Thunnus obesus*), por lo que a estas tallas ambas especies pueden ser confundidas (Collette y Nauen, 1983). Sin embargo, la falta de líneas o puntos distingue al atún blanco de otros túnidos. La aleta caudal es relativamente corta, amplia y terminada en forma de semicírculo muy marcado, con un estrecho borde blanco posterior que también es característico de esta especie. El pedúnculo caudal es más delgado que en otras especies del género. La segunda aleta dorsal está situada claramente por debajo de la primera aleta dorsal y, al igual que la aleta anal, posee radios blandos. Las aletas pélvicas son pequeñas. La primera aleta dorsal es de color amarillo fuerte, la segunda aleta dorsal y la aleta anal son de color amarillo pálido, y las pínulas anales de color oscuro. El atún blanco, al

igual que otras especies de túnidos (Collette *et al*., 2001; Graham y Dickson, 2004), posee un sistema muy perfeccionado de intercambio de calor a contracorriente (*rete mirabile*). Este sistema permite que el calor generado por la actividad muscular se conserve en el cuerpo en lugar de disiparse a través de las branquias, lo cual favorece que estas especies puedan explorar latitudes más altas y mayores profundidades oceánicas. La talla máxima fue establecida por Collette y Nauen (1983) en 127 cm, y se ha calculado una longevidad teórica de 15 años (Le Gall, 1974; Wells *et al*., 2013).

El atún blanco es una especie oceánica epipelágica y mesopelágica ampliamente distribuida en aguas tropicales, subtropicales y templadas de todos los océanos y en el mar Mediterráneo. Su distribución geográfica se extiende desde los 50-55°N a los 40-45°S aproximadamente, siendo menos abundante en latitudes comprendidas entre 10°N y10°S (Collette y Nauen, 1983). Su rango óptimo de temperatura es de 13.5° a 25.2°C, aunque puede tolerar aguas más frías, por debajo de 9.5°C, durante periodos cortos de tiempo. A nivel mundial se reconocen seis *stocks* de atún blanco: el *stock* del Pacífico norte, Pacífico sur, Atlántico norte, Atlántico sur, Índico y el del Mediterráneo.

Mar Mediterráneo, pesca y situación del *stock* Mediterráneo de atún blanco

El Mediterráneo es un mar semicerrado y relativamente pequeño. Su única conexión natural con otros océanos es a través del estrecho de Gibraltar, que lo comunica con el Atlántico norte. Su superficie total es de 2.51 millones de km^2 y su volumen de 3.7 x 10^6 km^3, lo que supone un 0.8% de la superficie y un 0.3% del volumen total de todos los océanos y mares de la Tierra. Se extiende desde los 6°W a los 36°E de longitud y desde los 30°N a los 45°N de

latitud, es decir, posee una longitud de oeste a este de 4000 km y de 1600 km de norte a sur (Fig. 2).

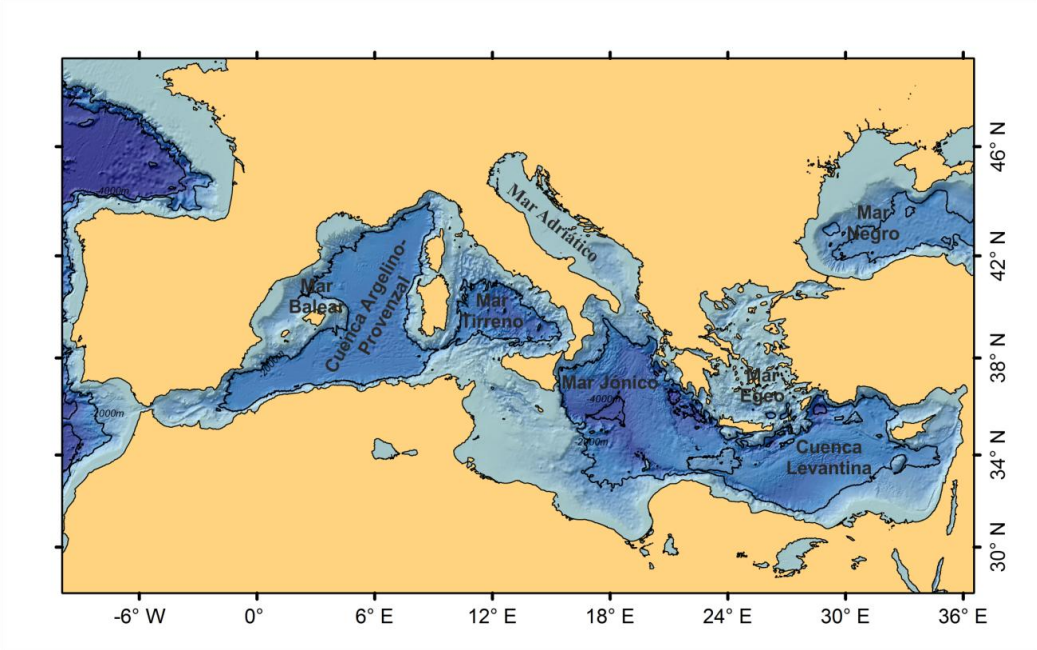

Figura 2. Mar Mediterráneo (imagen batimétrica generada a partir de la base de datos ETOPO).

El atún blanco está ampliamente distribuido en el Mediterráneo y es una especie objetivo de la pesca comercial y recreativa. La pesquería del atún blanco a lo largo del Mediterráneo es una actividad tradicional, pero la información sobre sus capturas es escasa y probablemente infradeclarada. El arte más utilizado en la pesquería del atún blanco en el Mediterráneo es el palangre de superficie (Fig 3). Otros artes de pesca utilizados son las redes de enmalle de deriva, el cerco, el currican y el cebo vivo. Las capturas de atún blanco con redes de enmalle de deriva en los últimos años son efectuadas principalmente por Turquía, ya que a partir del 1 de enero de 2008 el uso de este arte quedó prohibido para los países miembros de la Comunidad Europea (reglamentos (CE) nº 812/2004 del Consejo, de 26 de abril de 2004 y nº 809/2007 del Consejo, de 28 de junio de 2007) y desde 2012 para Marruecos. La captura

media declarada entre los años 2000 y 2014 está en torno a las 4 300 toneladas, sin embargo, las capturas en los dos últimos años han descendido respecto a las de principios de la década del 2000 (Fig. 4). De las capturas comunicadas en dicho periodo (2000–2014), el 67.3% corresponden a Italia, el 14.4% a Grecia, el 6.0% a Chipre, a España al igual que a Turquía corresponden el 5.8% y el resto a otros países ribereños (http://iccat.org/en/accesingdb.htm).

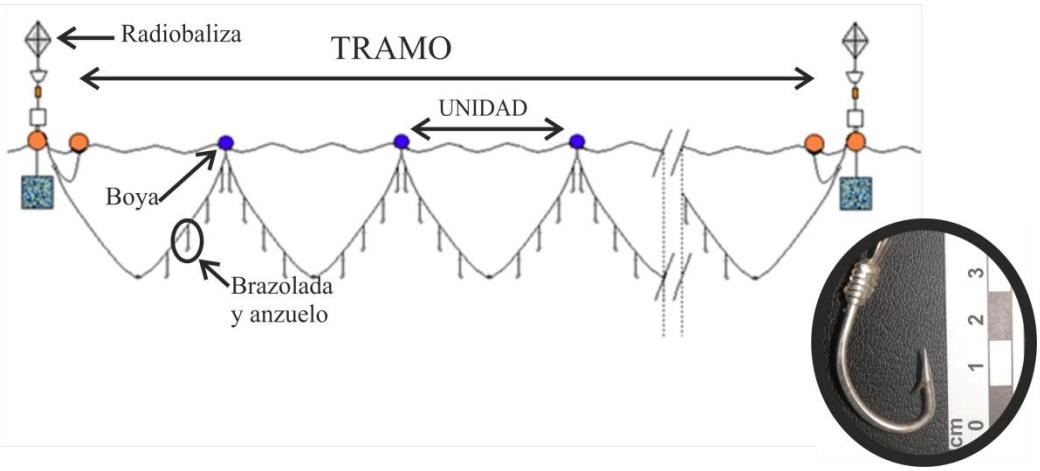

Figura 3. Esquema del palangre de superficie y fotografía de anzuelo tipo "J" empleado en el palangre de atún blanco.

