UNIVERSITY OF BERGEN

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DOCTORAL THESIS

2018

EXPERIMENTAL STUDIES ON GROWTH AND SURVIVAL IN ATLANTIC BLUEFIN TUNA *(Thunnus thynnus)* AND ATLANTIC BONITO *(Sarda sarda)* LARVAE: EFFECTS OF LIGHT, FOOD AVAILABILITY AND TEMPERATURE ON THEIR PHYSIOLOGY AND BEHAVIOR

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DECLARE:

That the thesis title "*Experimental studies on growth and survival in Atlantic bluefin tuna* (*Thunnus thynnus*) *and Atlantic bonito* (*Sarda sarda*) *larvae: effects of light, food availability and temperature on their physiology and behavior*", presented by Edurne Blanco Rodríguez to obtain a doctoral degree, has been completed under our supervision, and meets the requirements to opt for an International Doctorate.

For all intents and purposes, we hereby sign this document.

Signature

Palma de Mallorca, Bergen and Mazarrón, 8th of October, 2018

-Gurasoei eta Josuri-

“Los peces no se mueren, los mata la ignorancia de quien los cultiva”

De la Gándara, F. (2003)

Table of content

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List of publications

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Blanco, E., Reglero, P., Ortega, A., De la Gándara, F., Fiksen, Ø., Folkvord, A., 2017. The effects of light, darkness and intermittent feeding on the growth and survival of reared Atlantic bonito and Atlantic bluefin tuna larvae. Aquaculture 479, 233-239. (Chapter 5).

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Reglero, P., Blanco, E., Alemany, F., Ferrá, C., Alvarez-Berastegui, D., Ortega, A., De la Gándara, F., Aparicio-González, A., Folkvord, A., 2018. Vertical distribution of Atlantic bluefin tuna *Thunnus thynnus* and bonito *Sarda sarda* larvae is related to temperature preference. Marine Ecology Progress Series 594, 231-243. (Chapter 7).

The Ph.D. candidate has participated in four papers directly related to the line of research of this thesis, which have not been included in this thesis:

Reglero, P., Balbín, R., Abascal, F.J., Medina, A., Alvarez-Berastegui, D., Rasmuson, L., Mourre, B., Ortega, A., Blanco, E., De la Gándara, F., Alemany, F.J., Ingram Jr, G.E., Hidalgo, M. (accepted). Pelagic habitat and offspring survival in the Eastern stock of Atlantic bluefin tuna. ICES Journal of Marine Science.

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Reglero, P., Ortega, A., Blanco, E., Fiksen, Ø., Viguri, F.J., De la Gándara, F., Seoka, M., Folkvord, A., 2014. Size-related differences in growth and survival in piscivorous fish larvae fed different prey types. Aquaculture 433, 94-101

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List of acronyms and abbreviations

AB	Atlantic bonito
ABFT	Atlantic bluefin tuna
Alt.	Alternating treatment
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ATAME	Assessment of the Atlantic bluefin tuna population breeding in the western Mediterranean.
CSD	Cumulative size distribution
Cont.	Continuous treatment
DHA	Docosahexaenoic acid
DLI	Daily length increment
DNA	Deoxyribonucleic acid
Dph	Days post hatch
DW	Dry weight
DYSL	Delayed yolk-sac larva
EB	Ethidium bromide
Eday	Experimental day
EDTA	Ethylenediaminetetraacetic acid
Exp.	Experiment
F0	Pre-flexion stage
F1	First caudal fin rays stage
F2	Flexion stage
F3	Post-flexion stage
FAO	Food and Agriculture Organization of the United Nations
GAM	Generalized additive model
GLM	Generalized linear model
IBM	Individual-based models
ICCAT	International Commission for the Conservation of Atlantic Tunas
ICES	International Council for the Exploration of the Sea
IEO	Spanish Institute of Oceanography
IUCN	International Union for Conservation of Nature and Natural Resources
IUU	Illegal, Unreported and Unregulated
L:D	Light:Darkness
M	Instantaneous mortality

MMR	Maximum metabolic rate
MO_2	Metabolic rate
mRNA	Messenger RNA
NaCl	Sodium chloride
NW	Northwestern
OR	Otolith radii
PVC	Polyvinyl chlorid
R1, R2	Tank replicates 1, 2
RMR	Routine metabolic rate
RNA	Ribonucleic acid
RNase A	Ribonuclease A
SD	Standard deviation
SCR	Stomach content ratio
SDA	Specific dynamic action
SDS	Sodium dodecyl sulfate [$CH_3(CH_2)_{11}SO_4Na$]
SE	Southeast
SGR	Specific growth rate
SL	Standard length
SMR	Standard metabolic rate
SpCR	Spearman's rank correlation coefficient
TAC	Total allowed catches
TRIS	Tris(hydroxymethyl)aminomethane [$(HOCH_2)_3CNH_2$]
tRNA	Transfer RNA
HSD	Honestly significant difference
VO_2	Mass-specific metabolic rate
YSL	Yolk-sac larva

Summary

Understanding what determines the growth and survival of individuals among large pelagic fish populations, particularly during the larval stage, is crucial, because these factors will eventually determine recruitment variability and the number of survivors that reach the juvenile stage. Both Atlantic bluefin tuna and Atlantic bonito serve important ecological role as top predators, are targets of some of the most important fisheries in the world and have a fundamental influence on the structure and function of marine communities. Besides, they are the only two scombrid species in the Mediterranean Sea that have been successfully reared in captivity. However, there are still several unresolved problems in their rearing due to our current lack of knowledge regarding their development during these early life stages, knowledge that is difficult to obtain in the field. This thesis aims to improve current knowledge on the growth and survival of the larval stage of Atlantic bluefin tuna and Atlantic bonito under different light, food and temperature conditions. The results of this thesis are based on laboratory experiments (Chapters 3–6) and combined field and laboratory results (Chapter 7).

Chapter 1 provides an introduction with a background to the topic of study. Chapter 2 discusses the materials and methods used for the studies upon which this thesis is based. In Chapter 3, growth differences during the first days of life of Atlantic bluefin tuna and Atlantic bonito are discussed. The nutritional condition of the larvae, measured by RNA:DNA ratio, did not explain the differences in developmental stage in larvae that had been cultured under the same conditions. Chapter 4 examines how larval size is related to vulnerability to manipulation in the timing of onset of piscivory. This is an important aspect to examine, since mortality during the larval stage is considered to be size-selective. The effect that changing the feeding regime, from continuous to intermittent piscivorous feeding, had on larval growth and survival is explored in Chapter 5. In Chapter 6, the metabolic cost of different light and darkness conditions was estimated. The effect that temperature had on fish larval vertical distribution was analysed in Chapter 7 by a comparative laboratory analysis between field-sampled individuals at different depths and experimental thermocline columns. The results suggest that the vertical distribution of Atlantic bluefin tuna and Atlantic bonito larvae is spatially constrained to the upper warmer layers due to larval thermal tolerance.

The results of this thesis demonstrate the importance of studying the larval stages of these species, stages which are still not well understood. The characteristics of Atlantic bluefin tuna and bonito are very different from other commonly studied cold water species, such as herring, cod and salmon, for which many empirical studies have been conducted. The results presented in this thesis demonstrate the need to use experimentation in order to obtain knowledge on the processes driving growth and survival—knowledge that is difficult to obtain in the field. Culture of larvae could be improved using alternating light regimes instead of the common long light photoperiod regimes that are used in aquaculture to maximize larval ingestion rates. Field and experimental work can be combined in order to have a more complete view of the processes driving these species and their ecosystems.

Resumen

Comprender qué determina el crecimiento y la supervivencia de los individuos, particularmente durante la etapa larvaria, es de crucial importancia ya que va a determinar la variabilidad del reclutamiento y el número de supervivientes que van a alcanzar la etapa juvenil. El atún rojo del Atlántico (*Thunnus thynnus*) y el bonito del Atlántico (*Sarda sarda*) son grandes predadores que se encuentran en la parte alta de la cadena trófica, son objetivo de algunas de las mas importantes pesquerías del mundo y tienen una influencia fundamental en la estructura y función de las comunidades marinas. Además, son las dos únicas especies de escómbridos del mar Mediterráneo que han sido cultivadas con éxito en cautividad. Sin embargo, debido a nuestro gran desconocimiento acerca de su desarrollo durante el estadio larvario, el cultivo de ambas es una tarea muy complicada donde existen una gran cantidad de problemas aún sin resolver. La presente tesis tiene como objetivo mejorar el conocimiento actual sobre el crecimiento y la supervivencia larvaria del atún rojo y del bonito bajo diferentes condiciones de luz, alimentación y temperatura, con especial énfasis en su etapa de alimentación piscívora. Los resultados se han obtenido mediante experimentos de laboratorio (Capítulos 3-6) y resultados combinados de campo y de laboratorio (Capítulo 7).

El Capítulo 1 ofrece una introducción acerca del tema de estudio. El Capítulo 2 desarrolla el material y métodos utilizado a lo largo de la tesis. En el Capítulo 3 se muestran las diferencias de crecimiento durante los primeros días de vida del atún rojo y del bonito. La condición nutricional medida como la relación ARN:ADN no explicó las diferencias entre los diferentes estadios de desarrollo en larvas cultivadas bajo las mismas condiciones. El Capítulo 4 examina el espectro de tamaño larvario más vulnerable a la manipulación del inicio de la piscivoría. Este es un aspecto importante ya que la mortalidad durante la etapa larvaria se sabe que es selectiva con respecto al tamaño. El efecto de un cambio en el régimen de alimentación, de una alimentación continua a una intermitente durante la etapa piscívora, en el crecimiento y supervivencia larvaria se exploró en el Capítulo 5. En el Capítulo 6, se midieron los costes metabólicos de las larvas en condiciones de luz y oscuridad. l efecto de la temperatura en la distribución vertical de las larvas de ambas especies se analizó en el Capítulo 7 mediante un análisis comparativo de individuos muestreados en el mar a diferentes profundidades y en el laboratorio en columnas experimentales. Los resultados sugieren que la distribución vertical del atún rojo y del bonito se encuentra limitada espacialmente a las capas superficiales más cálidas debido a su tolerancia térmica.

Los resultados de la presente tesis demuestran la importancia del estudio de la etapa larvaria de las dos especies, etapa hasta ahora ampliamente desconocida. Las características del atún rojo y del bonito son muy diferentes a las de otras especies comúnmente estudiadas de aguas frías como son el arenque, el bacalao y el salmón, para las cuales se han realizado una gran cantidad de estudios experimentales a lo largo de los años. Los resultados presentados en esta tesis demuestran la necesidad de utilizar los experimentos de laboratorio para ampliar el conocimiento acerca de los procesos que afectan al crecimiento y supervivencia larvaria, y obtener así información difícil de obtener mediante muestreos en el medio marino. Además, la combinación de estudios de campo y experimentales proporcionan una visión más completa de los procesos que impulsan a estas especies y a sus ecosistemas.

Resum

Comprendre allò que determina el creixement i la supervivència dels individus, particularment durant l'etapa larval, és de crucial importància ja que determinarà la variabilitat del reclutament o el nombre de supervivents que assoliran l'etapa juvenil. La tonyina (*Thunnus thynnus*) i el bonítol (*Sarda sarda*) són de gran importància ecològica ja que es troben a la part alta de la cadena tròfica, són objectiu d'algunes de les pesqueres més importants del món i tenen una influència fonamental en l'estructura i funció de les comunitats marines. A més, són les dues úniques espècies d'escòmbrids de la mar Mediterrània que han estat cultivades amb èxit en captivitat. Tanmateix, a causa del nostre gran desconeixement sobre el seu desenvolupament durant l'estadi larval, el cultiu de les dues és una tasca molt complicada on existeixen una gran quantitat de problemes que encara estan sense resoldre. La present tesi té com a objectiu millorar el coneixement actual sobre el creixement i la supervivència larval de la tonyina i del bonítol sota diferents condicions de llum, alimentació i temperatura, amb especial èmfasi en la seva etapa d'alimentació piscívora. Els resultats s'han obtingut mitjançant experiments de laboratori (Capítols 3-6) i resultats combinats de camp i de laboratori (Capítol 7).

El primer capítol ofereix una introducció al tema d'estudi. El capítol 2 desenvolupa el material i mètodes utilitzat al llarg de la tesi. En el capítol 3 es mostren les diferències de creixement durant els primers dies de vida de la tonyina i del bonítol. La condició nutricional mesurada com la relació ARN:ADN no va explicar les diferències entre els diferents estadis de desenvolupament en larves sota les mateixes condicions de cultiu. El Capítol 4 examina l'espectre de talla larval més vulnerable a la manipulació a l'inici de la piscivoria. Aquest és un aspecte important ja que és ben sabut que la mortalitat durant l'etapa larval és selectiva per la grandària. L'efecte d'un canvi en el règim d'alimentació, d'una alimentació contínua a una intermitent durant l'etapa piscívora, en el creixement i supervivència larval es va explorar en el capítol 5. En el capítol 6, es van mesurar els costos metabòlics de les larves en condicions de llum i foscor. L'efecte de la temperatura en la distribució vertical de les larves de les dues espècies es va analitzar en el capítol 7 mitjançant una anàlisi comparativa d'individus mostrejats a la mar a diferents profunditats i al laboratori en columnes experimentals. Els resultats suggereixen que la distribució vertical de la tonyina i del bonítol es troba limitada espacialment a les capes superficials més càlides causa de la seva tolerància tèrmica.

Els resultats de la present tesi demostren la importància de l'estudi de l'etapa larval de les dues espècies, etapa fins ara àmpliament desconeguda. Les característiques de la tonyina i del bonítol són molt diferents a les d'altres espècies d'aigües fredes comunament estudiades com són l'arengada, el bacallà i el salmó, per a les quals s'han realitzat una gran quantitat d'estudis experimentals al llarg dels anys. Els resultats presentats en aquesta tesi demostren la necessitat d'utilitzar els experiments de laboratori per ampliar el coneixement sobre els processos que afecten el creixement i la supervivència larvària, i obtenir així informació difícil d'obtenir mitjançant mostrejos en el medi marí. A més, la combinació d'estudis de camp i experimentals proporcionen una visió més completa dels processos que impulsen a aquestes espècies i als seus ecosistemes.

CHAPTER 1

- GENERAL INTRODUCTION -

GENERAL INTRODUCTION

Large pelagic fish are fascinating organisms that have been studied by fisheries scientists and fish ecologists for many years, particularly adults. However, the early life stages of pelagic fish have received less attention. When considering large or medium pelagic species such as Atlantic bluefin tuna (*Thunnus thynnus*) or Atlantic bonito (*Sarda sarda*), we usually think of the adult individuals. However, they all pass through a planktonic egg and larval stage (millimetres in length) where survival rates are very low (McGurk, 1986; Peterson and Wroblewski, 1984). Decades of research have provided considerable information on adult bluefin tuna populations (though not as much on adult bonito) (*e.g.* Block *et al.*, 2001, 2005; Reglero *et al.*, 2012, 2017, 2018b; Rey *et al.*, 1984). However, relatively little is known about these populations' growth and survival during the early stages of life (De la Gándara *et al.*, 2012, 2016; Ortega, 2015). Both Atlantic bluefin tuna and Atlantic bonito serve important ecological role as top predators, are targets of some of the most important fisheries in the world and have a fundamental influence on the structure and function of marine communities (Shimose and Wells, 2015). Besides, are prey of other predators during the larval stage. Understanding the processes that drive growth and survival during the early life stages of these individuals has recently became a major objective in order to better manage fisheries and ensure the sustainable exploitation of these resources (Hjort, 1914; Houde, 2008). Stock recruitment variability, "the number of fishes surviving to enter the fishery or to some life history stage such as settlement or maturity", is known to be the result of complex interacting factors operating on different temporal and spatial scales and throughout the pre-recruit life stages of fishes. The growth of the aquaculture industry during recent years for Atlantic bluefin tuna (Mylonas *et al.*, 2010) and the expansion of experimental aquaculture for Atlantic bonito (De la Gándara *et al.*, 2012) provides us with a unique opportunity to improve process-based knowledge of the drivers of survival and growth during the egg and larval stages of these species, information that is difficult to obtain from field samplings.

How different processes influence growth and survival in fish larvae is complicated to understand if using only field data, since the effects of multiple variables acting at the same time are difficult to isolate. Consequently, field observations and laboratory experiments can be combined. This thesis is an attempt to generate new information on the processes that drive growth and survival during the larval stage of a large pelagic fish, Atlantic bluefin tuna, and a medium pelagic fish, Atlantic bonito, taking advantage of rearing techniques developed in the last few years. The knowledge of the ecology of the early life stages of fishes is crucial to understanding the population dynamics of fish stocks, and also the functioning of marine ecosystems.