La Comisión Internacional para la Conservación del Atún Atlántico (en inglés, *International Commission for the Conservation of Atlantic Tunas*, ICCAT) considera a efectos de ordenación tres *stocks* de atún blanco: Atlántico norte, Atlántico sur (separado del anterior por el paralelo 5° N) y Mediterráneo. La ICCAT, basándose en la existencia de zonas de puesta independientes, datos de marcado, y diferencias en la morfometría, en las tasas de crecimiento y en la edad de primera madurez, considera que los *stocks* Mediterráneo y Atlántico norte son independientes (ICCAT, 2010). El *stock* Mediterráneo de atún blanco fue evaluado por primera vez por la ICCAT en 2011 usando los datos recabados hasta 2010. Debido a la falta de datos, tanto pesqueros (de capturas y esfuerzo)

como biológicos, se constató que es un *stock* pobre en datos, por lo que los análisis realizados se tuvieron que adaptar a esta escasez de datos y no se pudieron llevar a cabo proyecciones del estado futuro del *stock*. Los resultados de la evaluación basada en la limitada información disponible y en análisis simples indicaron un patrón relativamente estable para la biomasa del atún blanco del Mediterráneo en el pasado reciente. Los niveles actuales de mortalidad por pesca parecen haberse reducido respecto a los de principios de los 2000, que probablemente superaban la mortalidad por pesca asociada al rendimiento máximo sostenible, y en la actualidad podrían estar a este nivel o por debajo (ICCAT, en prensa). Para poder hacer frente a esta considerable incertidumbre sobre el estado del *stock*, la ICCAT recomienda establecer medidas de ordenación destinadas a limitar aumentos en la captura y en el esfuerzo pesquero, así como recopilar datos de pesca históricos y/o recientes y llevar a cabo estudios que incrementen el conocimiento sobre los parámetros básicos del ciclo de vida y de la ecología del atún blanco en el Mediterráneo (Anon., 2012; ICCAT, en prensa).

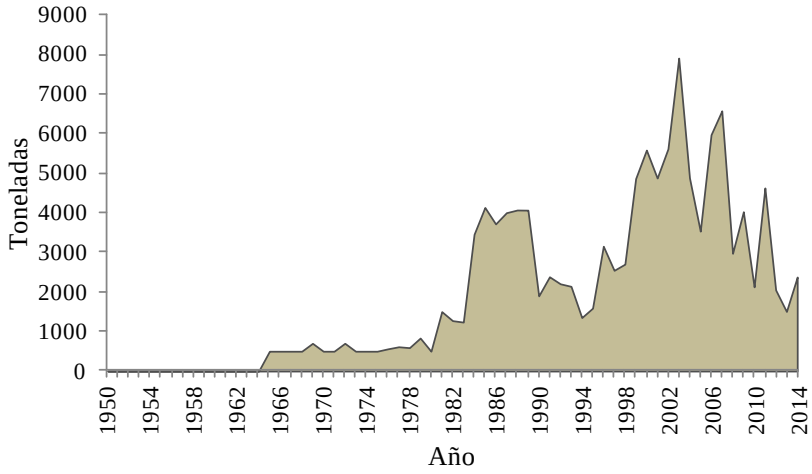

Figura 4. Capturas totales de atún blanco del *stock* del Mediterráneo comunicadas a ICCAT (Tarea I, disponible en: http://iccat.org/en/accesingdb.htm; actualizado el 2 de noviembre de 2015).

Biología de la reproducción del atún blanco. Antecedentes

El atún blanco, como el resto de túnidos, no muestra dimorfismo sexual aparente en el esquema de color o en los caracteres morfológicos externos. Al igual que las otras especies de túnidos, con la excepción *Allothunnus fallai*, se reproduce en aguas cálidas cuando la temperatura superficial supera los 24°C (Schaefer, 2001; Graham y Dickson, 2004). Son reproductores múltiples parciales en los que la reproducción se extiende durante un largo periodo y los huevos se liberan por tandas o lotes y el desarrollo de los ovarios es asincrónico por encontrarse simultáneamente ovocitos en diferentes fases de desarrollo (Wallace y Selman, 1981). La estrategia reproductiva es indeterminada (Schaefer, 2001), ya que la producción de ovocitos es continua durante la época de reproducción y el número de huevos que pondrán en la temporada no está determinado al principio de la puesta (Hunter *et al*., 1992). Para estimar la fecundidad potencial anual de las especies cuya estrategia reproductiva es indeterminada se requiere de las estimas de la fecundidad por tanda (en inglés, *batch fecundity*), de la fracción de hembras maduras en puesta por día (en inglés, *spawning fraction*) y de la duración del periodo de puesta (en inglés, *spawning period*) (Hunter *et al*., 1985; Murua *et al*., 2003).

Las áreas de puesta del atún blanco en el Mediterráneo se han descrito a partir de estudios de distribución larvaria. Se distinguen tres grandes áreas de puesta: la cuenca Levantina (Mediterráneo oriental) (Piccinetti *et al*., 1996); los mares Jónico, Adriático y Tirreno (Potoschi *et al*., 1994; Piccinetti *et al*., 1996; De Ruggieri *et al*., 1997) y el mar Balear (Dicenta *et al*., 1975; García *et al*., 2005; Alemany *et al*., 2010). La edad máxima estimada para individuos capturados en el Mediterráneo oriental es de 9 años (Karakulak *et al*., 2011) y para los capturados en el Mediterráneo occidental de 11 años (Quelle *et al*.,

2011). De los escasos estudios sobre la reproducción del atún blanco en el Mediterráneo el más relevante es el publicado por Arena *et al*. (1980), en el que se determinó que la edad a la que el 50% de la población alcanza la madurez sexual es de 2 años (talla media de 66.3 cm FL). Este trabajo se llevó a cabo con ejemplares capturados en el mar Tirreno y los autores determinaron la madurez de las hembras mediante el uso de técnicas histológicas. Megalofonou (1990), basándose en el examen de las gónadas en fresco, encontró que los atunes blancos capturados en el mar Egeo durante el otoño son inmaduros o están en postpuesta; mientras que Akayli *et al*. (2013), basándose en el examen microscópico de gónadas masculinas, determinaron que el atún blanco en el Mediterráneo oriental se reproduce principalmente entre mayo y julio. En este último estudio se observó que la talla mínima a la que los machos alcanzan la madurez sexual es de 63 cm LF. Sin embargo, no se encuentran trabajos relacionados con el estudio de la biología reproductiva del atún blanco en el área de puesta del mar Balear.

El conocimiento preciso de parámetros reproductivos tales como la talla de primera madurez, la fecundidad, y la duración y frecuencia de puesta son de gran importancia para comprender la dinámica de las poblaciones, evaluar su estatus de conservación y abordar una gestión pesquera racional. A diferencia del *stock* del Mediterráneo, la biología de la reproducción del atún blanco de las poblaciones del Pacífico (norte y sur) está bien documentada. La talla a la que el 50% de la población alcanza la madurez sexual (talla de primera madurez) ha sido estimada recientemente para los atunes blancos del Pacífico sur en 87 cm FL (a la edad de 4.5 años) (Farley *et al*., 2014). La edad máxima para los individuos de los dos *stocks* del Pacífico está estimada en 14 – 15 años (Chen *et al*., 2012; Williams *et al*., 2012; Wells *et al*., 2013). La duración de la época de puesta es de seis a siete meses y el intervalo de puesta de aproximadamente 1.3

– 1.7 días (Chen *et al*., 2010; Farley *et al*., 2013). La fecundidad por tandas, es decir, el número total de huevos liberados en un evento de puesta, ha sido estimada para los *stocks* del Pacífico norte y sur, Atlántico sur e Índico y está comprendida entre los 0.17 y los 2.83 millones de ovocitos (Ueyanagi, 1957; Otsu y Uchida, 1959; Wu y Kuo 1993; Chen *et al*., 2010; Anon., 2012; Farley *et al*., 2013).

Histología

En especies cuya estrategia reproductiva es indeterminada es necesario realizar un examen histológico de los ovarios antes de acometer las estimas de fecundidad por tanda (Murua *et al*., 2003), ya que solo las hembras en puesta cuyos ovarios contienen ovocitos en una de las dos últimas fases de desarrollo (ovocitos con núcleos migratorios u ovocitos hidratados) y sin folículos postovulatorios recientes son adecuadas para realizar dichas estimas (Hunter *et al*., 1985; Hunter *et al*., 1992; Schaefer, 1996).

El examen macroscópico (inspección visual) de las gónadas y el uso de índices gonadosomáticos son métodos rápidos y poco costosos para clasificar la madurez y el estado reproductivo de un gran número de individuos de una especie, pero si los usamos con la finalidad de estimar la talla o edad de primera madurez estos métodos pueden resultar inadecuados y producir sesgos importantes en la estimación del potencial reproductor de un *stock* (De Vlaming *et al*., 1982; Vitale *et al*., 2006). En cambio, aunque la histología es una técnica costosa, el examen microscópico de las gónadas ofrece una visión precisa de la estructura del ovario y de sus componentes, proporcionando una información inequívoca sobre el estado reproductivo en el que se encuentra el ejemplar

observado y, por tanto, se considera el método más preciso para este fin (Hunter y Macewicz, 1985; West, 1990). Además, los criterios histológicos son especialmente importantes cuando los individuos son capturados después del periodo de puesta, ya que mediante el uso de criterios macroscópicos o índices gonadosomáticos las hembras maduras e inactivas (gónadas en fase de pospuesta y reposo) podrían clasificarse como hembras inmaduras (Schaefer, 2001). Por otro lado, es sabido que la histología lleva asociado un cierto grado de encogimiento en los tejidos dependiendo del fijador y medio de inclusión usados. Por tanto, los datos de estimas de fecundidad o de distribución de talla de los ovocitos obtenidos para una misma especie en estudios que hubieran usado diferentes protocolos y en los que el encogimiento no haya sido estimado, no deberían ser contrastados o analizados en conjunto.

Métodos para la estima de la fecundidad por tandas

Las estimas de fecundidad proporcionan una información fundamental de la dinámica de las poblaciones y del potencial reproductor de un *stock* (Hunter *et al*., 1992). Para las especies con estrategia reproductiva indeterminada como los túnidos, la fecundidad por tandas es la única medida de fecundidad útil para poder estimar la fecundidad potencial anual (Hunter *et al*., 1985). Hoy en día existe una gran variedad de métodos que pueden ser aplicados para estimar la fecundidad. Algunos de ellos requieren el uso de histología y otros no. Entre estos últimos se encuentran métodos tradicionales como el del *Hydrated Oocyte* desarrollado por Hunter *et al*. (1985), que se basa en el conteo manual de ovocitos de una submuestra de peso conocido bajo una lupa, y métodos más actuales que consisten en automatizar el conteo de los ovocitos mediante el uso de *software* específico. La aplicación de métodos que

cuantifican el número de ovocitos a partir de cortes histológicos es más lenta y laboriosa, pero tiene la ventaja de poder evaluar y cuantificar la presencia de ovocitos atrésicos (ovocitos que entran en un proceso degenerativo y de reabsorción) o la de folículos postovulatorios (capa folicular externa que envuelve a los ovocitos y que permanece en el ovario durante un tiempo determinado después de la ovulación). Entre estos métodos están los métodos estereológicos de Weibel y Gomez (1962), el Oocyte *Packing Density* (OPD) (Kurita y Kjesbu, 2009) y el método libre de asunciones *o "3D"* denominado *Physical disector* (Sterio, 1984). Este último método en combinación con el del *fractionator* (Gundersen, 1986) eliminaría los efectos del encogimiento que sufre un tejido al ser fijado y procesado histológicamente pero en tejidos relativamente grandes, como las gónadas de los túnidos, solo se aplica de manera experimental.

Fundamentación y objetivos de la tesis

La gestión responsable de un recurso vivo implica la sostenibilidad de su explotación. La capacidad de respuesta de una determinada especie a la explotación depende de su productividad, resistencia o plasticidad y de su vulnerabilidad. De una manera simple se podría decir que el riesgo ecológico de una especie sometida a la explotación pesquera depende del balance entre su capacidad productiva (reproducción y crecimiento) y su vulnerabilidad (en nuestro caso capturas / valor económico). Las especies con altas tasas de crecimiento y reproducción, y con bajo valor económico presentarían riesgo más bajo. En cambio, aquellas especies con tasas muy bajas de crecimiento y reproducción y que sean objetivos específicos de las pesquerías por su alto valor económico, presentarían un alto riesgo y soportarían menores niveles de

explotación. Para estimar el efecto de la explotación sobre un recurso, los investigadores pesqueros hacen uso de modelos matemáticos que simulan la respuesta del *stock* a diferentes niveles y/o sistemas de explotación. Sin embargo, estos modelos dependen de la calidad de los datos de entrada, tanto biológicos (realidad biológica de los datos de producción del *stock*) como pesqueros (niveles de explotación). Por tanto, el desconocimiento de la biología de un *stock* pone en peligro la sostenibilidad de su explotación. En este sentido la explotación del atún blanco del Mediterráneo tendría un futuro incierto al ser un *stock* pobre en datos.

El objetivo general de esta tesis es doble: por un lado profundizar en el conocimiento de la biología de la reproducción del atún blanco, *Thunnus alalunga*, en el Mediterráneo occidental; y por otra parte tiene un objetivo metodológico cuyo fin es contrastar y valorar las estimas de la fecundidad por tanda procedentes del uso de los dos protocolos de inclusión más comunes (parafina y resina), y de la aplicación de diversos métodos de estima de fecundidad usando como caso de estudio el atún blanco.

Para ello se consideran los siguientes objetivos específicos:

1. Estimar diversas variables reproductivas útiles en modelos de evaluación: sex ratio, composición por tallas de la fracción reproductora del *stock*, talla mínima de madurez sexual y frecuencia de puesta.
2. Determinar la época y duración de la puesta.
3. Examinar la dinámica de reclutamiento de los ovocitos en ovarios en puesta.
4. Contrastar y valorar el efecto de encogimiento del tejido ovárico consecuencia del uso de dos protocolos de inclusión y su repercusión en las

estimas del número de ovocitos por gramo de ovario y en la fecundidad relativa por tandas.

5. Contrastar y valorar la aplicabilidad de diferentes métodos usados para la estima de la fecundidad por tanda.
6. Estimar la fecundidad por tanda, otra variable reproductiva útil en modelos de evaluación, y explorar las relaciones con variables biométricas (talla, peso corporal y peso gonadal).

Estructura de la tesis

Los diferentes objetivos y resultados de la presente tesis doctoral se han agrupado en tres capítulos (organizados en forma de artículos científicos):

En el primer capítulo titulado *"*Some aspects of the reproductive biology of albacore *Thunnus alalunga* from the western Mediterranean Sea*"*, se abordan aspectos relacionados con la biología reproductiva de la especie (objetivos específicos 1 y 2). En el segundo capítulo, titulado *"*Stereological comparison of oocyte recruitment and batch fecundity estimates from paraffin and resin sections using spawning albacore (*Thunnus alalunga*) ovaries as a case study*"*, se abordan dos objetivos metodológicos: por una parte el estudio de la dinámica de reclutamiento de los ovocitos en el ovario en puesta aplicando un método desarrollado para tal fin, el *Oocyte Packing Density* (objetivo 3) y, por otra, la comparación del efecto del encogimiento del tejido ovárico consecuencia de dos protocolos de inclusión en las estimas del número de los distintos tipos de ovocitos por gramo de ovario y en las estimas de la fecundidad (objetivo 4). En el tercer capítulo, titulado "Contrasting batch fecundity estimates of albacore (*Thunnus alalunga*), an indeterminate spawner, by different laboratory

techniques", se estima la fecundidad por tanta usando diferentes métodos y se contrastan los resultados además de valorar los pros y contras del uso de cada uno de ellos (objetivo 5). A partir de esta valoración uno de los métodos es seleccionado para estimar la fecundidad por tanda en un mayor número de hembras y se exploran las relaciones entre fecundidad y variables biométricas (talla, peso corporal y peso gonadal) (objetivo 6).

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Chapter 1.

Some aspects of the reproductive biology of albacore *Thunnus alalunga* from the Western Mediterranean Sea

Sámar Saber, Josetxu Ortiz de Urbina, María José Gómez-Vives, David Macías, 2015. Journal of Marine Biological Association of the United Kingdom 95 (8), 1705−1715
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ABSTRACT

Thunnus alalunga is an important commercial tuna species widely distributed in the three major oceans and the Mediterranean Sea. The Mediterranean population is currently classified as a data-poor stock and little is known about its basic life history parameters. This study provides the first detailed information on some aspects of the reproductive biology of *T. alalunga* from the western Mediterranean Sea. A total of 16104 specimens were measured between 2005 and 2012. The overall sex ratio of females to males was 1.1:1, although the ratio was female biased in fish <70 cm fork length (L_F) and male biased in those >75 cm L_F. Histological analysis of the ovaries (n = 587) and the monthly variation of the gonadosomatic index for both sexes showed that spawning occurred from June to August, which is a much shorter period than the seven months reported for *T. alalunga* in tropical oceanic waters. *Thunnus alalunga* caught during June and July are capable of spawning daily. The gonadosomatic index values for *T. alalunga* from the western Mediterranean were up to eight times higher than those of *T. alalunga* from other oceans. Histological examination of the ovaries showed that the minimum length at sexual maturity of females was 56 cm L_F, which is considerably smaller than those estimated for other stocks.