1.1 Phylogeny

The Scombridae is a family of 5 tribes and 51 species of epipelagic marine fishes (Collette *et al.*, 2001; Graham and Dickson, 2004). Mackerels, Spanish mackerels, bonitos and tunas form the basis of important commercial and recreational fisheries in tropical and temperate waters worldwide (Fig. 1.1).

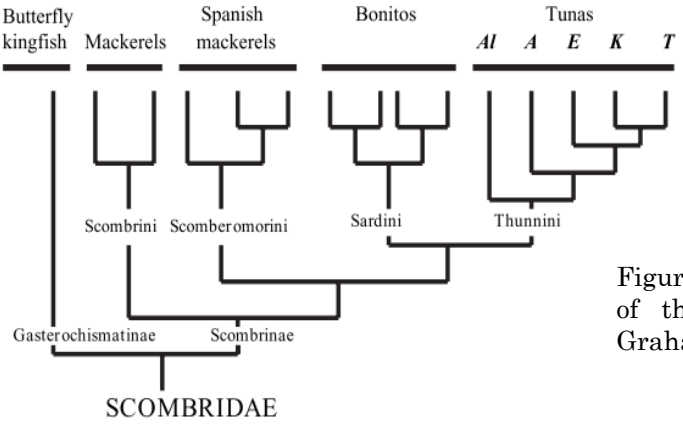


Figure 1.1. Morphological phylogeny of the family Scombridae. From Graham and Dickson (2004).

Bonitos and tunas are sister groups which differ both morphologically and physiologically (Collette *et al.*, 2001; Graham and Dickson, 2004). The derivation of tunas from a bonito-like ancestor occurred through selection for an integrated set of characteristics affecting locomotion and endothermy (Graham and Dickson, 2000). Selection for continuous, steady, and efficient swimming resulted in changes in body shape which increased streamlining and led to the adoption of the thunniform swimming mode unique to tunas. Alterations in blood supply necessitated by the anterior shift in red muscle led to the development of numerous arterial and venous branches, which set the stage for heat conservation. The evolution of endothermy, together with thunniform swimming, contributed significantly to the ecological radiation and diversification of tunas during the Early Tertiary Period (Graham and Dickson, 2000).

-Atlantic bluefin tuna

A total of 15 species of tuna comprise the Thunnini tribe, which is divided into 5 genera (Collete *et al.*, 2001): *Thunnus* (8 species), *Katsuwonus* (1 species), *Euthynnus* (3 species), *Auxis* (2 species) and *Allothunnus* (1 species). The genus *Thunnus* include tunas that are typically considered tropical and temperate fishes with a wide geographical distribution such as bigeye tuna (*Thunnus obesus*) and yellowfin tuna (*Thunnus albacares*); species that show a tropical but also a temperate distribution, such as albacore tuna (*Thunnus alalunga*); tunas with a more restricted distribution, such as longtail tuna (*Thunnus tonggol*) and blackfin tuna (*Thunnus atlanticus*); and some species that have adapted to colder waters, such as the three bluefin tuna species, southern bluefin tuna (*Thunnus maccoyii*), Atlantic bluefin tuna (*Thunnus thynnus*) and Pacific bluefin tuna (*Thunnus orientalis*).

Among the tuna species, bluefin tunas are the largest (Collete *et al.*, 2001). Atlantic bluefin tuna (*Thunnus thynnus*) (Linnaeus, 1758) grow up to 300 cm in length and attain masses up to 600 kg (Cort, 2007) (Fig. 1.2a). The heaviest documented Pacific bluefin tuna and southern bluefin tuna are 555 kg and 260 kg respectively (Foreman and Ishizuka, 1990; Nakamura, 1990). Along with the rest of the tunas and some sharks, bluefin tuna are unique among teleosts for their endothermic capacity and cardiovascular physiology (Blank *et al.*, 2004; Dickson and Graham, 2004; Kubo *et al.*, 2008). This allows them to maintain a higher body temperature than the surrounding environment and a constant metabolism enables them to penetrate cooler waters, thus expanding their thermal niche (2.8 to 30.6 °C) in both latitude and depth (Block *et al.*, 2001, 2005; Cermeño *et al.*, 2015; Reglero *et al.*, 2014b). Elevated body temperature is maintained by a *retia mirabile* vascular counter-current heat exchange system of warm venous blood and cold arterial blood (Graham and Dickson, 2000). This system allows bluefin tunas to retain 95% or more of the body heat produced by muscle activity, preventing it from dissipating externally.

Atlantic bluefin tuna adults are continuous swimmers that breathe using ram ventilation, a mechanism whereby forward swimming provides enough force to drive water into the mouth and through the branchial chamber (Wegner *et al.*, 2010, 2013). Atlantic bluefin tuna adults swim an average of 5.9 km h^{-1} and maximum sustained speeds of 20–31 km h^{-1} (Lutcavage *et al.*, 2000).

-Atlantic bonito

The Sardine tribe is comprised of 8 species of bonitos, which are divided among 4 genera: *Cybiosarda* (1 species), *Gymnosarda* (1 species), *Orcynopsis* (1 species) and *Sarda* (5 species). The genus *Sarda* comprises Atlantic bonito (*Sarda sarda*), striped bonito (*Sarda orientalis*), eastern Pacific bonito (*Sarda chiliensis*), Pacific bonito (*Sarda lineolate*) and Australian bonito (*Sarda australis*).

The Atlantic bonito (*Sarda sarda*) (Bloch 1973), considered a small scombrid, can reach 80 cm in length and weigh more than 5–6 kg, although the most common catches are of individuals of 1–3 kg, corresponding to fish up to three years old (Macías *et al.*, 2005; Rey *et al.*, 1984; Santamaría *et al.*, 2005) (Fig. 1.2b). Unlike tunas, bonitos do not have a counter-current heat exchange system, but like other scombrids (including Atlantic bluefin tuna), they are ram-ventilators, which implies that they swim continuously so that water is continually passing through their gills (Wegner *et al.*, 2013).

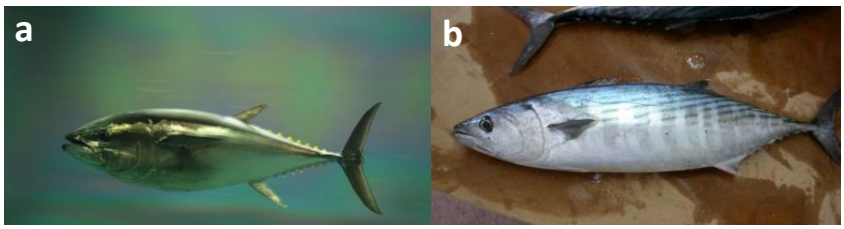


Figure 1.2. Image of adult a) Atlantic bluefin tuna and b) Atlantic bonito, targeted species in this study.

1.2 Distribution / Migration

-Atlantic bluefin tuna

Atlantic bluefin tuna is a highly migratory species with a range across vast expenses of the Atlantic Ocean and its adjacent seas–the Mediterranean Sea, the Black Sea, and the Gulf of Mexico. Atlantic bluefin tuna are able to do trans-Atlantic migrations (Fromentin and Powers, 2005; Teo and Boustany, 2016). Adult individuals are distributed widely, from the cold waters of Norway and Canada to more tropical equatorial regions (Fig. 1.3). The adults are also able to exploit the water column down to 1000 m but they generally spend most of their time in waters less than 100 m (Block *et al.*, 2001; Lutcavage *et al.*, 2000). Since 1982, Atlantic bluefin tuna are separated into the eastern and the western stocks for management purposes at the 45° W meridian by the International Commission for the Conservation of Atlantic Tunas (ICCAT), the international organism in charge of the management of this species. This separation is based on spatial observation and temporal differences in spawning and a genetic differentiation between the two populations (Boustany *et al.*, 2008; Carlsson *et al.*, 2007; Fromentin and Powers, 2005). However, several researchs show evidence of mixing of the two stocks in geographical areas, an element that needs to be considered for the correct management of the species (Block *et al.*, 2001; Dickhut *et al.*, 2009; Rooker *et al.*, 2008; Lutcavage *et al.*, 1999).

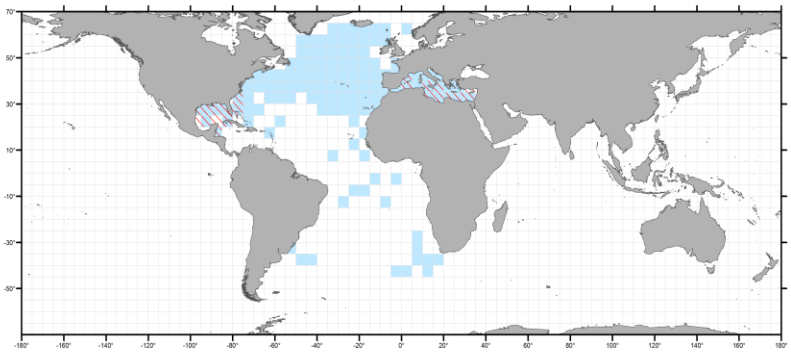


Figure 1.3. Larval *versus* adult habitat for Atlantic bluefin tuna. The presence of larvae is shown with red lines and the habitats of adult individuals is shown in blue. From Reglero *et al.* (2014b).

In general, adult Atlantic bluefin tuna performs two types of migrations. A reproductive migration in late spring, from the feeding areas in the North Atlantic to the breeding areas (Gulf of Mexico and the Mediterranean Sea) and post-reproductive or food migration in late summer towards the feeding areas, where both stocks mix (Fromentin and Powers, 2005; Reglero *et al.*, 2014b) (Fig. 1.4). Atlantic bluefin tuna of the eastern stock (east of the 45º meridian) migrates more than 10 000 km in May–June to reach the warm, confined areas of the Mediterranean Sea to spawn, and then return to their foraging grounds in the North Atlantic during July–August (Aranda *et al.*, 2013; Block *et al.*, 2005). Western Atlantic bluefin tuna (west of the 45º meridian) migrates from central and eastern Atlantic foraging grounds to spawn in April–June in the Gulf of Mexico (Block *et al.*, 2005). Several studies have indicated a no mixing on the spawning grounds with a strong natal homing (Block *et al.*, 2005; Dickhut *et al.*, 2009; Rooker *et al.*, 2008). However, the migration

patterns vary considerably among individual age and size, years and areas (Block *et al.*, 2001; Lutcavage *et al.*, 1999). Still, the relative magnitude of trans-Atlantic movement and mixing, and the timing and extent of natal homing are unresolved. Resident individuals are found in the Mediterranean Sea, where they move to productive areas (Gulf of Leon and Ligurian Sea) and can be found in deeper layers (Mather *et al.*, 1995; Tudela *et al.*, 2011).

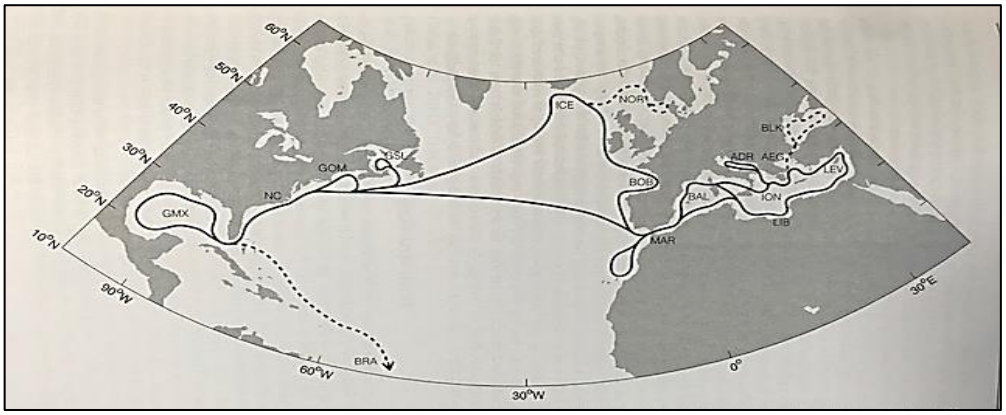


Figure 1.4. Migratory pathways for Atlantic bluefin tuna in the Atlantic Ocean. From Teo and Boustany (2016).

The Mediterranean Sea and the Gulf of Mexico are considered the two major spawning grounds for the eastern and western stocks of Atlantic bluefin tuna, respectively, where reproduction occurs during a short season (Gordoa *et al.*, 2015; Muhling *et al.*, 2017; Reglero *et al.*, 2014b, 2018b). Recently, however, the continental shelf of the Northeastern United States (Slope Sea) has been described as an alternative reproductive area used by smaller tunas than those observed in the Gulf of Mexico (Richardson *et al.*, 2016). Spawning here may occur two months later than in the Gulf of Mexico.

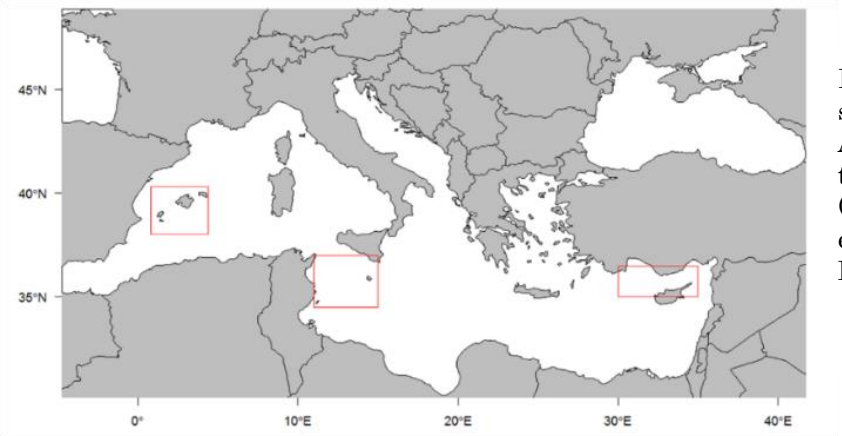


Figure 1.5. Three main spawning grounds of Atlantic bluefin tuna in the Mediterranean Sea (western, center and eastern zones). From Reglero *et al.* (2018b).

Some of the most important spawning areas in the Mediterranean Sea are around the Balearic Islands (western Mediterranean), Sicily, Malta and Tunisia (Central Mediterranean) and Cyprus (eastern Mediterranean) (Koched *et al.*, 2013; Rooker *et al.*, 2007; Sanzo, 1932; Sella, 1924; Zarrad *et al.*, 2013) (Fig. 1.5). The breeding season extends from May to June in the eastern Mediterranean and June to July in the central and western Mediterranean, in correlation with a progressive increase in sea surface temperature from east to west (Reglero *et al.*, 2018b). In general, tunas, including bluefin tunas, reproduce in warm waters with temperatures above 20 °C (Reglero *et al.*, 2014b). Another major characteristic of bluefin tuna spawning areas is their mesoscale oceanographic activity (Alemany *et al.*, 2010).

-Atlantic bonito

Atlantic bonito adult individuals are found along the tropical and temperate coasts of the Atlantic Ocean, the Mediterranean Sea and the Black Sea, and inhabit pelagic waters limited by the continental shelf (epipelagic and neritic) (FAO; Rey *et al.*, 1984) (Fig. 1.6). Among four allopatric species (*S. australis*, *S. chiliensis*, *S. orientalis* and *S. sarda*), *Sarda sarda*, the Atlantic bonito is the only species inhabiting both Atlantic (both sides) and Mediterranean waters. In general, *S. sarda* has a plurality of spawning areas, where the reproduction occurs at precise times (Relini *et al.*, 2005). The spawning season in the Mediterranean usually occurs between May and July, with a maximum in June (Sanzo, 1932; Rodríguez-Roda, 1966). In general, reproduce in water with temperatures above 18-19 ºC (Ortega, 2015). Spawning is well known in the eastern part of the Mediterranean, Black Sea and Marmara Sea (Demir, 1963). However, less information is known about the western Mediterranean, although eggs and larvae have been found around the Catalan coast, Balearic Islands and Algerian coast (see references from Sabatés and Recasens, 2001). Information regarding to migration patterns is scarce and contradictory with some studies suggesting migration to spawning areas in the Mediterranean through the Gibraltar Strait and a reverse migratory from June to September after reproduction (Rey and Cort, 1981). And others suggesting that is resident in the Mediterranean and mature specimens migrate from coastal areas to open sea to spawn, and return at the end of the summer (Sabatés and Recasens, 2001).

Figure 1.6. World distribution of Atlantic bonito. Source www.fao.org.

1.3 Fisheries

-Atlantic bluefin tuna

Tunas are the most valuable fishery resource exploited worldwide (Majkowski *et al.*, 2011) and with the exception of skipjack tuna (*Katsuwonus pelamis*), global stocks are either fully exploited, over-exploited, or depleted (IUCN, 2018). Seven species of tunas support the major part of the tuna market in terms of quantity and economic value in the world (~ 90%): skipjack tuna, albacore, yellowfin tuna, southern bluefin tuna, bigeye tuna, Pacific bluefin tuna and Atlantic bluefin tuna. Among these, the skipack tuna fishery is the most important in terms of volume of caches (primarily sold to the canning industry), and although bluefin tuna contributes relatively little in terms of landings, they are among the most valuable fish in the sea (Majkowski *et al.*, 2011). In the Mediterranean Sea, there are five species of tunas, all of commercial interest: Atlantic bluefin tuna, albacore, bullet tuna (*Auxis rochei*), Atlantic black skipjack (*Euthynnus alletteratus*) and skipjack tuna (Alemany *et al.*, 2010). Atlantic bluefin tuna is the most important tuna and one of the most important species in the Mediterranean, attracting the highest level of commercial interest from the fisheries industry.

Eastern and western stock of Atlantic bluefin tuna are managed by ICCAT. The high market value of tuna stocks has led to intensified fishing pressure that, in turn, has resulted in drastic population reductions (Fernandez-Polanco and Llorente, 2016) (Fig. 1.7). Long-line and mainly purse-seine fleets targeting breeding adults have complemented the traditional trap fisheries in the Mediterranean, increasing the overall effort, which resulted in higher pressure on the population. Also, during the last decade, most of the caught fishes are destined for fattening activities, in which wild specimens are transferred alive to rearing cages—an activity which has contributed to an increase in the misreporting of catches. This caused a deterioration of the spawning stock biomass from the 1970s that led to a full exploitation of the commercial stock (ICCAT, 2011). In 2007, ICCAT coordinated a strict recovery plan, decreasing the total allowable catches (TACs) for the first time, in line with the scientific recommendations—namely, having a minimum legal size of 30 kg (Anonymous, 2007) and following specific capture time limits (Cort and Martínez, 2010). Partly as a result of these protective measures, and possibly also due to an extraordinary good recruitment in 2003 (García *et al.*, 2013), the stock seems to be gradually recovering (Fig. 1.7) (Anonymous, 2017).

The management of the Atlantic bluefin tuna stock is a complicated matter. The eastern and western stocks are evaluated and managed independently by ICCAT. In general, the complexity of breeding migrations and spawning zones leads to a complex estimation of the abundance of the population, which may translate into lower estimates of the vulnerability of this species to exploitation (Fromentin and Powers, 2005). Additionally, ongoing farming (fattening) activities and the underreporting of total catches still affect the representativeness of catch statistics, which are crucial elements for the bluefin tuna stock assessment. There is therefore

an increasing effort to improve the quality and accuracy of fisheries-independent indicators in order to provide more reliable evaluations of the stock status. Recently, ICCAT is working on an alternative larval index, an independent method from the fisheries that will estimate the reproductive biomass by using fish larval abundance in spawning grounds (Ingram *et al.*, 2010, 2015, 2017).

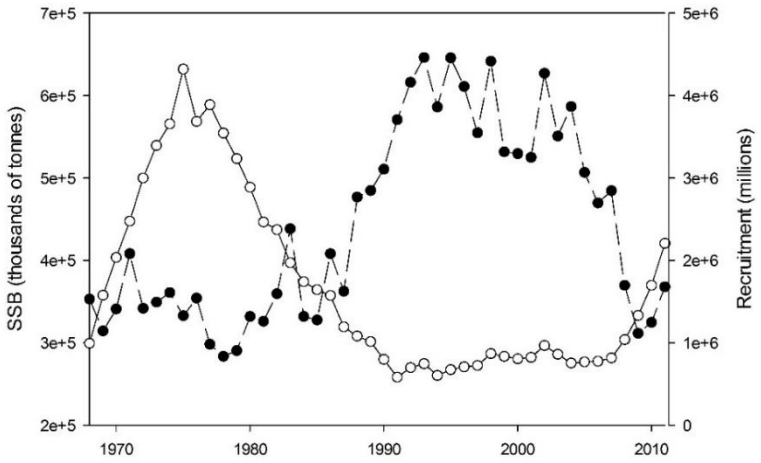


Figure 1.7. Estimates of spawning stock biomass in thousands of tons (white dots) and recruitment at age 1 in millions of fish (black dots) from the 2017 assessment for the eastern stock of Atlantic bluefin tuna (Anonymous, 2017).

-Atlantic bonito

Atlantic bonito is one of the most abundant small scombrid in the Mediterranean Sea. The species is particularly important in the Mediterranean, Black Sea and Marmara Sea where it is taken mainly by artisanal flets, trap net, ring net, gillnet, trammel net, purse seine, beach seine, and hook and line (Demir, 1963). Fishing in the Black Sea and Marmara Sea peaks between May and October, while in the Mediterranean it may vary from area to area or even extend throughout the year (FAO). In the western and central Mediterranean, Atlantic bonitos are mainly fished in coastal waters, but large specimens (60–85 cm fork length) are sporadically present offshore (observations; Relini *et al.*, 2005). Peak fishing of the Spanish fleet all around the peninsula is in late spring and fall. In the western Atlantic (Gulf of Maine), Atlantic bonito is fished between June and October (FAO). Total catches have fluctuated with no apparent increasing or decreasing trends (FAO). This species is also managed by ICCAT, although no proper assessment is conducted. There are no quote estimates or even an established fishing season for Atlantic bonito, and the stock is considered to be in good condition (IUCN, 2018).

1.4 Aquaculture

-Atlantic bluefin tuna

Fishing of different tuna species has been practiced for several millennia, however, tuna aquaculture is a relatively new industry (Benetti *et al.*, 2016; Mylonas *et al.*, 2010). Tuna aquaculture production comprised only 0.2% of the total world tuna production (wild fishery + aquaculture) in 2011 (FAO, 2015). Besides some production of yellowfin tuna in Mexico and Oman between 2004 and 2008, tuna aquaculture is fully focused on the three different species of bluefin tuna (Fernandez-Polanco and Llorente, 2016). Pacific bluefin tuna is cultured in Japan and Mexico, Atlantic bluefin tuna in several countries around the Mediterranean Sea and the Atlantic coast of Portugal (not in the Gulf on Mexico), and southern bluefin tuna only in south Australia. When the three bluefin tuna species are considered separately, their aquaculture production represented 18% of the total world bluefin tuna production (Fig. 1.8a and b).

First tuna aquaculture or farming efforts started in the late 1960s, however, it was not until the early 1990s when commercial-scale farming began. To date, the majority of tuna aquaculture production is reliant upon the capture of wild-caught adult and juvenile induviduals (ICCAT, 2014). This practice makes it difficult to consider the aquaculture independent from the fisheries sectors. Atlantic bluefin tuna fish targeted for farming in the Mediterranean are captured by purse seiners and traditional traps during the spawning migration to the Mediterranean and then they are fattened over 3–7 months (De la Gándara *et al.*, 2016; Karakulak *et al.*, 2016). In Croatia, juvenile tunas are caught and kept in cages for more than 1 year to reach marketable size (Mylonas *et al.*, 2010). There are some criticisms of this aquaculture system, which is strongly based on wild-caught tuna, because illegal, unreported, and unregulated (IUU) Atlantic bluefin tuna fishing is affecting the proper management of wild stocks (Mielgo, 2007).

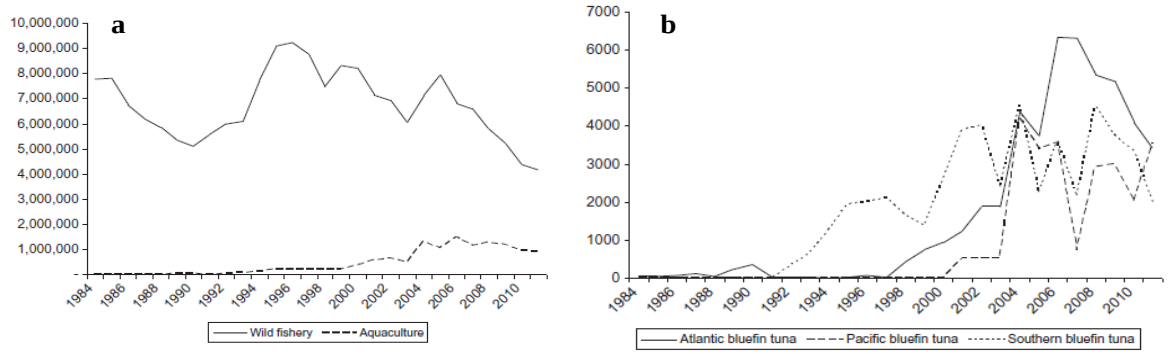


Figure 1.8. a) Compared production of bluefin tuna species. This production does not include the data from Japan. b) Aquaculture production of bluefin tuna. Quantities in tons. Souce FAO 2015, Fishstat plus.

In an effort to reduce the reliance on wild-caught adult individuals and enable consistent supply, there has been an intensive effort on closing the life cycle of Atlantic bluefin tuna in Europe to farming purposes since the early 2000s (De la Gándara *et al.*, 2016). In Japan, research into closed-cycle aquaculture of the relative Pacific bluefin tuna started in the 1970s, and the life cycle was closed in 2002 (Sawada *et al.*, 2005). The Japanese success with Pacific bluefin tuna raised European interest in developing similar spawning and larval rearing protocols to support the Atlantic bluefin tuna farming industry (De la Gándara *et al.*, 2012; Mylonas *et al.*, 2010; Ottolenghi, 2008). The largest land-based bluefin tuna broodstock facility in the world was completed in 2016 at the Spanish Institute of Oceanography (IEO) in Cartagena, Spain (Fig. 1.9). This facility will allow Atlantic bluefin tuna to be spawned in captivity under controlled conditions and represents a significant advancement in the closed-cycle production of this species (De la Gándara *et al.*, 2016). In 2011 the IEO put 3000 juvenile Atlantic bluefin tuna to sea, and in July 2016 these tuna spawned in sea cages, generating the complete closed cycle of Atlantic bluefin tuna (Ortega and De la Gándara, 2017). Turkey, Malta and Croatia are also improving in Atlantic bluefin tuna aquaculture. The closure of the life cycle of southern bluefin tuna has not been succefully achieved yet.

Figure 1.9. Image of the newly built land-based Atlantic bluefin tuna aquaculture facility in Cartagena, Spain.

-Atlantic bonito

The reproduction in captivity and production of juveniles of Atlantic bonito are only carried out experimentally (Chapter 5; Blanco *et al.*, 2017; De la Gándara *et al.*, 2012; Ortega, 2015; Reglero *et al.*, 2014a). There are few research projects that have undertaken the study of captive reproduction, obtaining the development of larval cultures, the feeding of larvae during the first days of life, etc. The completion of the Atlantic bonito life cycle in captivity was succeeded in 2010 (Ortega *et al.*, 2013). Atlantic bonito could be a new candidate to aquaculture for several reasons: it has a quick growth, reaching 1kg in a few months of life (Santamaria *et al.*, 2005), it is capable of reproducing at the end of its first year of life (Rey *et al.*, 1984; Macías *et al.*, 2005) and it is also a valuable and well-known species.

-Challenges in aquaculture

The culture of Atlantic bluefin tuna and Atlantic bonito is a very difficult task and still fluctuates due to the significant mortality events occurring during every life stage. These can be summarized as (i) floating death and sinking death during the first 8 days of life (Tanaka *et al.*, 2009) (ii) cannibalism and collision death during the post-flexion stages, and (iii) transfer-related mortalities after they are moved from land-based hatcheries to sea cages. Mortality rates during each period can approach 90% and the overall average survival of tuna larvae ranges from 0.01% to 4.5% (De la Gándara *et al.*, 2012).

Feeding tuna larvae also represents a challenge not experienced in the culture of other marine fish, as they must be fed on the yolk-sac larvae of other marine fish, with feeding on *Artemia* alone resulting in "growth failure" (Seoka *et al.*, 2007). The different feeding transition phases, planktivory and piscivory, have been seen to generate two bottlenecks in the survival of both species where a delay in the timing of onset of the different diet can caused significant mortalities (see section 1.5) (Reglero *et al.*, 2014a). Arguably, cannibalism remains the most serious problem causing low survival rates of all species of tuna larvae. To minimize the effects of cannibalism, it is important to switch the live prey offered, from planktivory to newly hatched fish larvae such as sea bream (*Sparus aurata*) (De la Gándara *et al.*, 2010; Sawada *et al.*, 2005).

1.5 Life cycle of Atlantic bluefin tuna and Atlantic bonito

Atlantic bluefin tuna and Atlantic bonito share similar life history at early developmental stages, despite differing life history traits as adults (Fig. 1.10). Both species spawn pelagic eggs that hatch into larvae within a few days (~3–4 mm long), grow fast and develop foraging and swimming organs, then metamorphose into juveniles during the first month of life (De la Gándara *et al.*, 2012; Kaji *et al.*, 1996; Margulies *et al.*, 2007; Miyashita *et al.*, 2001). Both species share spawning grounds in oligotrophic areas where the abundance of plankton prey is often low, although bonito can also spawn in more coastal areas (Mena *et al.*, 2016; Reglero *et al.*, 2018b; Rey *et al.*, 1984). The growth rate in Atlantic bonito larvae is higher than in Atlantic bluefin tuna (Chapter 5; Blanco *et al.*, 2017; De la Gándara *et al.*, 2012; Reglero *et al.*, 2014a) (Fig. 1.11).

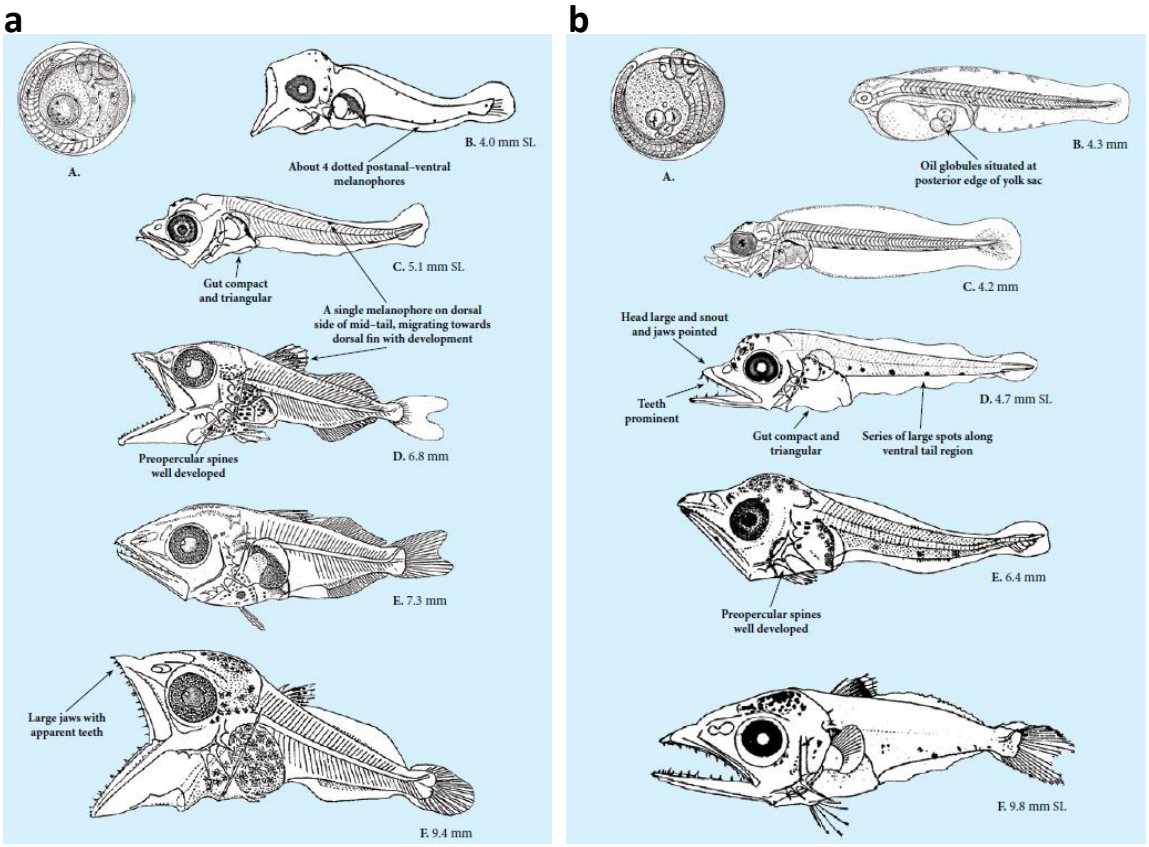


Figure 1.10. Morphological and developmental differences during the larval stage of Atlantic bluefin tuna (a) and Atlantic bonito (b). From Rodríguez *et al.* (2017).