Keywords

Size distribution; Sex ratio; Gonadosomatic index; Histology; Sexual maturity; Spawning season

Chapter 2.

Stereological comparison of oocyte recruitment and batch fecundity estimates from paraffin and resin sections using spawning albacore (*Thunnus alalunga*) ovaries as a case study

Sámar Saber, David Macías Josetxu Ortiz de Urbina, Olav Sigurd Kjesbu, 2015.
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ABSTRACT

Traditional histological protocols in marine fish reproductive laboratories using paraffin as the embedding medium are now increasingly being replaced with protocols using resin instead. These procedures entail different degrees of tissue shrinkage complicating direct comparisons of measurement results across laboratories or articles. In this work we selected ovaries of spawning Mediterranean albacore (*Thunnus alalunga*) as the subject of our study to address the issue of structural changes, by contrasting values on oocyte recruitment and final batch fecundity given from the same tissue samples in both paraffin and resin. A modern stereological method, the Oocyte Packing Density (OPD) theory, was used supported by initial studies on ovarian tissue sampling and measurement design. Examples of differences in the volume fraction of oocyte stages, free space and connective tissue were found between the embedding media. Mean oocyte diameters were smaller in paraffin than in resin with differences ranging between 0.5 % in primary growth and 24.3 % in hydration (HYD) stage oocytes. Fresh oocyte measurements showed that oocytes shrank as a consequence of the embedding process, reaching the maximal degree of shrinkage for oocytes in the HYD stage (45.8 % in paraffin and 26.5% in resin). In order to assess the effect of oocyte shrinkage on the OPD result, and thereby on relative batch fecundity (F_r), oocyte diameters corrected and uncorrected for shrinkage, were used for estimations. Statistical significant differences were found ($P<0.05$) between these two approaches in both embedding media. The average F_r was numerically smaller in paraffin compared to resin (86 ± 61 vs. 106 ± 54 oocytes per gram of body mass (mean ± SD)). For both embedding media statistical significant differences ($P<0.05$) were seen

between F_r results based on either oocytes in the germinal vesicle migration stage or HYD stage. As a valuable adjunct, the present use of the OPD theory made it possible to document that the oocyte recruitment of spawning ovaries of Mediterranean albacore followed the typical pattern of an asynchronous oocyte development and indeterminate fecundity.

Keywords

Shrinkage; Paraffin; Resin; Oocyte Packing Density; Fecundity; Albacore

Chapter 3.

Contrasting batch fecundity estimates of albacore (*Thunnus alalunga*), an indeterminate spawner, by different laboratory techniques

Sámar Saber, David Macías Josetxu Ortiz de Urbina, Olav Sigurd Kjesbu, 2016.
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ABSTRACT

A range of methods can be applied to estimate the batch fecundity of species with an indeterminate reproductive strategy. The traditional Hydrated Oocyte (HO) method based on direct counts of hydrated oocytes is the easiest and most accurate method but the main problem with this method is the shortage of hydrated ovaries in sampled fish such as tuna species. Batch fecundity estimates of albacore *Thunnus alalunga* resulting from counts of migratory nucleus (MG) oocytes using the application of the Weibel and Gomez (W&G), Physical Disector (PD), Oocyte Packing Density (OPD), and HO methods were compared using the last method as "control". Postovulatory follicles (POFs) were also counted using the PD method. Correction factors due to shrinkage were considered in the application of the different methods. Our results showed the highest batch fecundity estimates were obtained with the design-based PD method. The outputs from the assumption-based W&G and the theoretical OPD methods were closest to the HO method. Annotations of POFs instead of MG oocytes gave markedly lower values. The new OPD method was used to estimate batch and relative fecundity on a larger sample of fish (selected according to their length). The relationships between batch and relative fecundity estimates of albacore and the associated biological metrics (length, body weight and ovary weight) were investigated. Batch fecundity estimates ranged from 0.42 to 2.16 million oocytes with a mean relative batch fecundity of 136 oocytes per gram of body weight. The batch fecundity was shown to increase with fish size (length and weight) and gonad weight, while relative batch fecundity (g^{-1}) was related only to gonad weight.

Keywords

Assumption-based stereology; Physical Disector; Oocyte Packing Density; Indeterminate species; Fecundity

General discussion

An understanding of the reproductive biology of any species is important for the purpose of stock assessment. The Mediterranean albacore stock was first assessed in 2011. Taking into account the incomplete fishing statistics, and the lack of knowledge on the life cycle and biological population parameters, the stock was classified as data-poor. Information on Mediterranean stock maturity was provided in a study conducted with samples obtained from 1978 to 1980 in the Tyrrhenian Sea; it was found that 50% of 2-year-old albacore with a mean fork length (FL) of 66.3 cm were sexually mature (Arena *et al*., 1980). Since then, few studies have addressed the reproductive biology of albacore (Marano *et al*., 1999; Akayli *et al*., 2013), and thus the reproductive biology of the Mediterranean population of albacore remains poorly understood (Juan-Jordá *et al*., 2013). Studies on the maturity and fecundity of this species were recommended by the *International Commission for the Conservation of Atlantic Tunas* (ICCAT) (Anon., 2011). Such studies could be of value for future stock assessment. In fact, a stock assessment project has been planned for 2017. The present PhD thesis not only attempts to improve knowledge of the reproductive biology of albacore *Thunnus alalunga* (Chapters 1, 2, and 3) — a species for which there are serious gaps in information on its life history traits in the Mediterranean Sea — but also addresses methodological issues related to fecundity estimates that are of interest to fish reproductive researchers (Chapters 2 and 3).

The aim of management strategies should be to achieve the long-term sustainable exploitation of marine resources. Currently, there are no ICCAT regulations aimed at managing this stock (http://iccat.org/en/RecsRegs.asp). Accurate life history information (maximum size, growth, longevity, maturity, sex ratio, fecundity, spawning duration, and spawning interval) is needed to

develop realistic models to assess fish stocks; these models can then be used as the basis for the sustainable exploitation, management, and conservation of fish species. As mentioned, Mediterranean albacore is considered a data-poor stock, and so suitable methods have been used to assess its status (Anon., 2012). The present PhD thesis presents data on the reproductive biology of albacore from the western Mediterranean Sea, which is an important spawning area for many species, including tuna species (Atlantic bluefin tuna *Thunnus thynnus*, little tunny *Euthynnus alletteratus*, Atlantic bonito *Sarda sarda*, bullet tuna *Auxis rochei*, skipjack tuna *Katsuwonus pelamis*, and albacore) (Alemany *et al*., 2010; Reglero *et al*., 2012).

Life history traits can vary between populations of the same species (Morgan 2008), as has been shown for albacore (Chapters 1 and 3). The maximum sizes of albacore in the Mediterranean Sea range between 92 and 111 cm FL (Megalofonou, 2000; Karakulak *et al*., 2011; Di Natale *et al*., 2011; Chapter 1), whereas larger maximum sizes (from 110 to 130 cm FL) are frequently found in the Pacific, Indian, and Atlantic ocean albacore populations (Otsu and Hansen, 1962; Wu and Kuo, 1993; Ramon and Bailey, 1996; Zhu *et al*., 2008; Chen *et al*., 2010; Farley *et al*., 2013). We found that the overall estimated sex ratio for albacore during the spawning season in the western Mediterranean was 1.1♀:1♂. However, the analysis of sex ratio by length indicated that the sex ratio was close to 1:1 for length classes between 71 and 74 cm FL; females predominated in the smaller length classes and males in the larger length classes. Similarly, in the eastern Mediterranean, 75 cm FL was the length at which males became predominant (Karakulak *et al*., 2011). A preponderance of albacore males in larger length classes has also been found in the Pacific Ocean, although the length at which this occurs is greater than 95 cm FL (Chen *et al*., 2010; Farley *et al*., 2013). The same phenomenon of male

preponderance in larger length classes has also been found in other tuna species, such as yellowfin tuna *Thunnus albacares*, blackfin tuna *Thunnus atlanticus*, Atlantic bluefin tuna, bigeye tuna *Thunnus obesus* (Schaefer, 1998; Zhu *et al*., 2010; Aranda *et al*., 2013a; Bezerra *et al*., 2013). According to Schaefer (2001) and Schaefer *et al*. (2005), the almost complete absence of females in tuna species within larger size classes seems to be related to differences in natural mortality and vulnerability to capture rather than to differential growth. However, it has been found that male albacore grow faster and reach greater sizes at age than females (Megalofonou 2000; Karakulak *et al*., 2011; Chen *et al*., 2012a; Williams *et al*., 2012), suggesting that once the size at maturity is reached, reproductive investment rather than somatic growth is higher in females than in males (Chen *et al*., 2012a; Williams *et al*., 2012; Farley *et al*., 2013).