The nomenclature of the different early developmental stages of both species in this thesis follow the one suggested by Kendall *et al.* (1984). They divided the early life history of fishes into three stages: egg, from spawning to hatching; larva, from hatching to attainment of complete fin-ray counts and beginning of squamation (juvenile); and juvenile, young fish, fundamentally like the adult in the meristic characters (excluding squamation) but smaller and reproductively inactive. Kendall *et al.* (1984) also divided the larval stage into four sub-stages: yolk-sac larva, from hatching to exhausting of yolk reserves (endogenous feeding); pre-flexion larva, from yolk exhausting (start of exogenous feeding) to the beginning of upward flexion of the notochord; flexion larva, ending when the urostyle is in its final position, 90 º; and post-flexion larva, ending when metamorphosis begins.

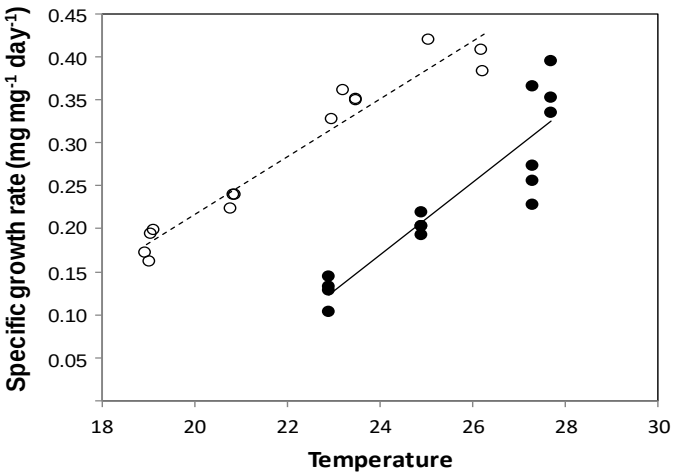


Figure 1.11. Relationship between specific growth rate of larvae and water temperature in planktivorous Atlantic bluefin tuna (black dots) and Atlantic bonito (white dots). The specific growth rate with temperature was estimated from the slope of an exponential curve between age (experimental day) and dry weight (Reglero *et al.*, unpublished).

1.5.1 Eggs, endogenously and exogenously planktivorous feeding life stages

Both Atlantic bluefin tuna and Atlantic bonito spawn spherical eggs with an average diameter of 1–1.3 mm (Ortega, 2015) (Fig. 1.12a, b). Atlantic bluefin tuna eggs normally have a single oil globule, but some eggs can present two or even three globules. At hatching, Atlantic bluefin tuna larvae had a total length of 3.3 ± 115.7 mm (Fig. 1.12c). Atlantic bonito eggs had on average 3.4 ± 0.6 oil globules and the larvae hatch at a total length of 4.3 ± 105.4 mm (Ortega, 2015) (Fig. 1.12b, d).

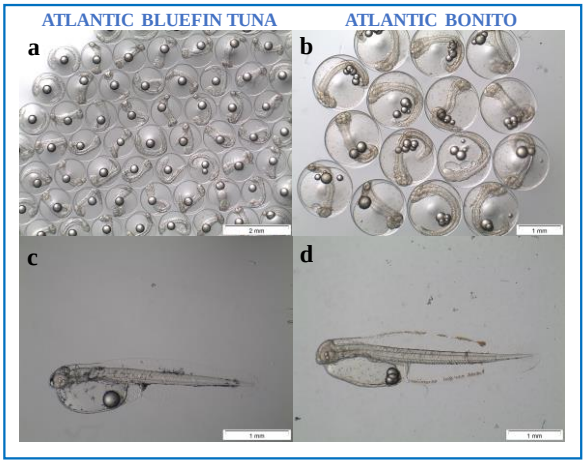


Figure 1.12. Picture of the eggs and newly hatched Atlantic bluefin tuna (a and c) and Atlantic bonito (b and d).

The thermal range for viable egg hatching is slightly colder in Atlantic bonito than Atlantic bluefin tuna—ranging from 14–27 ºC and 19–32 ºC, respectively (Fig. 1.13a). According to Ortega (2015), Atlantic bluefin tuna eggs do not hatch below 19 ºC and above 32 ºC. Between 21–29 ºC hatching success is above 50%, and between 23–26 ºC hatching success is about 75%. In Atlantic bonito, no hatching occurs at temperatures below 14 ºC and above 28 ºC, the probability of hatching success is more than 50% between 16–26 ºC, and there is about a 75% chance at the optimum temperature, between 18–23 ºC. The developmental time to hatch the eggs decreases when the temperature increases. At lower temperatures, the developmental time for hatching of Atlantic bonito eggs is higher than for Atlantic bluefin tuna, with 10 hours of difference at 20 ºC. However, at higher temperatures, from 25 ºC, similar egg developmental time to hatch occurs in both species (Fig. 1.13b).

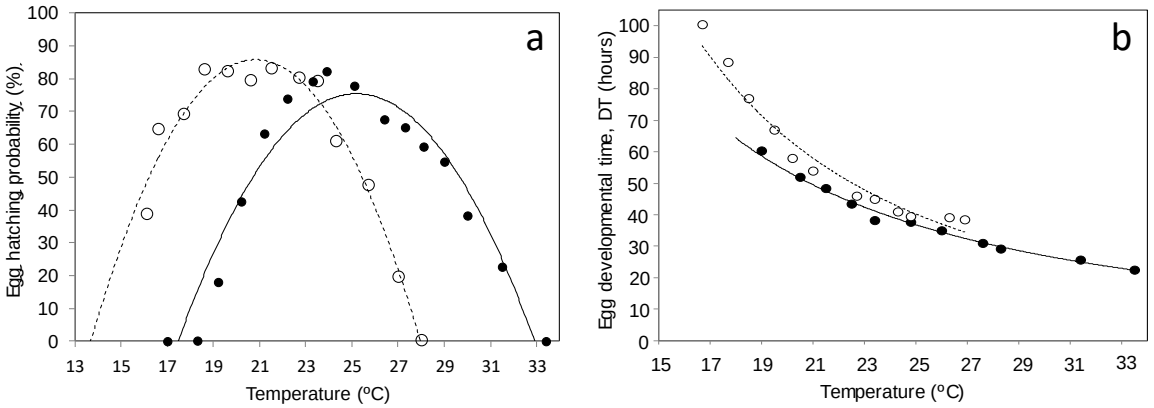


Figure 1.13. a) Relationship between egg hatching probability and water temperature in Atlantic bluefin tuna and Atlantic bonito, b) relationship between egg developmental time and water temperature in Atlantic bluefin tuna and Atlantic bonito. Black dots for Atlantic bluefin tuna and white dots for Atlantic bonito (Ortega, 2015).

The transition from endogenous to exogenous feeding is considered one main bottleneck affecting larval survival in Atlantic bonito and Atlantic bluefin tuna as observed for other fish species (Hjort,1914). Nearly all offspring produced (>99.9%) in most marine fish species, will not survive their first year of life, and the strength of the recruitment is determined shortly after yolk-sac absorption, at the beginning of exogenous feeding, when the larvae must find suitable prey in sufficient amounts (Houde, 2008) (Fig. 1.14). Failing to find adequate feeding conditions would lead to massive larval mortality in a short period of time, making successful "first-feeding" a prerequisite for survival (Hjort, 1914). Given the large number of offspring produced, subtle differences in mortality rates can cause order-of-magnitude differences in recruitment from year to year (Hjort, 1914; Leggett and Deblois, 1994). This stage has been associated with massive mortalities both in nature and in laboratory populations. After the exhaustion of yolk reserves, deprivation of food in nature as well as inappropriate food quality and feeding procedure in reared populations reduce survival substantially in the first days or weeks.

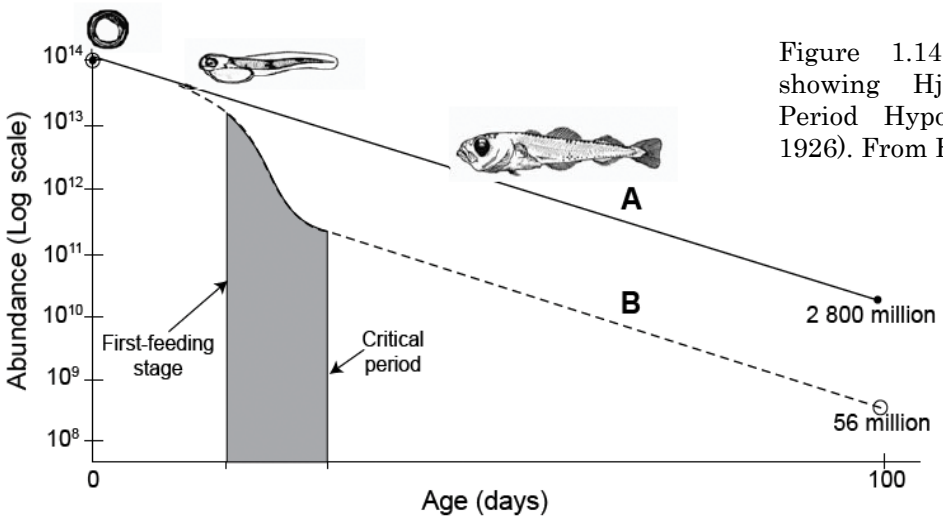


Figure 1.14. Illustration showing Hjort's Critical Period Hypothesis (1914, 1926). From Houde (2008).

In Atlantic bonito and Atlantic bluefin tuna larvae, the short duration of the yolk-sac (~ 2 days after hatching) and the fast exhaustion of the inner reserves forces the ingestion of prey items just one or two days after hatching (De la Gándara *et al.*, 2012; Ortega, 2015). Successful feeding during the first days of life is key for larval survival as high ingestion rates are necessary to maintain growth and metabolic needs. High mortalities after only one day of starvation have been seen in planktivorous Pacific bluefin tuna (Tanaka *et al.*, 2008). In the laboratory, first prey items usually consist of enriched rotifer (see all chapters in this Ph.D. thesis) since culture with copepods (their natural prey in the field) is still under development (Ortega, 2015).

Although there is still limited knowledge of the two species, the planktivorous feeding stage is the most studied period in the laboratory, and a detailed description of organ development has been described (Yúfera *et al.*, 2014). In the field the planktivorous feeding stage is also the most common stage captured during the ichthyoplankton surveys, which have provided data to help understand larval spatial distribution and timing (Alemany *et al.*, 2010; Reglero *et al.*, 2012). However, there are still major gaps in our knowledge of basic ecology during this developmental stage, such as the role that temperature has on driving the vertical distribution of the larvae (see Chapter 7) or the mechanisms that explain why larvae attains the flexion stage at different ages (see Chapter 3). The flexion of the notochord is considered an important event in the early life stage of the life of fishes (Kendall *et al.*, 1984). It is a particularly important development in Atlantic bonito and in Atlantic bluefin tuna because it coincides with the development of an adult-type digestive system (Kaji *et al.*, 1996, 1999; Yúfera *et al.*, 2014) that leads to a transition to a piscivorous diet during the larval stage (Chapter 4–6; Blanco *et al.*, 2017, 2018; Reglero *et al.*, 2014a, 2015). This development of an adult-type digestive system occurs earlier in Atlantic bluefin tuna and Atlantic bonito relative to other marine fishes. The completion of the flexion allows the larvae to improve their swimming and reach new prey (Kendall *et al.*, 1984; Osse *et al.*, 1995; Roy *et al.*, 2014). Growth variability to flexion time has been observed in Pacific bluefin tuna (Takebe *et al.*, 2012; Tanaka *et al.*, 2006), however an explanation for it has not yet been found in larvae reared under the same abiotic and *ad libitum* feeding conditions (Chapter 3).

1.5.2 Exogenously piscivorous feeding life stage

Piscivorous feeding during the larval stage is known to be a second bottleneck in the survival of both Atlantic bonito and Atlantic bluefin tuna (Reglero *et al.*, 2014a). Other species of scombrids also pass through a piscivorous feeding stage. An early transition to piscivory has previously been shown to benefit survival and increase growth in cultured Atlantic bluefin tuna and Pacific bluefin tuna with high survival and growth rates only achievable when fish larvae were included in diets (Reglero *et al.*, 2014a; Tanaka *et al.*, 2014a). Evidence for the onset of piscivorous behavior in Atlantic bonito and in Atlantic bluefin tuna has been observed in the laboratory (Fig. 1.15) at larval lengths between 7–9 mm and 8–10 mm respectively.

Figure 1.15. Atlantic bonito larva feeding on sea bream yolk-sac larva. Source: IEO Murcia.

In the field, evidence of a piscivorous diet has already been reported during the early larval stages for some tuna species (*Thunnus alalunga* Catalán *et al.*, 2011; *Euthynnus alleteratus*, *Katsuwonus pelamis*, *Axis rochei* Llopiz *et al.*, 2010; *A. rochei* Morote *et al.*, 2008; *Thunnus macoyii* and *K. pelamis* Young and Davis, 1990). However, it has not yet been documented in the field for Pacific bluefin tuna larvae. High rates of piscivory and even cannibalism in Atlantic bluefin tuna larvae has been reported in the Gulf of Mexico at lengths 8–10 mm (Llopiz and Hobday, 2015; Llopiz *et al.*, 2015). Based on modelling work, it has been shown that Atlantic bluefin tuna larvae cannot solely survive on zooplankton diets and consequently must prey on fish larvae at post-flexion stages to provide the sufficient energy for growth and therefore survival (Reglero *et al.*, 2011). However, it was not until recently that piscivory in Atlantic bluefin tuna larvae in the Mediterranean Sea was found in larvae > 7 mm (Uriarte *et al.*, 2017). The lack of evidence of piscivory in the field could be due to the capturability of large larvae since individuals longer than 7 mm are scarcely captured during ichthyoplankton surveys by the oblique Bongo 60 and surface Bongo 90 plankton tows, mainly due to net avoidance (Satoh *et al.*, 2008; Tilley *et al.*, 2016). For Atlantic bonito larvae there is no knowledge of their diet in the field, since their larvae are usually very scarce in ichthyoplankton surveys and no trophic studies have been conducted yet.

The start of the piscivory is associated with an increase in the growth rate of the larvae (Reglero *et al.*, 2014b; Tanaka *et al.*, 2010, 2015). The fast growth, along with the mortalities caused by the dietary transition, can cause size differences among individuals of the same cohort (Hecht and Pienaar, 1993) due to suggested size-selective mortality during the larval stage (McGurk, 1986). However, knowledge is scarce regarding how mortality affects the size spectrum of the larvae that survive through piscivory and become part of the juvenile population (Chapter 4; Blanco *et al.*, 2018).

1.6 Methods to study larval growth and survival

The knowledge of the ecology of the early life stages of fishes is crucial to understanding the population dynamics of fish stocks, and also, the functioning of marine ecosystems. Laboratory studies are a perfect tool to understand how different variables drive fish physiology and behavior and eventually influence growth and survival by manipulating abiotic and biotic factors. Those responses are difficult to estimate from field-sampled individuals, as fish individuals are exposed to multidimensional environmental conditions that simultaneously vary and interact, and by the time the fishes reach the boat they are often dead or not fit for study. Even though the complexity of the aquatic environment makes it difficult to replicate adequately in the laboratory, it is the best and more appropriate alternative in order to obtain those data that are unknown in the field.

A wide array of technological advances and new analytical techniques developed in the last several decades have allowed for significant advances in the knowledge of the growth process and the causes of mortality during the early life stages of fishes (*e.g.* Fiksen *et al.*, 2007; Moyano *et al.*, 2017; Reglero *et al.*, 2014a). In this Ph.D. thesis study, we have focused on the use of microstructure analysis of otoliths (Chapter 4; Panella, 1971) and of the biomolecular analysis of condition (Chapters 3 and 6; Buckley, 1984) as proxies for growth and survival in fish larvae.

1.6.1 Microstructure analysis of otoliths

Otoliths (ear stones) are small calcified structures found in the head of the fish which assist in detecting sound and are used for balance and orientation (Fig. 1.16). Otoliths are natural data loggers that record information in their microstructure and chemistry at different temporal scales (Campana, 1999). The presence of these daily structures enables not only a size-independent estimate of age, but also of growth and mortality of larvae and juvenile fish, assuming a relationship between otoliths' length increments and somatic growth (Campana and Jones, 1992; Folkvord *et al.*, 2004). Previously formed increment widths-at-age in an otolith, will remain unchanged as the fish continues to grow over time. If the temporal significance of these increments has been established, comparison of increment widths at given ages of fish sampled at later stages will have the potential to reveal any changes in the increment width pattern (or otolith size distribution) among fish from the sampled population (Folkvord *et*

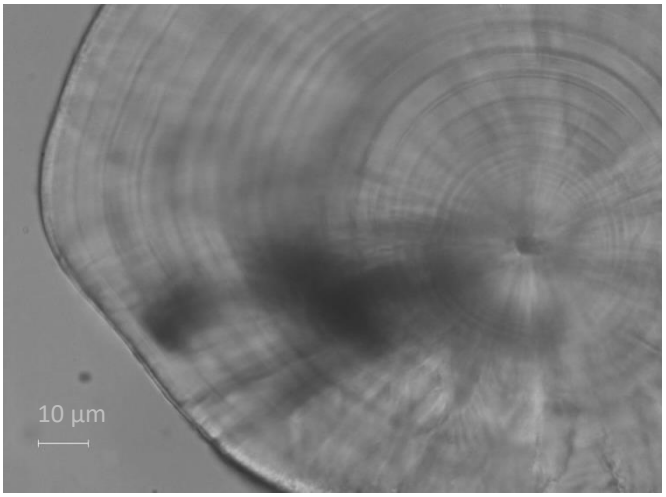


Figure 1.16. Image of the otolith of Atlantic bluefin tuna 27 days post-hatch.

al., 2010; Mosegaard *et al.*, 2002). The analysis of the otoliths' microstructure also allows for assessing the environmental influence of larval survival and dating early life events with high precision and relating these to environmental conditions.

1.6.2 Biochemical analysis of condition

Another indicator useful to determining nutritional status and recent growth rate in fish larvae is nucleic acid analysis (RNA, DNA) (Buckley, 1984). In general, the nutritional condition describes the "state of being" of an organism on different levels of biological organization, from cell to population, and throughout ontogeny and habitats using morphological, histological, biochemical or ethological proxies. Studies of condition of individuals has been widely used to assess whether they were suffering from food- limitation and/or were at risk of mortality due to starvation (*e.g.* Foley *et al.*, 2016).

The nucleic acid biochemical method uses tissue components, such as ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), to indirectly measure increases in somatic tissue that result in size change. Briefly, the amount of DNA in cells is constant and the amount in a sample reflects the size of the sample (numbers of cells being analysed). Whereas the amount of the RNA is variable and reflects the amount of recent protein synthesis (Buckley *et al.*, 1999). Nucleic acid-derived indices such as DNA:dry weight or RNA:DNA are sensate to nutritional status. Fishes in good condition are relatively fat and well-fed and their future prospects for continued growth are good (Chícharo *et al.*, 1998; Clemmesen, 1987).

The biggest advantage of biochemical indices is, next to their accuracy, objectivity and fast processing of large sample numbers that they quickly respond to changes in nutritional condition. Depending on temperature, changes can already be detected after 1-3 days of starvation (Clemmesen, 1994; Buckley *et al.*, 1999).

1.7 Aims of the study

This thesis aims to improve knowledge of the growth and survival of the larval stage of Atlantic bluefin tuna and Atlantic bonito under different light, food and temperature conditions. The results of this thesis are based on laboratory experiments (Chapters 3-6) and on combined field and laboratory results (Chapter 7).

The flexion of the notochord is considered a crucial point in the growth and mortality of Atlantic bluefin tuna and Atlantic bonito larvae. The flexion increases the swimming capacity of the larvae and aids in the capture of mobile prey. The flexion coincides with the timing of the start of piscivory, which is considered a second bottleneck in these species. Chapter 3 analyses the relationship between the flexion of the notochord and the nutritional condition of the larvae in order to find a possible explanation for the different flexion timings for individuals that are of the same age and cultured under the same conditions. Once the larvae complete the notochord flexion and therefore start with a piscivorous diet, growth is highly increased in

comparison with the previous phase. A delay in the start of piscivory is known to generate high mortalities. Chapter 4 examines which size spectrum of the larvae will be more affected by a manipulation of the timing of onset of piscivory, an important aspect since mortality during the larval stage is assumed to be size-selective.

Atlantic bluefin tuna and Atlantic bonito larvae are visual feeders (Chapter 5; Blanco *et al.*, 2017). The high growth rates resulting from the piscivorous phase are in part due to the optimal nutritional condition of the fish prey and of the amount of prey they feed on during the light periods. Chapter 5 investigates if a change in the feeding regime, from continuous to intermittent piscivorous feeding, has an effect upon larval growth and survival. The intermittent feeding schedule was manipulated by the use of light, with larvae feeding in light and stopping while in darkness. Besides the interruption of feeding, in darkness fish larvae are assumed to reduce their swimming and metabolism. Chapter 6 estimates the metabolic cost (oxygen consumption) of Atlantic bluefin tuna larvae with light and darkness conditions.

The effect of temperature in fish larval vertical distribution is analysed in Chapter 7. Temperature may be a dominant cue influencing the vertical distribution of Atlantic bluefin tuna and Atlantic bonito larvae however, there is a lack information about how the thermal tolerance of these species restricts their vertical distribution. This study combines laboratory and field studies to improve the understanding of the environmental requirements for Atlantic bluefin tuna and Atlantic bonito larvae.

CHAPTER 2

- MATERIALS AND METHODS -

MATERIALS AND METHODS

2.1 General characteristics of the facility

Most of the data in this thesis comes from eggs and larvae obtained from captive breeding animals. Only in the analyses described in Chapter 7 were larvae sampled under natural conditions, in addition to laboratory conditions. The rearing and the experimental work were carried out in a facility owned by the Spanish Institute of Oceanography (IEO), a few meters from the sea in Puerto de Mazarrón (Murcia, southeastern (SE) Spain, along the Mediterranean coast) (Fig. 2.1).

Figure 2.1. a) Localization of the facility in the southeast of Spain shown with a yellow arrow, b) aerial picture of the location of the facility from the coast and c) close picture of the whole facility. Images a and b obtained from @Google earth.

The facility consists of three sheds equipped with tanks to culture a variety of marine fish species, including sea bream (*Sparus aurata*), shi drum (*Umbrina cirrosa*), sea bass (*Dicentrarchus labrax*), and common dentex (*Dentex dentex*), along with Atlantic bluefin tuna (ABFT) (*Thunnus thynnus*) and Atlantic bonito (AB) (*Sarda sarda*), the two targeted species for this thesis. The egg and larval culture experiments outlined in this Ph.D. thesis took place in the part of the facility with seven circular tanks of 5000 L (2.5 m diameter and 1 m depth) and eighteen circular tanks of 1500 L (1.5 m diameter and 0.85 m depth), in addition to one experimental module with 16 cylindrical tanks of 150 L (0.55 m diameter and 0.65 m depth). The tanks were all placed together in one of the sheds, except the 150 L tanks, which were located in a separated room (Fig. 2.2). Also, two tronco-conical incubation tanks of 400 L (1 m diameter) were used occasionally for egg rearing for very short periods (hours) (Chapter 3). For some experimental work, specific tanks had to be designed and constructed, for example the experimental columns used in Chapter 7. The facility also includes the equipment needed to produce the phytoplankton, enriched rotifers and enriched *Artemia franciscana* nauplii (AF, INVE AQUACULTURE, Belgium) that are used to feed the larvae during the first life stages (Chapters 3–7) and to produce sea bream yolk-sac larvae that are used for feeding ABFT and AB during the post-flexion larval stage (see Chapters 4–6) (Fig. 2.2). There is also a well-equipped laboratory and a -80 ºC freezer to preserve the samples. A specific piece of equipment consisting of a respirometer system was used to measure fish larval oxygen consumption, detailed in Chapter 6.

Figure 2.2. Several images of the equipment of the facility used during this Ph.D. thesis. The pictures inside the red box show the auxiliary cultures of phytoplankton, rotifers, *Artemia* and sea bream breeders. Their yolk-sac larvae were used in the Atlantic bluefin tuna and Atlantic bonito experiments for feeding purposes. Inside the green box the different type of tanks used during experimentation are shown.

2.2 This thesis

Figure 2.3. Picture of the oceanographic cruise "Bluefin 2011" where part of the larvae used in the field part of the Chapter 7 were obtained. Pic by Pierre Vandenbussche.

The experiments in this thesis were conducted during the summers of the period from 2012 to 2016. This period coincides with the technical development and the expertise needed to succeed on the culture of ABFT and AB in the laboratory as well as with the beginning of collaboration between experts on tuna to find synergies between laboratory and field work. In 2012–2013, previous to the formal start of the Ph.D. grant, the experiments targeting the hypotheses proposed in this Ph.D. thesis were conducted in the framework of the research project "Assessment of the Atlantic bluefin TunA population breeding in the western MEditerranean" (acronym ATAME) funded by the Spanish Government, to which this Ph.D. grant was associated. AB eggs were only available in 2013 and 2016 and therefore the experiments in this thesis related to bonito only refer to activities conducted during those two years (Table 2.1). In contrast, ABFT eggs were available every year and experiments were conducted yearly, except in 2013 when no experiments with ABFT included in this Ph.D. were conducted. In 2012, the experiments were conducted to test size-selective mortality in ABFT (Chapter 4). In

2013, the experiments testing the vertical distribution of AB larvae were conducted. These experiments were complemented by the same type of experiments on ABFT in 2014 (Chapter 7) (Fig. 2.4a). This chapter also included field-sampled larvae obtained during a scientific cruise that was conducted in 2011 and 2012 in the Balearic Sea, the major spawning area for this species in the western Mediterranean Sea. I participated as a Master's student in the 2011 cruise (Chapter 7) (Fig. 2.3). In 2013, the experiments on intermittent feeding on AB were conducted and complemented in 2014 for ABFT (Chapter 5). Summer 2015 was the year when the experiment to test flexion in ABFT was conducted, with the same experiment with AB conducted in 2016 (Chapter 3). Finally, in 2016 the experiments to measure the metabolism in ABFT were performed (Chapter 6) (Fig. 2.4b, c and d). See Table 2.1 for a summary of this information.

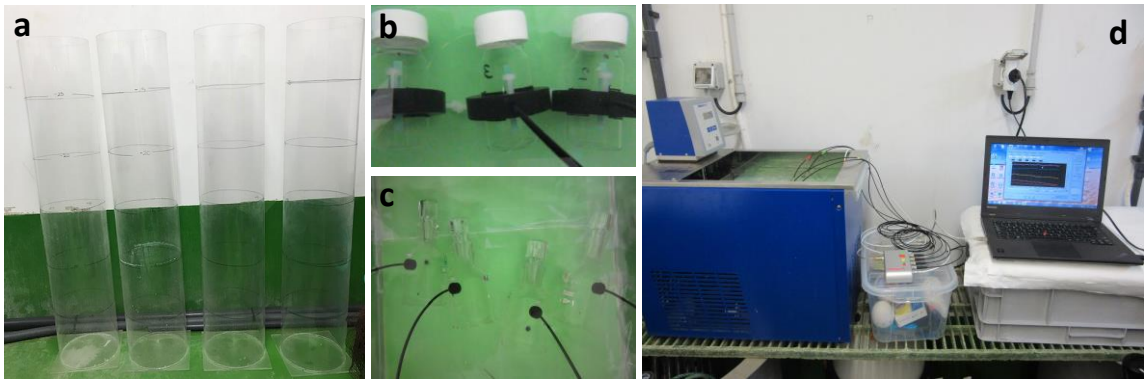


Figure 2.4. Images of the laboratory part of the experiments described in Chapter 7 (a), and the material and set-up used in the experiments described in Chapter 6 (b, c and d). a) Vertical columns used for Atlantic bluefin tuna and Atlantic bonito, where thermocline and isothermal conditions were generated for the experiments described in Chapter 7. Glass chambers of b) 20 mL and c) 100 mL used for the measurement of oxygen consumption. d) Set-up of the equipment used to measure oxygen consumption in Atlantic bluefin tuna larvae.

Table 2.1. Chronogram explaining how and when the data used in this Ph.D. thesis was generated. This thesis contains data obtained in 2011 and 2012 during an oceanographic cruise (described in Chapter 7). The rest of the data were gathered by running experiments during the summer, when Atlantic bluefin tuna (Bluefin) and Atlantic bonito (Bonito) larvae are available. The biochemical laboratory work was done at the Spanish Institute of Oceanography in Gijón, and some of the analysis was performed at the University of Bergen.

Collaborations			Ph.D. grant			
2011	2012	2013	2014	2015	2016	2017
OCEANOGRAPHIC CRUISE				BIOCHEMICAL ANALYSIS IN GIJÓN		STAY AT THE UNIVERISTY OF BERGEN
	EXPERIMENTS*					
Species: **BLUEFIN** Experiment: **vertical field sampling (part I)** Chapter: **7**	Species: **BLUEFIN** Experiment: **vertical field sampling (part II)** Chapter: **7**	Species: **BONITO** Experiment: **vertical laboratory (part III)** Chapter: **7**	Species: **BLUEFIN** Experiment: **vertical laboratory (part IV)** Chapter: **7**	Species: **BLUEFIN** Experiment: **flexion (part I)** Chapter: **3**	Species: **BONITO** Experiment: **flexion (part II)** Chapter: **3**	
	Species: **BLUEFIN** Experiment: **size-selective mortality** Chapter: **4**	Species: **BONITO** Experiment: **pulse feeding (part I)** Chapter: **5**	Species: **BLUEFIN** Experiment: **pulse feeding (part II)** Chapter: **5**		Species: **BLUEFIN** Experiment: **metabolism** Chapter: **6**	

*All experiments were conducted in June and July, coinciding with the spawning season, when larvae are available.

2.3 Obtainment of the eggs

Batches of fertilized eggs were obtained differently for ABFT and AB. Both species have a narrow environmental window for spawning (Reglero *et al.*, 2018b), which reduces the days available to work with these species. In addition, the difficult task of obtaining eggs, along with the difficulty of the culture process (see Chapter 1), does not guarantee the ability to carry out experiments every year. Whereas ABFT eggs were obtained from naturally spawning captive adults in different farming facilities, AB eggs were obtained from the natural spawning of captive adults reared at the Spanish Institute of Oceanography in Puerto de Mazarrón (in 2013) and from hand stripping adult individuals captured in an almadraba trap in La Azohía, Cartagena (SE Spain) (in 2016).

-Atlantic bluefin tuna

From 2012 to 2014, ABFT eggs were obtained from two cages with naturally spawning captive adult individuals captured in waters off the Balearic Islands (western Mediterranean), in the farming facilities at El Gorguel, Cartagena (SE Spain), owned by the private company Caladeros del Mediterráneo S.L. In 2012, one broodstock circular cage (25 m in diameter and 20 m in average depth) was monitored to obtain ABFT eggs. Due to sabotage, this cage was later lost. During 2013 and 2014, the other broodstock, maintained in a circular cage with the same dimensions, was used for the egg collection. In 2015 and 2016, ABFT eggs were obtained from a broodstock lot coming from newly captured adult individuals by purse-seine fishing, captive in circular cages (50 m in diameter and 16 m in average depth) in the farming facility at San Pedro (SE Spain) owned by Tuna Graso S.A. Broodstocks in the cages were fed to satiety once a day, from Monday to Saturday, on a diet of raw fish (De la Gándara *et al.*, 2016).

During 2012–2014, during the spawning season (end of May to mid-July), sea cages were covered by a vertical PVC curtain that surrounded the entire cage perimeter from 50 cm above water to 6 m below the surface (Fig. 2.5a). The curtain was not completely closed and the opening was located facing the current in order to allow water exchange inside the cage. Every night during the spawning season (from 2:00–5:00 a.m.), if the weather conditions allowed it, two workers travelled to the cages by boat to collect the eggs. ABFT floating eggs are normally accumulated in the opposite part of the cage from the curtain opening, and were collected by a landing net of 500 µm mesh size (Fig. 2.5b). In 2015 and 2016, the eggs were captured using the same landing net from the outside of the commercial cages, where no PVC curtain was placed (De la Gándara *et al.*, 2010, 2016).

After the egg collection, they were transferred to 20 L cubitainers. A maximum of 200 000 eggs were introduced into each one. The cubitainers were filled with 10 L of sea water and compressed oxygen was added in order to maintain high oxygen levels during the transportation to the facility of the Spanish Institute of Oceanography in Puerto de Mazarrón.

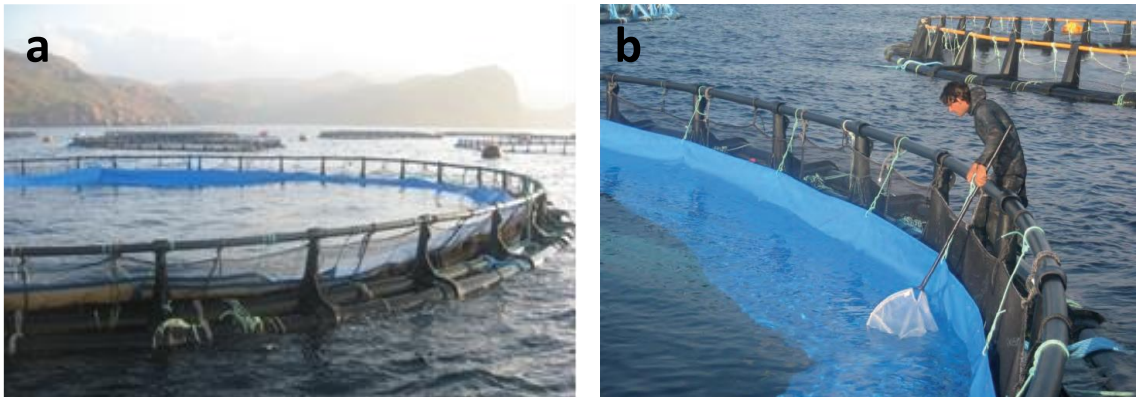


Figure 2.5. Images of the captive cages used from 2012–2014. a) Farming facility at El Gorguel with the PVC curtain placed in the monitored cage and b) collection of the eggs by a worker from the inside of the cages by a landing net of 500 µm.