Another reproductive trait that differs between Mediterranean and oceanic albacore populations is length/age at first maturity (L_{50} / A_{50}). In Mediterranean albacore, the A_{50} has been estimated as 2 years with a mean FL of 66.3 cm (Arena *et al.*, 1980); in contrast, in South Pacific albacore, the L_{50} has been estimated as 87 cm FL (Farley *et al*., 2014). In the present study, only five immature individuals were found and so it was not possible to estimate the L_{50} by fitting the proportion of mature fish by age-length class to a logistic equation (the most common method). However, we found that the minimum size at maturity for females was 56 cm FL, which is similar to that observed in the central (62 cm FL) and eastern (63 cm FL for males) Mediterranean Sea (Arena *et al.*, 1980; Akayli *et al*., 2013). Nevertheless, a larger minimum size at maturity (between 71 and 96 cm FL) has been found in albacore in the North Pacific, South Pacific, and Indian oceans (Otsu and Uchida, 1959; Otsu and

Hansen, 1962; Kikawa and Ferraro, 1967; Ratty *et al*., 1990; Wu and Kuo, 1993; Ramon and Bailey, 1996; Chen *et al*., 2010; Farley *et al*., 2013).

It is known that tuna species have an indeterminate reproductive strategy (Schaefer, 2001). Indeterminate fecundity refers to species whose annual potential fecundity is not fixed prior to the start of the spawning season (Hunter *et al*., 1992). In order to estimate the potential annual fecundity of these species, three measurements are required: batch fecundity (number of eggs released per spawning event), spawning fraction (fraction of females spawning per day), and the length of the spawning season (Hunter *et al*., 1985; Murua and Saborido-Rey, 2003).

Our results of the histological examination of ovaries and the gonadosomatic index values indicated that the spawning season for albacore in the western Mediterranean Sea is from June to August. Similar results were found for albacore adults in the eastern Mediterranean Sea (Akayli *et al*., 2013). Both sets of results were consistent with larval surveys (Piccinetti *et al*., 1996; Alemany *et al*., 2010). In contrast, the albacore spawning season in oceanic tropical waters is between 6 and 7 months (Chen *et al*., 2010; Anon., 2012; Farley *et al*., 2013). This difference is unsurprising given that tuna spawn in water temperatures of about 24°C and higher (Schaefer, 2001) and that these temperatures are only found in the western Mediterranean Sea during the summer months (Vargas-Yáñez *et al*., 2010). This disparity between the spawning season in the Mediterranean and that in the oceans has also been found in other migratory species such as little tunny and swordfish *Xiphias gladius* (Collette and Nauen, 1983; Arocha and Lee, 1996; De la Serna *et al*., 1996; Arocha, 2007; Hajjej *et al*., 2010). Unfortunately, the individual duration of spawning is difficult to measure (Murua *et al*., 2003) and there is no simple

way of estimating this reproductive trait in the field in the absence of new technologies, such as electronic tagging, which not only provide valuable data on the individual spawning period but also on reproductive behaviour, spawning habitat preferences, and spatial-temporal patterns (Aranda *et al*., 2013b; Cosgrove *et al*., 2014).

The estimation of the spawning frequency of female albacore from the western Mediterranean Sea was calculated using the postovulatory follicle method developed by Hunter and Macewicz (1985). This method is commonly used in tuna studies. We assumed that the postovulatory follicles (POFs) of albacore ovaries are resorbed at the same rate as other tunas (e.g., skipjack tuna, yellowfin tuna, and South Pacific albacore) spawning in water temperatures above 24ºC (Hunter *et al*., 1986; Schaefer, 1996; Farley *et al*., 2013), given that water temperatures appear to have a significant effect on POF resorption rates (Fitzhugh and Hettler, 1995; Ganias *et al*., 2007; Kurita *et al*., 2011). The estimated spawning fraction of females classified as reproductively active was 0.99, resulting in a mean spawning interval of 1.01 days (i.e., females would be capable of spawning almost daily). Similar results have been reported for other tuna species (active females alone were considered): skipjack tuna, bigeye tuna, yellowfin tuna, Atlantic bluefin tuna, Southern bluefin tuna *Thunnus maccoyii*, and Pacific bluefin tuna *Thunnus orientalis* (Hunter *et al*., 1986; Nikaido *et al*., 1991; Schaefer, 1996; Farley and Davis, 1998; Medina *et al*., 2002; Aranda *et al*., 2013a, 2013b; Farley *et al*., 2015; Okochi *et al*., 2016). However, the spawning interval estimated during the peak spawning activity of North and South Pacific *T. alalunga* was somewhat longer (1.7 days and 1.3 days, respectively) (Chen *et al.*, 2010; Farley *et al.*, 2013).

The gonadosomatic index (Chapter 1) was estimated using two

alternative formulations in order to compare our results with those reported in other albacore studies. The gonadosomatic index values for female albacore from the western Mediterranean were between two and four times higher than those estimated for oceanic albacore (Wu and Kuo, 1993; Ramon and Bailey, 1996; Chen *et al*., 2010; Farley *et al.*, 2013). The estimated gonadosomatic indexes for male albacore from the western and eastern Mediterranean were similar (Akayli *et al*., 2013, Chapter 1), whereas the highest gonadosomatic index was eight times higher than that reported by Wu and Kuo (1993) for albacore in the Indian Ocean. These results reflect that the Mediterranean albacore mature at smaller size than the oceanic albacore populations.

Reproductive information, such as fecundity, can be used to improve the assessment of fish stocks and therefore their management. Information on the fecundity strategy is needed to correctly estimate fecundity and has to be investigated before applying any fecundity estimation method (Murua and Saborido-Rey, 2003; Kjesbu, 2009). Two types of fecundity, determinate and indeterminate, have been defined in fish according to the strategy of recruitment of oocytes to the stock of mature oocytes (Hunter *et al*., 1985; Hunter *et al*., 1992; Murua and Saborido-Rey, 2003). It is known that tuna species have indeterminate fecundity (Schaefer, 2001). Evidence of this type of fecundity (see Hunter *et al*., 1989; Greer Walker *et al*., 1994; Murua and Saborido-Rey, 2003) has been shown in albacore spawning ovaries by applying the Oocyte Packing Density (OPD) method (Kurita and Kjesbu, 2009) (Chapter 2). The estimated number of previtellogenic stage oocytes per gram of ovary was very high; that is, there is a new (de novo) recruitment of oocytes from previtellogenic into vitellogenic stage oocytes during the spawning period. Moreover, as expected for indeterminate species, there was no gap in oocyte size between the previtellogenic and vitellogenic stages; however, there was a

gap (hiatus) between the vitellogenic stage oocytes and the most advanced group of oocytes in each gonad subphase (advanced vitellogenic, migratory nucleus, or hydrated oocytes). This distinct hiatus differentiates the spawning batch from the standing stock of oocytes (Schaefer, 2001; Kjesbu, 2009).

Histology is considered the most accurate approach to correctly classify the reproductive female and male gonad stages (Hunter and Macewicz, 1985; West, 1990; Schaefer, 2001). Histological sections are used for estimating the number and size of different structures (oocyte developmental stages, POFs and atretic oocytes) in fish and some invertebrate reproductive studies. The two most common embedding media used in reproductive-related studies on teleost ovarian tissue are paraffin and resin. It is well known that the process of fixation and histological processing entails a certain degree of tissue shrinkage, which is higher or lower depending on both the nature of the tissue and the protocol used (Davis, 1982; Johnson *et al*., 1997; Dorph-Petersen *et al*., 2001; Chen *et al*., 2012b). Consequently, any estimates of oocyte size and numbers should be considered in the light of the method used, in particular when contrasting values across different protocols. When comparing the differences in mean diameter between fresh and embedded oocytes, it was clear that oocytes shrank as a consequence of the embedding process, reaching the maximal degree of shrinkage for hydrated oocytes (45.8 % in paraffin and 26.5% in resin) (Chapter 2). Our results were very similar to those of Kraus *et al*. (2008), who found that cod *Gadus morhua* hydrated eggs embedded in paraffin shrank 48 ± 7%. Differences in shrinkage between fresh and embedded oocytes, depending on the oocyte stage and embedding medium, have also been found when contrasting two studies of cod. Whereas shrinkage of late vitellogenic oocytes embedded in resin was about 7% (calculated according to Kjesbu *et al*., 2011), in paraffin the shrinkage was 30 ± 9% (Kraus *et al*., 2008).

The OPD theory (Kurita and Kjesbu, 2009), a new methodology originally developed to study recruitment dynamics of small oocytes, has been applied on both determinate and indeterminate fecundity fish (Kurita and Kjesbu, 2009; Korta *et al*., 2010; Schismenou *et al*., 2012). In this PhD study, the theoretical OPD was used in order to detail oocyte batch recruitment in albacore spawning ovaries and to examine any differences in the number of stage-specific oocytes per gram of ovary between the two embedding media, i.e., paraffin and resin (Chapter 2). Five different oocyte developmental stages were observed in each ovarian spawning subphase. When comparing the differences in mean oocyte diameter between embedding media, we found that embedded paraffin oocytes of all stages were smaller in comparison with those embedded in resin, with differences ranging between 0.5 % in primary growth (previtellogenic oocytes) and 24.3 % in hydration stage oocytes. These results were in line with studies that compared the size of POFs of sardine *Sardina pilchardus* (Ganias *et al*., 2007) and the size of human adipocyte (Verhoef *et al*., 2013) in the two embedding media. Estimates of volume fraction of the earlier oocyte stages were generally higher in paraffin than in resin; in contrast, the volume fraction of the later oocyte stages was lower. In order to assess the effect of oocyte shrinkage on the OPD results, and thereby on relative batch fecundity (BF_{rel}), oocyte diameters corrected and uncorrected for shrinkage were used for estimations. The results demonstrated that oocyte shrinkage corrections must be used in the OPD formula for both embedding media. After proper oocyte shrinkage corrections, only statistical differences were found in the estimated number of cortical alveoli (lipid-stage oocyte) and hydrated oocyte stage per gram of ovary between embedded media. The theoretical OPD results in ovaries of the spawning albacore (for both embedding media) were comparable with values seen in the ovary of spawning European hake *Merluccius merluccius*

(Korta *et al*., 2010). The estimated number of previtellogenic oocytes per gram of ovary was very high for both species, and then the number of oocytes per gram of ovary decreased as the volume-based oocyte diameter increased, that is, with the oocyte developmental stage (abruptly in the case of albacore ovaries), and finally slightly increased for the most advanced group of oocytes (advanced vitellogenic, migratory nucleus or hydrated oocytes) in each gonad subphase.

For species that exhibit indeterminate fecundity, spawning ovaries with final stages of oocyte maturation, i.e. migratory nucleus (MG) or hydrated oocytes, should be used for the accurate estimation of batch fecundity (Hunter *et al*., 1992; Schaefer, 1996). Our results showed that in spite of the higher degree of oocyte shrinkage in the MG and hydrated oocyte stages in paraffin, no significant differences in BF_{rel} were found between the two embedding media, suggesting that the OPD formula could equally well be used for batch fecundity (BF) estimations in paraffin as well as in resin (Chapter 2). Nevertheless, when BF_{rel} was estimated using only hydrated oocytes, BF_{rel} was significantly lower than when using MG oocytes, for both embedding media. Similarly, Schismenou *et al*. (2012) found that as a consequence of the high degree of shrinkage of the hydrated oocytes, stereological methods for measuring BF based on ovaries with hydrated oocytes are less precise than using earlier oocyte stages. Thus, MG oocytes appear to be better candidates for stereological BF estimations. However, it should be mentioned that if the purpose is restricted to estimating BF only instead of presenting a detailed picture of the formation of a batch of oocytes, then much simpler methodologies exist such as the 'Hydrated Oocyte Method'. In this quick method introduced several decades ago (see Hunter *et al*., 1985), hydrated oocytes are simply counted under the stereomicroscope and the number in each subsample is raised to the whole ovary and thereafter averaged. However, specimens with hydrated ovaries are seldom

found in the samples (Schaefer, 2001), including albacore individuals caught in the western Mediterranean Sea (Chapter 1).

In the western Mediterranean Sea, albacore are mainly caught during the summer months by commercial (albacore longline fishery) and recreational (sport vessels) fisheries. The scarcity of hydrated ovaries in samples of albacore fish (Chapter 1) could not only be due to tuna species being nocturnal spawners (Hunter *et al*., 1986; Schaefer, 2001; Farley *et al*., 2013; Gordoa *et al*., 2015), but also to warm waters appearing to having a significant effect on the limited time course of oocyte hydration (Kurita *et al*., 2011). A higher proportion of fish with hydrated ovaries was collected from the longline fishery (n = 11) in comparison with those collected from the recreational fishery (n = 6) (Chapter 1). This could be due to the fact that sports vessels fish during the day, whereas sets from the longline fishery can also be placed during the night. Another explanation for these results may be due to the differences in the potential time-lapse between the moment the fish bite the hook and their death, i.e., the fish die almost immediately in a sports vessel, whereas tuna species such as albacore could be caught hours before eventually dying on the longline, and thus the stress caused by the capture could induce the hydration process (Hunter *et al*., 1986).

Fecundity is a very important reproductive trait to be estimated. Nowadays, a variety of methods can be applied to estimate fecundity, and the method chosen by researchers depends not only on the reproductive strategy of the fish species but also on the available resources of each laboratory (see Murua *et al*., 2003; Kjesbu, 2009; Ganias *et al*., 2014). In chapter 3, batch fecundity estimates of albacore resulting from counts of MG oocytes using the application of the Weibel and Gomez (W&G), Physical Disector (PD), Oocyte

Packing Density (OPD), and Hydrated Oocyte (HO) methods were compared using the last method as "control". Given that small POFs coexisted with MG oocytes (see Chapter 1), the PD method (design-based method) was also applied to estimate the BF based on counts of POFs. Correction factors due to shrinkage were considered in the application of the different methods (Chapter 3). For the application of the methods currently considered (Chapter 3), the necessary equipment should be widely available in virtually every routine histology lab. Then, in order to choose the best method for estimating BF in an indeterminate species, the advantages and disadvantages in terms of time-cost associated with each method, the number of samples needed for a certain study, and the level of accuracy required in that particular study were evaluated.

Our results showed the highest BF estimates were obtained with the PD method used for counting MG oocytes. The BF estimates from the W&G and OPD methods were close to those given by the HO method. However, counting POFs instead of MG oocytes gave highly significant lower values (Chapter 3). Lower means of BF estimates from the counts of POFs have been also reported by Aragón *et al*. (2010) and Aranda *et al*. (2013a) for bluefin tuna spawning in the western Mediterranean Sea, but in these cases with no statistical difference between BF estimates obtained from counts of POFs and MG oocytes. Both albacore and bluefin tuna spawn around the Balearic Islands (western Mediterranean Sea); however, the peak albacore spawning season occurs later in the summer season when the water temperature is higher (Alemany *et al*., 2010). As mentioned, it was assumed that the POFs of albacore from the western Mediterranean are resorbed within 24 hours after spawning as in other tunas (Hunter *et al*., 1986; Schaefer, 1996; Farley *et al*., 2013), given that warm water temperatures appear to have a significant effect on POF resorption rates (Fitzhugh and Hettler, 1995; Ganias *et al*., 2007; Kurita *et al*., 2011). So our

results could indicate that the rate of reabsorption of POFs could be faster for albacore than for the bluefin tuna. Thus, counting the POFs in present albacore ovaries might not well represent the actual number of eggs released, at least when these structures coexist with MG oocytes.

In the biomedical field a large number of researchers are interested in evaluating the different available methods for counting particles. In this field, authors consider the design-based methods unbiased (Howard and Reed, 2010), but they are not unanimous in this view because their use should be validated (Baddeley 2001; von Bartheld 2002; Delaloye *et al*., 2009). Although nowadays many medical studies use design-based methods (e.g. both physical and optical disector, physical disector in combination with the fractionator), the fact is that a small percentage of studies use these methods in comparison with those that use conventional assumption-based methods (Geuna and Herrera-Rincon, 2015). In fish reproductive studies the PD method has been used to quantify irregular particles because, unlike the W&G and OPD methods, the PD method does not require any assumptions about particle shape and size distribution. Hence, the PD method has been used as an appropriate method to estimate the number of atretic oocytes and POFs (Kurita et al., 2003; Kraus et al., 2008; Aragón et al., 2010; Kjesbu et al., 2010; Aranda et al., 2011, 2013a). However, the number of studies estimating the number of oocytes with this method is limited (Aragón et al., 2010; Korta, 2010; Aranda et al., 2013a; Bucholtz et al., 2013) mainly because it is time-consuming. Based on our results (Chapter 3), no method should be dismissed for fecundity estimation purposes. However, if we consider a reasonable trade-off between workload/economic cost and data quality, the OPD or W&G methods stand out as the best candidates. If hydrated ovaries could be commonly collected, we would argue for the HO method, exploring the

possibility of using automatic counting procedures in whole mounts, i.e. freely available image analysis software.