-Atlantic bonito

In 2013, fertilized eggs were obtained from seventeen natural spawnings of AB, 9 females and 8 males with an average weight of 2 kg, reared in captivity in an indoor 55 m^3 rectangular tank at the facilities of the Spanish Institute of Oceanography in Puerto de Mazarrón. The adults died during that winter and no more captive AB were available. In 2016, fertilized eggs of AB were obtained from adult individuals captured in an almadraba trap in La Azohía after hand stripping on-board (Fig. 2.6). The fertilization was performed by collecting the eggs of one female and the sperm of two males that were mixed in a bowl. After two minutes a bit of sea water was added, and after fifteen minutes the fertilized eggs were placed into a cubitainer with fresh sea water and moved to the Spanish Institute of Oceanography in Puerto de Mazarrón as indicated above. The eggs were observed for a few hours, after which time it was possible to observe that the fertilization had succeeded.

Figure 2.6. Picture of the traditional almadraba trap in La Azohía where from fertilized Atlantic bonito eggs were obtained in 2016 by hand stripping several captured adult individuals.

2.4 From eggs to hatching

Once in the facility, the same protocol was used for ABFT and AB eggs. All eggs were transferred to a 5 L glass chamber, where floating (live fertilized) and sinking (dead fertilized) eggs were separated by gravimetry (density) after removing the aeration in the glass chamber. Unfertilized eggs are assumed to sink soon after spawning and most of the eggs that reach the installations are fertilized eggs that die for unknown reasons (De la Gándara *et al.*, 2014). The fertilized eggs were counted and transferred to incubation tanks, where after several hours the eggs or the hatched larvae were transferred to rearing tanks.

Sometimes incubation tanks (400 L) were used until larvae hatching (Chapter 3). Eggs were then inoculated at densities lower than 1000 eggs L^{-1}, at light conditions of 24 hours of light, moderated aeration and a water flow of 100–200 L $hour^{-1}$. ABFT needs around 38–40 hours in order to hatch at 23–26 ºC, and 30 hours at 27–28 ºC (see Chapter 1: Fig. 1.13b). AB needs 50 hours to hatch at 23 ºC.

In other occasions (Chapters 3 to 7), the eggs, at densities lower than 10 eggs L^{-1}, were placed in tanks of 5000 L volume. The conditions in these rearing tanks during egg rearing were similar to those in the 400 L incubation tanks, although the photoperiod was set at 15L:9D and the water flow was increased. In these tanks, the larvae were maintained after hatching until the juvenile stage, when they were moved to weaning tanks.

2.5 Larval rearing

The diet during rearing of the larvae consisted of different prey as the larvae increased in size. From 2 to 14 days post hatch (dph), ABFT larvae were fed on enriched rotifers (*Brachionus plicatilis*), from 12 to 16 dph enriched Instar II *Artemia* nauplii (*Artemia franciscana*) (AF, INVE AQUACULTURE, Belgium), from 16 to 30 dph sea bream yolk-sac larvae (*Sparus aurata*) and once they are juvenile, from 25 to 30 dph onwards they are fed on dry food or minced frozen small pelagic fishes (De la Gándara *et al.*, 2012, 2016; Ortega, 2015).

After two days of endogenous feeding the AB larvae started exogenous feeding, consisting of enriched rotifers (*Brachionus plicatilis*) from 2 to 9 dph, and then enriched Instar II *Artemia* nauplii (*Artemia franciscana*) (AF, INVE AQUACULTURE, Belgium) from 5 to 12 dph. From 8 to 22 dph they fed piscivorously with sea bream yolk-sac larvae (*Sparus aurata*) and from 18 dph onwards they were fed dry food or minced frozen small pelagic fishes (Ortega, 2015).

In general, rotifers were added in the tanks, up to 5–10 rotifers mL^{-1} a couple of times every day. Rotifers were fed on DHA-enriched *Chlorella* (Super fresh Chlorella SV-12, Chlorella Industry CO., Ltd., Japan), and three hours before being offered to the larvae, they were enriched with Origreen (Skretting, Nutreco). *Artemia* nauplii were also added twice a day. Before, Instar II stage of *Artemia* were enriched over a minimum of a 14 hours period with Origreen and then offered to larvae at densities

of 0.1–0.5 *Artemia* mL^{-1} according to larvae survival. 0–2 dph sea bream yolk-sac larvae were added twice every day to reach 300 yolk-sac preys per predator larvae. During this period, *Chlorella* or cultivated microalgae of *Nannochloropsis gaditana* were added to the tanks to maintain pseudo-green water conditions.

2.6 From the facility to the laboratory

Larvae in Chapters 3 and 6 were analysed at the biochemical laboratory of the Spanish Institute of Oceanography in Gijón. The concept of condition in fish larvae was analysed. Among the most frequently used biochemical condition proxies for larval fish are the RNA:DNA and DNA:dry weight ratios.

The RNA:DNA ratio is an index that determines individual recent growth capacity and recent nutritional condition which can be useful to examine survival processes (Clemmesen, 1994; Folkvord *et al.*, 1996). The RNA:DNA ratio also provides information on the feeding environment of the larvae within a time frame of days prior to sampling. Well-fed larvae are metabolically more active, grow better and have a relatively higher RNA:DNA ratio compared to poor-fed larvae with less active metabolism (Clemmesen, 1987, 1994). The DNA:dry weight ratio shows larval cell condition. During starvation, cell weight decreases while DNA concentration stays constant. The DNA:dry weight ratio increases when conditions decrease, because less weight is attributed per cell number (Chícharo and Chícharo, 2008).

RNA and DNA contents were determined using a modification of the method described by ICES (2004). The method analysis of the bulk content of all RNA and DNA contained in a specific tissue or whole-body crude homogenate and expresses them as a ratio. RNA in a fish cell is mainly made up of mRNA and tRNA, which are both involved in protein biosynthesis. The quantity of both RNAs changes with biosynthesis rate, and thus growth, because growth in fish larvae is mainly realized by protein build-up and it is therefore the enumerator of the RNA:DNA ratio. The DNA content is a function of cell number. DNA is therefore the denominator in the RNA:DNA ratio, because it normalizes RNA quantity to body mass. The amount of DNA is stable under changing environmental situations reflecting the number of cells of an individual (Buckley *et al.*, 1999), whereas the amount of RNA is directly proportional to the protein synthesis capacity in the cell.

RNA and DNA contents were individually measured using the whole larval body and all the reagents were prepared using Tris-EDTA buffer (0.05 M TRIS, 0.1M NaCl, 0.01 M EDTA, adjusted to pH 8.0 with HCl). First, the larvae conserved in RNAlater® (Sigma-Aldrich R0901) were rinsed with milliQ water and subsequently the larva was rehydrated by transferring to a mixture of Tris-EDTA buffer and sodium dodecyl sulfate 0.7% (SDS) for 15 minutes at 4 °C. Once rehydrated in the vial, the larvae were completely disintegrated by applying two pulses of 10 sec of ultrasound (Bandelin Sonoplus) (Fig. 2.7a). An increase in the temperature of the homogenates was avoided by keeping all the vials on ice. The homogenate was centrifuged at 3800 x *g* for 8 minutes at 4 °C. Two supernatant aliquots were taken, one for the measurements of the total nucleic acids (RNA + DNA) and another one

for the measurement of the DNA content. The DNA measurement was carried out by incubating the samples with RNase A (type I-AS, Sigma-Aldrich) at 37 °C during 30 minutes. Acid nucleic fluorescence was determined fluorometrically with a Perking-Elmer LS-5 (excitation: 327 nm and emission: 614 nm) by adding Ethidium Bromide buffer solution (0.1 mg mL^{-1}) (Fig. 2.7b).

DNA and RNA contents were estimated by means of a calibrated standard curves of calf thymus DNA (Sigma-Aldrich D1501) and baker yeasts RNA (Sigma-Aldrich R7125) respectively. All biochemical analyses of larvae reported in this study were completed within 4–5 months after sampling.

A more detailed description of the methodology used in each experiment is specifically described in the materials and methods sections of Chapters 3 to 7.

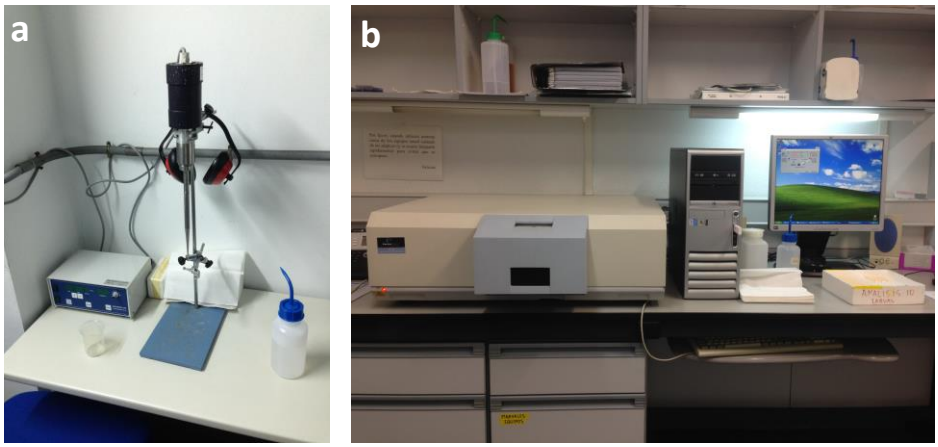


Figure 2.7. Image of some of the equipment used in the nucleic acid measurement. a) A sonicator used to disintegrate the larvae by ultrasound and b) the fluorometer used to obtain the fluorescence generated by the Ethidium bromide.

2.7 Animal ethics

All experiments and animal handling were carried out in accordance with the relevant guidelines on animal experimentation on fish. The methods used in the current study were accepted by the Ministry of Economy, Industry and Competitiveness of Spain and the Steering Committee of the project CTM2011-29525-C04-02-MAR. The ethics committee of the facility also supervised the research.

CHAPTER 3

- THE EFFECT OF NUTRITIONAL CONDITION ON THE GROWTH TO POST-FLEXION OF ATLANTIC BLUEFIN TUNA AND ATLANTIC BONITO -

The effect of nutritional condition on the growth to post-flexion of Atlantic bluefin tuna and Atlantic bonito larvae

ABSTRACT

The flexion of the notochord is considered a crucial point in the growth and mortality of scombrid larvae. Notochord flexion, which is associated with caudal fin development, increases the swimming capacity of the larvae and aids in the capture of mobile preys. Also, it coincides with the timing of when the physiological capacities of larvae begin to develop significantly, allowing an early shift to piscivory. Therefore, reaching the flexion stage as soon as possible can be considered beneficial for the growth and survival of the larvae and the timing of this stage likely determines which larvae reach the next crucial point, piscivory. In this study, we examined if the nutritional status of the larvae, measured by the RNA:DNA and DNA:dry weight ratios, explains the differences in the development of notochord flexion in laboratory reared Atlantic bluefin tuna (*Thunnus thynnus*) and Atlantic bonito (*Sarda sarda*) larvae. Flexion was first observed in Atlantic bluefin tuna larvae at a length of 6.1 mm and a dry weight of 0.24 mg, with post-flexion at 6.5 mm and 0.43 mg, whereas in Atlantic bonito, flexion larvae where observed at 6.3 mm and 0.24 mg and post-flexion larvae at 8.2 mm and 0.80 mg. In Atlantic bluefin tuna, an increasing trend in RNA:DNA ratio with age and developmental stage was a result of decreasing DNA concentration and stable RNA concentration (μg mg^{-1}). In Atlantic bonito, RNA:DNA and DNA and RNA concentrations did not change with age and development stage. The RNA:DNA ratio explained variations in somatic growth in Atlantic bonito and Atlantic bluefin tuna larvae but did not explain differences among the different developmental stages. The cell condition of the larvae in the flexion stage was better than in the post-flexion stage in Atlantic bluefin tuna, as measured by the DNA:dry weight ratio. No trend was found in Atlantic bonito. In Atlantic bluefin tuna, the DNA:dry weight ratio, not the RNA:DNA ratio, reflected growth differences. Our findings suggest that under cultured conditions, nutritional condition might not be enough to explain developmental differences in fast growing species.

3.1 Introduction

The nutritional condition of marine fish reflects the energy reserves available for maintenance, growth and activity, and the individual responses to variability in food supply and feeding success are ultimately related to survival (Anderson, 1988; Cushing, 1990; Hjort, 1914). During their first weeks of life —a period characterized by high mortality rates (Bailey and Houde, 1989; Leggett and Deblois, 1994)— and after they have consumed all the lipids of the yolk, fish larvae have low energy reserves, and their tissues and organs are under progressive and intense differentiation and development (Fuiman, 1983; Kendall *et al.*, 1984; Osse *et al.*, 1995; Pittman *et al.*, 2013).

Suboptimal feeding may lead to the death of larvae directly, by starvation, or indirectly, through prolonged stage duration and higher vulnerability to predation (Fiksen and Jørgensen, 2011; Folkvord *et al.*, 2015). Differences in nutritional condition can also generate growth variability, and larvae of the same age may show individual differences, consequently influencing their success during this critical early life stage (Takebe *et al.*, 2012; Tanaka *et al.*, 2010).

One important event in the early life of fish is the flexion of the notochord, which accompanies the hypocordal development of the homocercal caudal fin during the larval stage (Kendall *et al.*, 1984). This physiologically stressful moment is associated with changes in body shape, resulting in improvements in swimming capacity and feeding techniques (Kendall *et al.*, 1984; Osse *et al.*, 1995). It is also considered an inflexion point in the early stages of the life cycle of a fish, where growth and survival rates increase due to enhanced access to a wider spectrum of prey and increased predation avoidance (Kaji, 2003; McFarlane *et al.*, 2000; Somarakis and Nikolioudakis, 2010).

Atlantic bluefin tuna (*Thunnus thynnus*), and Atlantic bonito (*Sarda sarda*), are the only scombrid species in the Mediterranean Sea that have been successfully reared in captivity (De la Gándara *et al.*, 2012; Ortega, 2015). Experimental studies have shown that these two species become piscivorous already during the larval stage, once the flexion of the notochord has occurred (Chapter 5; Blanco *et al.*, 2017; Reglero *et al.*, 2014a). This change in diet increases survival and is required for sustaining growth and the high metabolic requirements of these species (Reglero *et al.*, 2014a). The flexion coincides with the start of the development of an adult-type digestive system, with blind sac, gastric glands, pyloric caeca and digestive enzymes that allow for the digestion of fish prey (Kaji, 2003; Miyashita *et al.*, 1998; Yúfera *et al.*, 2014). On the other hand, the completion of the flexion is externally accompanied by the development of the fin rays and the development of the caudal muscle fiber, which will allow for improved swimming (Kendall *et al.*, 1984; Osse *et al.*, 1995; Roy *et al.*, 2014). In general, bluefin tunas are also characterized by having a very low tolerance to starvation, with up to 50% of the larvae dying one day after starved conditions and showing an immediate growth retardation. Therefore, those in the worst nutritional condition will rapidly die (*e.g.* Pacific bluefin tuna, Tanaka *et al.*, 2008).

Growth variability has been observed among individuals of Pacific bluefin tuna before reaching the flexion stage using otolith back-calculation analyses in laboratory-reared and field-captured larvae (Takebe *et al.*, 2012; Tanaka *et al.*, 2006). However, little is known about the processes explaining such growth differences in this and other tuna species.

Potential nutritional differences between flexion and pre-flexion larvae have been widely overlooked. The collection of post-flexion larvae in the field is a difficult task, mainly due to their ability to evade sampling nets once their swimming capacity has improved (Satoh *et al.*, 2008). Additionally, assessing individual nutritional condition from field samples is difficult, as extrinsic factors experienced by larval fish are often changing, and different larvae of the same cohort might experience different environmental conditions in a single day (Peck *et al.*, 2012). Food availability and water temperature are the main factors affecting the nutritional condition of fish larvae (*e.g.* Buckley *et al.*, 1984; Clemmesen, 1994; Foley *et al.*, 2016; Folkvord *et al.*, 1996). Laboratory studies allow us to obtain larval sizes rarely captured in the field and to identify individual responses in the nutritional condition to different feeding and thermal controlled conditions.