In this PhD study, a total of 61 albacore fish were selected according to their fork length to estimate BF and BF_{rel} using the OPD method (Chapter 3). The length of the specimens ranged from 57.2 to 85.5 cm FL. A high variability in BF estimates among specimens within this size range and for specimens of similar length was found. These results are in line with those found in other tuna species (Thorogood, 1986; Farley and Davis, 1998; Chen *et al*., 2010; Zudaire *et al*., 2013). Batch fecundity increased with fish size (length and body weight) and with gonad weight (Chapter 3). Gonad weight was found to be the most powerful predictor of BF, indicating that fish with larger ovaries produce more eggs per batch. In support of this, the GAM analysis showed that not only large females but also small ones are capable of spawning a high number of oocytes. The only significant relationship between the BF_{rel} and the target biological metrics was found for gonad weight. The individual BF estimates of albacore fish from the western Mediterranean Sea varied from 0.42 to 2.16 million oocytes (Chapter 3), which is similar to those estimated for Pacific and Indian albacore (0.17 − 2.83 million oocytes for a 76 − 113 cm FL fish) (Ueyanagi, 1957; Otsu and Uchida, 1959; Wu and Kuo, 1993; Chen *et al*., 2010; Farley *et al*., 2013). In contrast, our estimate of mean BF_{rel} for albacore of 136 (± 36) oocytes per gram of body weight is twice that estimated in North and South Pacific albacore (51 − 64 oocytes per gram of body weight) (Otsu and Uchida, 1959; Chen *et al*., 2010; Farley *et al*., 2013).

Reproductive traits for a given species have been shown to vary with environmental influences and geographically (Pawson *et al*., 2000; Ewing and Lyle, 2009; Rodrigues *et al*., 2015). The results found in this PhD thesis show a

great difference in the reproductive traits between the albacore population (from the western Mediterranean Sea) and the other global populations (oceanic ones): higher gonadosomatic index values (during the spawning season), lower minimum length at maturity, shorter spawning season, lower spawning interval and higher relative batch fecundity. These reproductive variables may be genetic or environmentally determined, or an interaction between both (Lawrence, 2000 in Kjesbu, 2009). Genetic studies confirm that the Mediterranean albacore population is genetically distinguishable from the other populations (Montes *et al*., 2012; Albaina *et al*., 2013; Laconcha *et al*., 2015); even within the Mediterranean Sea genetic heterogeneity has been observed (Davies *et al.*, 2011; Montes *et al*., 2012). It can be suggested that the different reproductive traits of Mediterranean albacore is an adaptive response to the environmental conditions (Mediterranean climate) that together with the genetic variability provide evidence of geographic and reproductive isolation of the Mediterranean population.

The incorporation of the batch fecundity estimates and sex ratio in the estimation of limit reference points could be of value for future stock assessment. A long-term sampling within the Mediterranean incorporating adult and larvae abundance estimates, electronic tagging, and genetic studies should lead to a more complete understanding of the reproductive dynamics of albacore in the Mediterranean Sea.

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Conclusions / Conclusiones

CONCLUSIONS

Methodological conclusions.-

1. The mean oocyte diameters were smaller in paraffin than in resin with differences ranging between 0.5% for previtellogenic oocytes and 24.3% for hydrated oocytes; this finding was probably due to the higher water content in hydrated oocytes.
2. Significant differences were found between the two embedding media in terms of volume fraction of 'free space' (extracellular space) and connective tissue, showing that the ovarian tissue morphology is better preserved in resin, especially in ovaries with hydrated oocytes.
3. The Oocyte Packing Density formula can equally well be used for estimating the number of migratory nucleus oocytes in paraffin as well as in resin histological sections. However, the mean relative batch fecundity estimated from hydrated oocytes was significantly lower than when using migratory nucleus oocytes, for both embedding media.
4. Batch fecundity estimates resulting from the counts of postovulatory follicles gave highly significantly lower values compared to estimates obtained from migratory nucleus oocytes, probably due to fast postovulatory follicle degeneration.
5. Batch fecundity estimates resulting from counts of migratory nucleus oocytes when applying the Physical Disector were significantly higher than those obtained with the other methods applied. The batch fecundity estimates from the Weibel & Gomez and the Oocyte Packing Density methods were close to those given by the Hydrated Oocyte method, which was used as the control.
6. Based on the present results, no method should be dismissed for fecundity estimation purposes. Therefore, if we consider the time-cost associated with each method, and the shortage of hydrated ovaries in sampled fish, the Weibel

& Gomez or the Oocyte Packing Density methods should be used in routine studies.

Conclusions regarding the Reproductive biology of albacore *Thunnus alalunga*.-

7. The maximum length recorded in the western Mediterranean was 109 cm. Few individuals over 90 cm were observed.

8. The estimated overall sex ratio during the spawning season in the western Mediterranean was 1.1♀:1♂. The analysis of sex ratio by length indicated that females predominated in the smaller length classes (< 70 cm) and males in the larger length classes (> 75 cm).

9. The minimum length at maturity for albacore in the western Mediterranean Sea is 56 cm. The length of spawning season is around three months (from June to August). The spawning interval of active females was 1.01, indicating that albacore in the western Mediterranean Sea are capable of spawning nearly every day.

10. Batch fecundity estimates ranged from 0.42 to 2.16 million oocytes, with a mean relative batch fecundity of 136 oocytes per gram of body weight. Batch fecundity increased with fish size (length and weight) and gonad weight.

11. Therefore, albacore from the western Mediterranean Sea show lower maximum length, lower minimum length at maturity, shorter spawning season, lower spawning interval, and higher relative batch fecundity in comparison with the oceanic albacore populations. This variation in expression of reproductive traits of Mediterranean albacore indicates that its reproductive tactic differs from those observed in the oceanic populations, which could represent an adaptive response to the environmental conditions in the Mediterranean.

CONCLUSIONES

Conclusiones metodológicas.-

1. El diámetro medio de los ovocitos fue menor en parafina que en resina. Las diferencias variaron entre el 5 y el 24.3% en los ovocitos previtelogénicos e hidratados, respectivamente. La mayor diferencia en los ovocitos hidratados probablemente se deba a su alto contenido de agua.
2. Se encontraron diferencias significativas en la fracción de volumen del "espacio libre" (espacio extracelular) y del tejido conectivo entre los dos medios de inclusión, lo que muestra que la morfología del tejido ovárico está mejor preservada en resina, especialmente en los ovarios con ovocitos hidratados.
3. El método del *Oocyte Packing Density* es adecuado para la cuantificación de ovocitos con núcleo migratorio tanto en resina como en parafina. Sin embargo, para ambos medios de inclusión la fecundidad relativa por tanda estimada a partir de los ovocitos hidratados fue significativamente menor que a partir de los ovocitos con núcleo migratorio.
4. Las estimas de la fecundidad por tanda a partir del conteo de folículos postovulatorios fueron significativamente menores que las obtenidas a partir del conteo de los ovocitos con núcleo migratorio, probablemente debido a la rápida degeneración de los folículos postovulatorios.
5. Las estimas de fecundidad por tanda a partir del conteo de ovocitos con núcleo migratorio obtenidas mediante el método *Physical Disector* fueron significativamente mayores que las obtenidas con el resto de métodos empleados. Las estimas obtenidas con los métodos de Weibel y Gomez y del *Oocyte Packing Density* fueron las más cercanas a las obtenidas con el método tradicional del *Hydrated Oocyte*, el cual se utilizó como control.

6. Basándonos en los resultados obtenidos, ningún método de los empleados para estimar la fecundidad debería ser descartado. Por tanto, teniendo en cuenta el tiempo y coste que conlleva la aplicación de cada uno de los métodos así como la escasez de ovarios con ovocitos hidratados en los ejemplares muestreados, utilizaríamos el método de Weibel y Gomez o el del *Oocyte Packing Density* para trabajos rutinarios.

Conclusiones sobre la biología reproductiva del atún blanco *Thunnus alalunga*.-

7. La talla máxima observada en el Mediterráneo occidental fue de 109 cm. Pocos individuos sobrepasaron los 90 cm.

8. Durante la época de puesta, el sex ratio en conjunto es de 1.1♀:1♂. El análisis del sex ratio por talla mostró que las hembras predominan en tallas inferiores a 70 cm y los machos en tallas superiores a 75 cm.

9. La talla mínima a la que el atún blanco alcanza la madurez en el Mediterráneo occidental es de 56 cm. La duración de la época de puesta es de cerca de tres meses (de junio a agosto). El intervalo de puesta de las hembras activas es de 1.01, lo cual indica que el atún blanco es capaz de poner casi a diario.