In this study, we examined if the nutritional status of laboratory reared larvae in Atlantic bluefin tuna and Atlantic bonito larvae could explain individual differences in the timing of notochord flexion. We use two different indices to determine fish larval nutritional condition: the RNA:DNA and the DNA:dry weight ratios. The RNA:DNA ratio is an index of cell metabolic intensity and it is used as an approach for recent growth and recent nutritional condition of fish larvae (Clemmesen, 1994; Folkvord *et al.*, 1996). The amount of DNA is stable under changing environmental situations, reflecting the number of cells of an individual, whereas the amount of RNA is directly proportional to the protein synthesis capacity in the cell. The RNA is highly dependent on food quantity and varies with age, life stage, organism size, disease state and changing environmental conditions (Buckley *et al.*, 1999). During food deprivation, the nutritional condition, and therefore the RNA:DNA ratio, decreases, reflecting the cessation of protein synthesis and somatic growth (*e.g.* Clemmesen, 1994). Therefore, well-fed larvae are metabolically more active, grow better and have relatively higher RNA:DNA ratios compared to poor-fed larvae with less active metabolism (Clemmesen, 1987, 1994). The RNA:DNA ratio is sensitive to changes in specific growth rates, both in terms of length and weight, and provides information on the feeding environment of the larvae within a time frame of days prior to sampling. This can be interpreted as recent growth capacity and can be useful to examine the survival processes (Bergeron, 1997; Rooker and Holt, 1996). On the other hand, the DNA:dry weight ratio show larval cell condition, increasing when condition decreases, because less weight is attributed per cell number (Chícharo and Chícharo, 2008).

Relatively little information is available concerning the relationship of growth and nutritional status using Atlantic bluefin tuna larvae biochemistry, and no information exists for Atlantic bonito larvae. Larval nutrition has been suggested as

a possible cause of the high mortality in laboratory-reared Atlantic bluefin tuna and Atlantic bonito (Reglero *et al.*, 2014a) but this theory has never been tested. To date, lipid content and histology of the organs has been used as a proxy of nutritional condition in Atlantic bluefin tuna (Ortega and Mourente, 2010; Yúfera *et al.*, 2014). For Pacific bluefin tuna, the ontogenetic changes in nutritional condition have been analyzed in the laboratory and in the field (Tanaka *et al.*, 2007, 2008), whereas data for Atlantic bluefin tuna is only available for field-captured larvae (García *et al.*, 2006). Nutritional condition is associated with the food supply and feeding success of the fish and therefore variability in the trophic environment is reflected in nutritional condition. The main objective of this to understand whether nutritional condition allows us to identify different developmental timings in Atlantic bluefin tuna and Atlantic bonito larvae and avoid size hierarchy under culture conditions.

3.2 Materials and methods

3.2.1 Atlantic bluefin tuna experiment

Fertilized eggs of Atlantic bluefin tuna (ABFT) were collected from naturally-spawning captive adults in the farming facilities at El Gorguel, Cartagena (SE Spain), owned by Caladeros del Mediterráneo S.L. In the laboratory, floating and sinking eggs (at natural seawater salinity of 37) were separated in a 5 L bucket. Floating ABFT eggs were incubated in 400 L tanks at 28 °C under a continuous light regime. A few hours after incubation, 60 000 ABFT eggs were transferred to four 1500 L cylindrical tanks, with 15 000 eggs per tank and the sea water temperature set at 28 °C. Water temperature in the incubators and in the rearing tanks was controlled using heaters—isolated to avoid larval mortality—inside the tanks. The water temperature was measured continuously by a HOBO data logger (www.onsetcomp.com). Rearing was conducted with a photoperiod of 14L:10D, similar to natural conditions in the area.

Cultivated microalgae of *Nannochloropsis gaditana* were added twice each day from 0 to 3 days post hatch (dph). Afterwards, a paste of concentrated *Chlorella* (Super fresh Chlorella SV-12, Chlorella Industry Co., Ltd., Japan) was added three times per day in each tank. The larvae were fed following the technique described in De la Gándara *et al.* (2012). Enriched rotifers (*Brachionus plicatilis)* were added from 3 dph at a concentration of 5 rotifers mL^{-1} to guarantee *ad libitum* conditions. From 14 dph onwards, sea bream (*Sparus aurata*) yolk-sac larvae of 0–2 dph (3.4 ± 0.04 mm) were added, providing up to 300 preys per individual twice daily. Pseudo–green water technique was used during the entire rearing period to avoid the depletion of the nutritional condition of the rotifers and the resulting effect on larval development (Yamamoto *et al.*, 2009).

Several small samplings were carried out every day before 8 dph in Atlantic bluefin tuna and before 5 dph in Atlantic bonito, to follow the development of the larvae and to accurately identify the first day any larvae started to flexion (data not included in this chapter). Experimental samplings started as soon as we found flexion stage larvae and finished when at least 50% of the larvae were found to be in the post-

flexion stage to avoid the possible cannibalism of those that first reached the post-flexion stage. The experiment lasted for 20 days, from 0 to 20 dph. 40 larvae per tank were randomly sampled at 8, 10, 11, 12 and 13 dph. At 13 dph, all the remaining ABFT larvae in the tanks were counted to estimate survival and a total of 1845 larvae were transferred to three new 1500 L cylindrical tanks, with up to 615 larvae randomly distributed in each tank. Hereafter, the larvae were cultivated until they were 20 dph, at which point 40 larvae per tank were sampled and all the remaining larvae were counted for survival estimates. Larval sampling was always carried out every day at the same time, early in the morning and in darkness.

3.2.2 Atlantic bonito experiment

Fertilized eggs of Atlantic bonito (AB) were obtained from stripped spawning adult individuals collected in an almadraba trap in La Azohía (Murcia, SE Spain). In the laboratory, floating and sinking eggs (at natural seawater salinity of 37) were separated in a 5 L bucket. Floating AB eggs were incubated in 400 L tanks at 26 °C under a continuous light regime. Just after hatching, at 0 dph, 11 250 AB larvae were transferred to three 1500 L cylindrical tanks, with 3750 larvae in each tank and the water temperature set at 26 °C. Water temperature in the incubators and in the rearing tanks was controlled using heaters—isolated to avoid larval mortality—inside the tanks. The water temperature in each tank was measured continuously by a HOBO data logger (www.onsetcomp.com). Rearing was conducted with a photoperiod of 14L:10D, similar to natural conditions in the area.

Cultivated microalgae of *Nannochloropsis gaditana* were added twice each day, from 0 to 2 dph. Afterwards, a paste of concentrated *Chlorella* (Super fresh Chlorella SV-12, Chlorella Industry Co., Ltd., Japan) was added three times per day in each tank. The larvae were fed following the technique described in De la Gándara *et al.* (2012). Enriched rotifers (*Brachionus plicatilis)* were added from 2 dph at a concentration of 5 rotifers mL^{-1} to guarantee *ad libitum* conditions. Pseudo green water technique was used during the entire rearing period to avoid depletion of the nutritional condition of the rotifers and the resulting effect on larval development (Yamamoto *et al.*, 2009).

Experimental samplings started as soon as we found flexion stage larvae and finished when at least 50% of the larvae were found to be in the post-flexion stage, to avoid the possible cannibalism of those that first reached the post-flexion stage. The experiment lasted for 9 days, from 0 to 9 dph. 20 larvae were randomly sampled in each tank at 5, 7 and 8 dph while 30 larvae per tank were sampled at 6 dph (Table 3.1). The last day, 9 dph, all the remaining larvae in the tanks were counted and sampled. Larval sampling was carried out every day at the same time, early in the morning and in darkness.

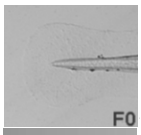
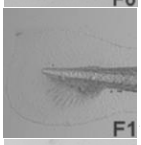
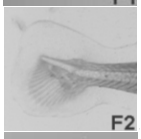
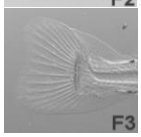
Table 3.1. Summary of the differences between Atlantic bluefin tuna and Atlantic bonito experiments. Daily total number of larvae sampled (n) in each tank replicate (R1, R2, R3 and R4) per larval day post hatch (dph). Average temperature in °C during the corresponding days and the average daily standard length (SL, mm) and dry weight (DW, mg) of the sampled larvae is shown in the table. In Atlantic bluefin tuna at 20 dph, the standard length is shown as a range due to the high size variability. Daily percentage of larvae in flexion (F2) and in post-flexion (F3) is shown.

Species	Dph	Sampling (n)				SL (mm)	DW (mg)	% Flexion (F2)	% Post-flexion (F3)
		R1	R2	R3	R4				
Atlantic bluefin tuna	8	40	40	40	40	5.1 ± 0.5	0.13 ± 0.05	0.0	0.0
27.7 ± 0.4 °C	10	40	40	40	40	6.2 ± 0.7	0.32 ± 0.15	38.0	1.9
	11	40	40	40	40	6.7 ± 0.6	0.42 ± 0.18	36.8	31.0
	12	40	40	40	40	7.0 ± 0.7	0.58 ± 0.26	30.2	53.0
	13	40	40	40	40	7.5 ± 0.6	0.77 ± 0.26	13.4	86.0
	20	40	40	40	-	11.3 - 24.6	4.46 - 50.1	0.0	100.0
Atlantic bonito	5	20	20	20	-	5.7 ± 0.3	0.16 ± 0.03	0.0	0.0
26.2 ± 0.9 °C	6	30	30	30	-	6.6 ± 0.5	0.28 ± 0.06	4.4	0.0
	7	20	20	20	-	7.3 ± 0.4	0.45 ± 0.09	50.0	0.0
	8	20	20	20	-	8.0 ± 0.3	0.68 ± 0.13	96.6	3.4
	9	all	all	all	-	8.2 ± 0.3	0.77 ± 0.13	54.1	45.9

3.2.3 Laboratory analyses

Immediately after sampling, the larvae were anesthetized using clove oil (Guinama© Spain), individually photographed using an image analysis system connected to a microscope (Leica Microsystem, Inc, Bannockburn, IL) and individually frozen in vials at -80 °C. ABFT larvae were submerged in RNAlater® before preserving at -80 °C. Later, in the laboratory, the larvae conserved in RNAlater® (Sigma-Aldrich R0901) were rinsed with milliQ water and lyophilized to estimate individual dry weight (DW) (to the nearest 0.01 mg) and larval nutritional condition (see section 3.2.4). The standard length (SL) of the sampled fish was measured to the nearest 0.1 mm from the anterior margin of the snout to the posterior margin of the hypural plate of the notochord. Four different developmental phases based on morphological characteristics of the notochord and caudal fin were determined following a modified version of the criteria of De la Gándara *et al.* (2013), a modified version for *Thunnus thynnus* of Kendall *et al.* (1984) and Kaji *et al.* (1996) (Table 3.2).

Table 3.2. Description of the four different developmental stages used in this article, based on morphological characteristics of the notochord and caudal fin rays.

Stage	Nomenclature	Description	Example
Pre-flexion	F0	Straight notochord	
First caudal fin rays	F1	Straight notochord with some rays in the ventral side	
Flexion	F2	Bending upward of the notochord tip in a very clear angle with an increase in the amount of fin rays	
Post-flexion	F3	The final tip of the notochord disappears. Definition of the hypural plate and caudal fork. The posterior margin of the upper hypural plate is at 90 ° from the notochord axis	

3.2.4 Nutritional condition: nucleic acid analyses

RNA and DNA content were determined using a modification of the method described by ICES (2004). RNA and DNA contents were individually measured using the whole larval body and all the reagents were prepared using Tris-EDTA buffer (0.05 M TRIS, 0.1 M NaCl, 0.01 M EDTA, adjusted to pH 8.0 with HCl). First, lyophilized larvae were rehydrated by transferring to a mixture of Tris-EDTA buffer and sodium dodecyl sulfate 0.7% (SDS) for 15 minutes at 4 °C. Once rehydrated in the vial, the larvae were completely disintegrated by applying two 10-second ultrasound pulses (Bandelin Sonoplus). An increase in the temperature of the homogenates was avoided by keeping all the vials on ice. The homogenate was centrifuged at 3800 x *g* during 8 minutes at 4 °C. Two supernatant aliquots were taken, one for the measurements of the total nucleic acids (RNA + DNA) and another one for the measurement of the DNA content. The DNA measurement was carried out by incubating the samples with RNase A (type I-AS, Sigma-Aldrich) at 37 °C during 30 minutes. The difference between the total acid nucleic fluorescence and the DNA fluorescence was corrected to determine the RNA fluorescence as suggested by Caldarone *et al.* (2006) assuming for DNA, a ratio of 2.4 RNA content.

Acid nucleic fluorescence was determined fluorometrically with a Perking-Elmer LS-5 (excitation: 327 nm and emission: 614 nm) by adding 200 µL Ethidium Bromide buffer solution (0.1 mg mL^{-1}). DNA and RNA content were estimated by means of calibrated standards curves of calf thymus DNA (Sigma-Aldrich) and baker yeasts RNA (Sigma-Aldric), respectively. All biochemical analyses of larvae reported in this study were completed within 4–5 months after sampling.

3.2.5 Statistical analyses

All the statistical analyses were carried out using the R statistical software (www.r-project.org). Final survivals and daily mortalities were estimated from the numbers of initial eggs and the number of larvae counted out at the end of the experiment, subtracting the number of larvae sampled on each sampling day. All sized data (SL, DW) were analyzed for heterogeneity of variance (Levene's test) and checked for normality with a Kolmogorov–Smirnov test.

Differences in larval sizes (SL, DW) among replicates within each species were tested using one-way ANOVA, and Bonferroni correction was applied to avoid type I error. A two-way ANOVA test was performed in each one of the 7 studied variables (larval RNA content, larval DNA content, RNA:DNA ratio, RNA:dry weight (concentration, ug mg^{-1}), DNA:dry weight (concentration, ug mg^{-1}), standard length (mm) and dry weight (ug)) with stage and dph as factors. And when significant, Tukey HSD tests were used for post-hoc comparisons. All test results were considered significant at a level of 0.05.

Cumulative size distributions (CSD) in standard length and dry weight were estimates as described in Folkvord *et al.* (2009). Assuming static ranking of fish sizes within a cohort is unlikely to change much in the short term, cumulative size distributions were used for visualizing variabilities within cohorts over time in a single graph. Stage developmental cumulative distribution was also estimated in order to obtain a cumulative approach of the duration of the different developmental stages in each species.

The residuals from the RNA and DNA relationship (RNA-DNA residuals), as a proxy for short term growth, were compared to the size-at-age residuals (DW-DPH residuals), as a proxy for long term growth, for the two species. The RNA:DNA ratio is very sensitive to feeding levels, and any change is reflected in the amount of available RNA almost immediately, after about 1 to 5 days (Buckley *et al.*, 1999; Clemmesen, 1987). Size-at-age (DW:DPH) integrated the feeding history and energetic utilization over the whole life-time of an organism. A significant positive (negative) correlation indicates faster growing larvae are in better (worse) nutritional condition than slower growing larvae. In order to determine if at a specific size development was affected by larval nutritional condition, DNA-DW residuals were analyzed against STAGE-DW residuals. DNA:dry weight has been also used as a proxy for larval cell nutritional status, since cell weight decreases while DNA concentration remains constant during a reduction in nutritional condition (Bergeron, 1997). The DNA:dry weight ratio increases when nutritional condition decreases, since more cells are present for the same weight of tissue of starved larvae (Bergeron, 1997; Chícharo and Chícharo, 2008). Residuals were analyzed using linear regression and ANCOVA analyses were carried out for stage effect.

3.3 Results

There were no significant differences in the daily larval sizes among tank replicates in both species (ANOVA, *p-adj.*>0.05); therefore, replicates were combined for further analyses. Survival rates of ABFT at 13 dph and AB at 9 dph were 1.1 ± 0.2% and 4.1 ± 0.2% respectively. Further, 27.7 ± 2.5% of the ABFT larvae survived from 13 to 20 dph. On average, every day 23.6 ± 6.4% of ABFT larvae died up to 13 dph, and 16.7 ± 1.0% up to 20 dph. In AB, 39.2 ± 1.6% died daily until 9 dph.

Near-parallel CSDs among subsequent sampling days showed similar growth rates of different size-ranked ABFT and AB larvae both in length (Fig. 3.1a, c) and dry weight (Fig. 3.1b, d). However, AB larvae showed lower growth rates from 8 to 9 dph than at other age intervals, as indicated by the almost overlapping cumulative curves (Fig. 3.1c, d).

There was no significant overlap in the larval length and weight among developmental stages both in ABFT and AB (Fig. 3.2, Table 3.3). ABFT larvae showed the first signs of flexion (stage F2) from 6.1 mm in length and 0.24 mg in weight and completed flexion (stage F3) from 6.5 mm in length and 0.43 mg in weight (Table 3.3). In AB first flexion (stage F2) larvae were found from 6.3 mm in length and 0.24 mg in weight and completed flexion from 8.2 mm in length and 0.80 mg in weight (stage F3) (Table 3.3).