10. Las estimas de la fecundidad por tanda variaron entre los 420000 y los 2 160 000 ovocitos con núcleo migratorio, con una fecundidad media relativa por tanda de 136 ovocitos por gramo de peso corporal. La fecundidad por tanda aumenta con el peso gonadal, la talla y el peso del individuo.

11. Por tanto, el atún blanco del Mediterráneo occidental en comparación con las poblaciones oceánicas muestra tallas máximas más pequeñas, menor talla mínima de madurez, época de puesta más corta, menor intervalo de puesta y mayor fecundidad relativa por tanda. Esta variación en la expresión de las características reproductivas del atún blanco del Mediterráneo indica que su

táctica reproductiva difiere de las observadas en las poblaciones oceánicas, lo cual representaría una respuesta adaptativa a las condiciones ambientales del Mediterráneo.

Annex

GONAD EMBEDDING, SECTIONING AND STAINING PROCEDURE

In this section is described the gonad embedding, sectioning and staining procedures followed in the chapters of this PhD thesis.

A 2−3 cm cross-section from the central part of the right or left lobe (Figure) was fixed in Bouin's fluid for four hours, and preserved in 70% ethanol (for at least three months prior to embedding).

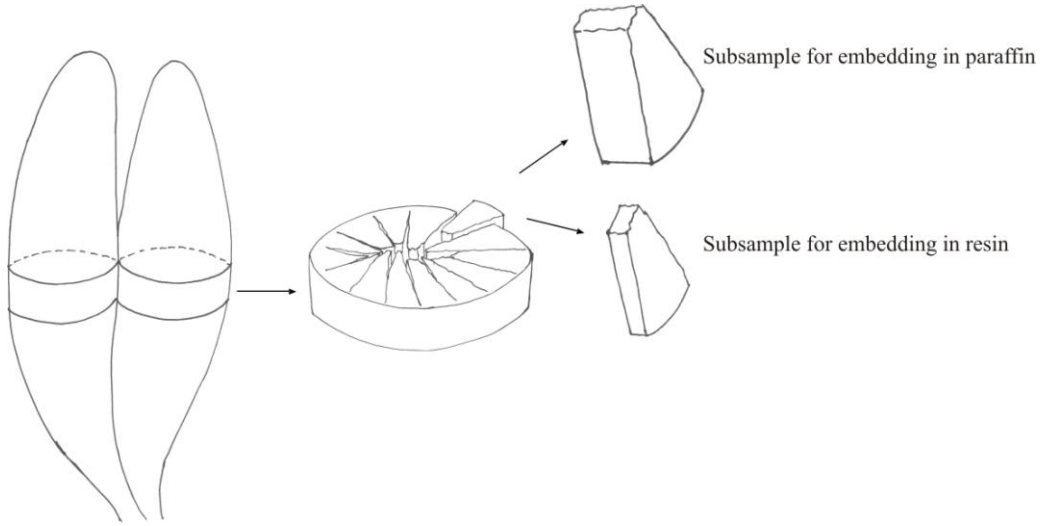

Figure. Schematic of albacore ovaries, showing the full cross section from the central part of one of the lobes which is fixed in Bouin's fluid for four hours, and preserved in 70% ethanol.

A. Gonad embedding, sectioning and staining procedure: paraffin

(IEO, Centro Oceanográfico de Málaga)

A representative subsample (from the *tunica albuginea* to the ovarian lumen, see Figure) was taken from the preserved ovarian tissue and processed, using gentle agitation (from step 1 to step 7), as follows:

If samples are fixed and preserved in phosphate buffered formaldehyde

Distilled water (2 x 30 min)

50% ethanol (1 hour)

70% ethanol (1 hour)

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

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

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

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

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

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

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

* butanol used one time in step 7

* paraffin used one time in steps 9 and 10

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

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

Paraffin should solidify in 30−60 minutes depending on the room temperature. Once the tissue is embedded, it is stable for many years.

Sectioning

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

Staining procedure

- Deparaffinize and rehydrate sections

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

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

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

- Dehydrate and clear sections

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

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

B. Gonad embedding, sectioning and staining procedure: paraffin

(Departamento de Biología, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz)

A representative subsample (from the *tunica albuginea* to the ovarian lumen, see Figure) was taken from the preserved ovarian tissue and processed as follows:

If samples are fixed and preserved in phosphate buffered formaldehyde

Rinse distilled water

70% ethanol (over night)

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

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

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

6. Clearing agent → Xylene (30 min)
7. Clearing agent → Xylene (30 min)

Immersion time in xylene (30 min) (steps 6 and 7) should be not exceeded

8. Paraffin* at ≈ 60°C (1 hours)

9. Paraffin at ≈ 60°C (2 hours)
10. Paraffin at ≈ 60°C (2 hours)

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

The infiltrated ovarian tissues are then embedded into wax blocks (same procedure as previously described, see section A).

Sectioning

Cut at 10 µm. Pick the sections up with forceps or a fine paint brush and float them on the surface of the 37°C distilled water bath. Pick up sections from water by placing the slides under the sections. Dry the slides overnight at room temperature.

Staining procedure

- Deparaffinize and rehydrate sections

  Xylene (2 x 10 min) (blot excess xylene before going into ethanol)
  Ethanol 99.6% (2 x 10 min)
  Ethanol 90% (10 min)
  Ethanol 70% (10 min)
  Ethanol 50% (5 min)
  Water (5 min)

- Staining (times should be not exceeded)

  Haematoxylin (6 min)
  Rinse in running tap water (≈10 min)
  Chlorhydric acid water 0.5% (1 second)
  Rinse in running tap water (≈10 min)
  Ammonia water (20 seconds)
  Rinse in running tap water (≈10 min)
  VOF stain (3 min)
  Rinse water (3−4 drips)

- Dehydrate and clear sections

  Ethanol 70% (2 seconds)

Ethanol 90% (2 min 30 seconds)
Ethanol 99.6% (2 x 10 min)
Xylene (2 x 10 min) (subsamples can kept more time in the final step)

Cover the sections using mountex/cover glasses. Dry the slides overnight at room temperature.

C. Gonad embedding, sectioning and staining procedure: resin (2-hydroxyethyl methacrylate)

(from Dr. Olav Sigurd Kjesbu, Institute of Marine research, Bergen, Norway)

A representative subsample (from the *tunica albuginea* to the ovarian lumen, see Figure) was taken from the preserved ovarian tissue and processed, using gentle agitation (from step 1 to step 6), as follows:

If samples are fixed and preserved in phosphate buffered formaldehyde

70% ethanol (over night)

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

1. 90% ethanol (1 hour)
2. 90% ethanol (1 hour)
3. 96% ethanol (1 hour)
4. One part activated resin / One part 96% ethanol (X2*) (2 hours)
5. Activated resin (X1*) (24 hours)
6. Activated resin (new) (24 hours)

* X2 resin used two times (from step 6) + ethanol

* X1 resin used one time in step 7

Note: all used resin has to be filtered though a filter paper.

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

Polymerisation: mix resin and harder in the ratio 15ml:1ml, mix well and put it in the form. Put the tissue in the form and put it on a cooling plate (4°C). The cooling plate can be turned off after half an hour, but leave the forms on the plate till the next day.

Mounting: mix mounting medium (fluid + powder) in a tube. Use a pipette to transfer it to the top of the resin and immediately put on a marked block. Leave it for at least 15 minutes before you take them out of the forms.

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

Keep the blocks in a cup in a box that contains 70% (volume) glycerol, the humidity should be around 50−55%.

Sectioning

Cut at 4 µm. Pick the sections up with forceps or a fine paint brush and float them on the surface of distilled water which contains a few drops of ammonia. Pick up sections from water by placing the slides under the sections. Dry the slide on a hot plate (≈100°C) during few seconds and then keep it in a hot plate at ≈ 60°C during 30−60 minutes approximately.

Staining

2% toluidine blue and 1% sodium tetraborate (borax). The borax is dissolved in distilled water and then the dye added with constant stirring. Filter the solution before use. Cover the section with the solution and rinse with hot tap water. Dry the slides on a hot plate at ≈ 60°C.

Cover the sections using mountex/cover glasses. Use a weight on top of the sections and leave it to dry overnight at room temperature.

Remark: This procedure was acquired from the Institute of Marine Research. In the IEO lab., the cooling plate was replaced by ice sheets kept in a plastic container.

FINAL REMARK: Distance between sections should exceeds the target particle, for instance, if the target particle is a migratory nucleus oocyte distance between sections should be more than 600 and 700 µm in paraffin and resin sections, respectively.