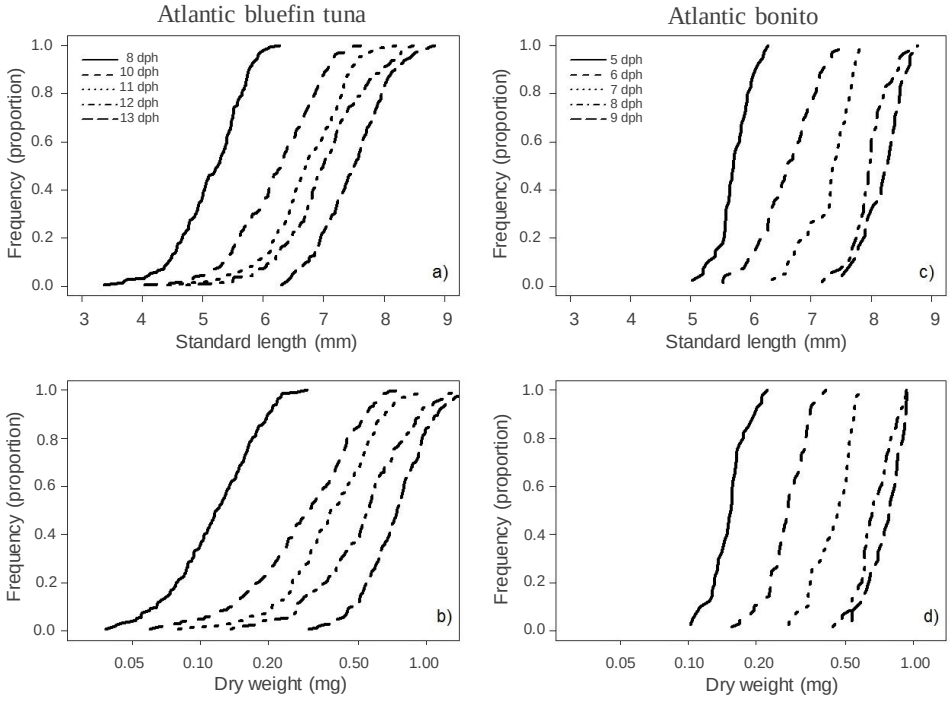


Figure 3.1. Cumulative size distributions on standard length (SL, mm) and dry weight (DW, mg). a) Atlantic bluefin tuna SL, b) Atlantic bluefin tuna DW, c) Atlantic bonito SL, and d) Atlantic bonito DW. Different experimental samplings days are shown with different cumulative line type. Note: The x-axis is natural-log transformed for DW in panels b) and d).

Figure 3.2. Boxplots for size rank of standard length and dry weight of each classified developmental stage in Atlantic bluefin tuna and Atlantic bonito larvae.

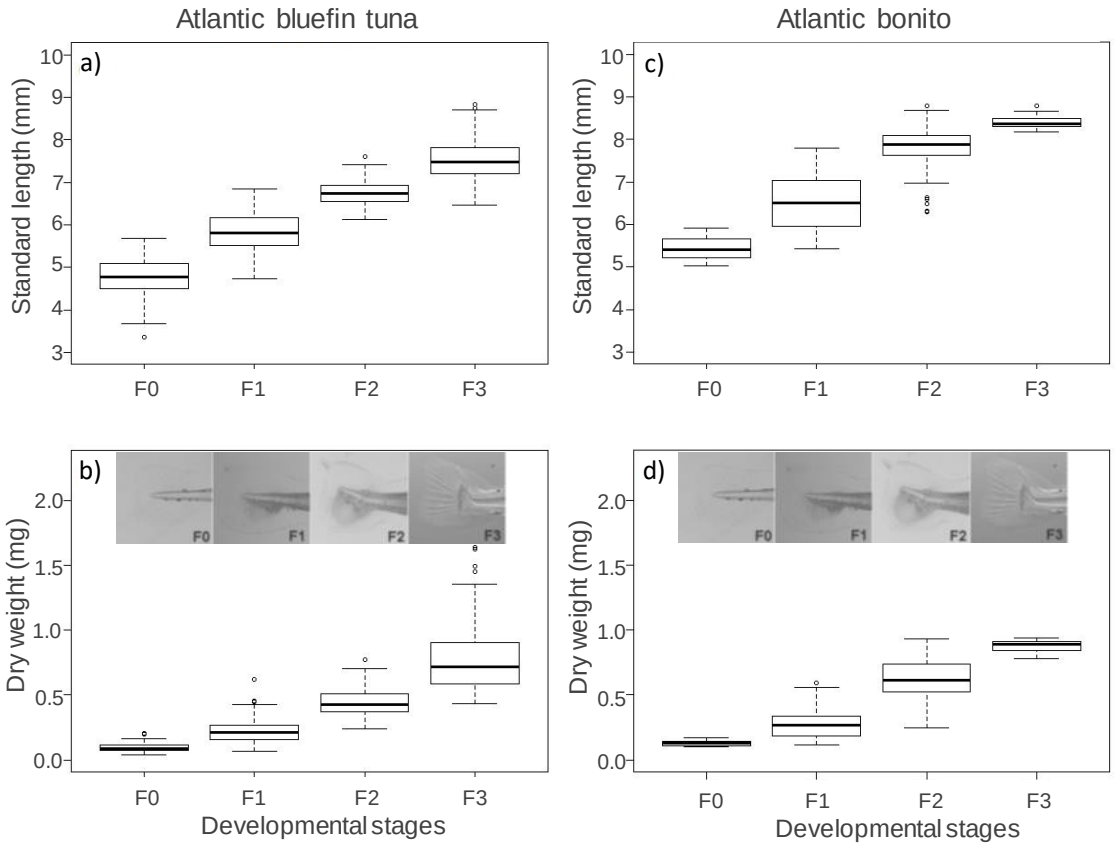


Table 3.3. Mean (± SD) standard length (SL, mm) and dry weight (DW, mg) measures of each developmental stage (F0, F1, F2 and F3) found of Atlantic bluefin tuna and Atlantic bonito larvae during our experiment. The exact moment the first larva in flexion (F2) and in post-flexion (F3) was seen is well documented, and the standard length and dry weight of that larva is show as *first signs*.

Species	Measurements	F0	F1	F2		F3	
		average	average	average	first signs	average	first signs
Atlantic bluefin tuna	SL (mm)	4.8 ± 0.5	5.8 ± 0.4	6.7 ± 0.3	6.1	7.5 ± 0.5	6.5
	DW (mg)	0.10 ± 0.03	0.22 ± 0.08	0.43 ± 0.10	0.24	0.77 ± 0.24	0.43
Atlantic bonito	SL (mm)	5.4 ± 0.3	6.5 ± 0.6	7.8 ± 0.5	6.3	8.4 ± 0.2	8.2
	DW (mg)	0.13 ± 0.02	0.30 ± 0.10	0.61 ± 0.20	0.24	0.90 ± 0.05	0.80

There was a significant overlap in the age at which different developmental stages were observed (Fig. 3.3). In ABFT, F0 stage larvae lasted to 11 dph, while in AB F0 larvae lasted only to 6 dph (Fig. 3.3). The start of F1 stage was not determined but the development of the rays in the last caudal fin was observed until 13 dph in ABFT and 8 dph in AB. Initial flexion (F2) in ABFT was observed from 8 dph and post-flexion from 9 dph (Fig. 3.3a). In AB, F0 stage finished around 6–7 dph and F1 at 8 dph. First flexion AB larvae started from 5 dph and post-flexion AB larvae from 7 dph (Fig. 3.3b). In ABFT, at 10 and 11 dph, larvae of all the stages co-existed, while in AB, at day 6 dph the larvae of first three the stages co-existed.

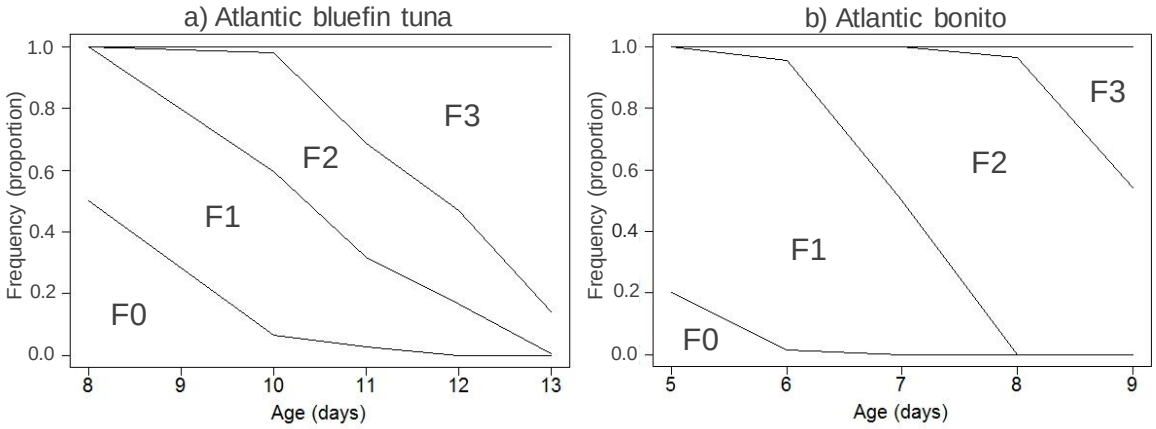


Figure 3.3. Cumulative developmental stage distribution showing the duration of the different developmental stages during the a) Atlantic bluefin tuna and b) Atlantic bonito ages. F0: larvae in pre-flexion, F1: larvae with development of the first caudal fin rays, F2: larvae in flexion and F3: larvae in post-flexion.

We found an increasing trend in RNA:DNA ratio with age in ABFT, whereas a steady tendency was observed in AB independent of age (Fig. 3.4). Average DNA concentration of the larvae was related to mean RNA:DNA ratio, since RNA concentration did not change among the larval development. The RNA concentration in ABFT remained steady and did not vary significantly among the stages (Table 3.4, ANOVA, $p>0.05$, Fig. 3.4a). The DNA concentration significantly decreased with age and developmental stage in ABFT (Fig. 3.4b), resulting in a significant increase of the RNA:DNA ratio with age and developmental stage (Table 3.4, ANOVA, Tukey HSD, $p<0.05$, Fig. 3.4c). In AB, RNA:DNA ratio and RNA and DNA concentrations were significantly different throughout the experiment (Table 3.4, ANOVA, Tukey HSD, $p<0.05$, Fig. 3.4d, f) although they did not vary significantly with developmental stage (Table 3.4, ANOVA, $p>0.05$, Fig. 3.4d, f).

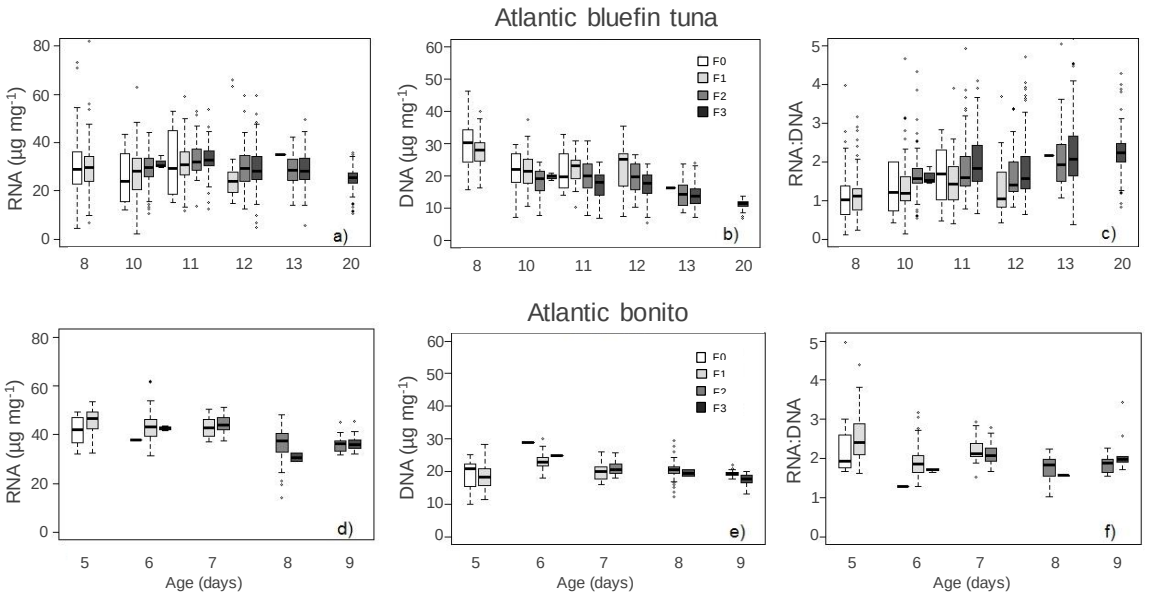


Figure 3.4. Boxplots of the larval content of: RNA (µg mg^{-1}), DNA (µg mg^{-1}) and RNA:DNA in Atlantic bluefin tuna (a–c) and Atlantic bonito (d–f). Different stages are shown using grey-scale. Atlantic bluefin tuna larvae were sampled from 8 to 20 dph, while Atlantic bonito larvae were sampled from 5 to 9 dph.

	Atlantic bluefin tuna Pr (>F)	Atlantic Bonito Pr (>F)
Larval RNA content		
dph	<0.001	<0.001
stage	<0.001	<0.001
Larval DNA content		
dph	<0.001	<0.001
stage	<0.001	0.001
Larval RNA:DNA		
dph	<0.001	<0.001
stage	<0.001	0.335
RNA (µg mg^{-1})		
dph	0.323	<0.001
stage	0.023	0.378
DNA (µg mg^{-1})		
dph	<0.001	<0.001
stage	<0.001	0.099
Standard length (mm)		
dph	<0.001	<0.001
stage	<0.001	<0.001
Dry weight (mg)		
dph	<0.001	<0.001
stage	<0.001	<0.001

Table 3.4. Summary table of the results from all two-way analysis of variance (ANOVA) among the studied variables. In Atlantic bluefin tuna, analyses were done from 8 to 13 dph and in Atlantic bonito from 5 to 9 dph.

There was a positive correlation between short-term growth and long-term growth in AB and ABFT larvae that was independent of the developmental stage (ANCOVA, $p<0.05$, Fig. 3.5a, b). Those larvae with low short-term growth capacity also had low long-term growth capacity, and when the short-term growth increased so did the long-term growth (Fig. 3.5a, b). At a given size, a negative correlation was obtained between DNA:dry weight and those larvae in a more developed stage in ABFT (ANCOVA, $p<0.05$, Fig. 3.6a) whereas no correlation was found in AB (ANCOVA, $p>0.05$, Fig. 3.6b).

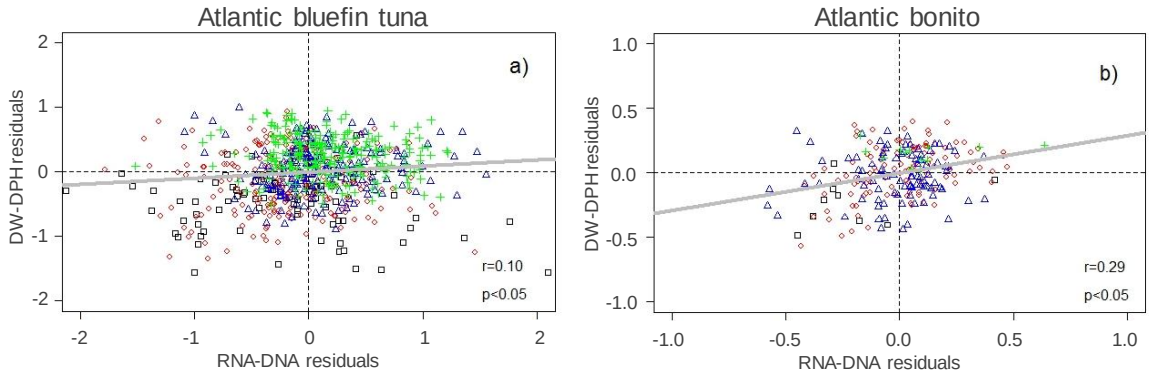


Figure 3.5. Growth residuals. Short-term growth residuals (RNA-DNA residuals) plotted against long-term growth residuals (DW-DPH residuals) in a) Atlantic bluefin tuna and b) Atlantic bonito larvae. Different stages are shown with different symbols and colors: F0: □, F1: ◊, F2: Δ and F3: +. A significant relationship was found in Atlantic bluefin tuna a): $y = -0.005 + 0.092 \cdot x$, $R^2=0.01$, $p<0.01$, $n=778$ and in Atlantic bonito b): $y = -0.002 + 0.289 \cdot x$, $R^2=0.08$, $p<0.01$, $n=230$. The average relationship is represented by the grey line.

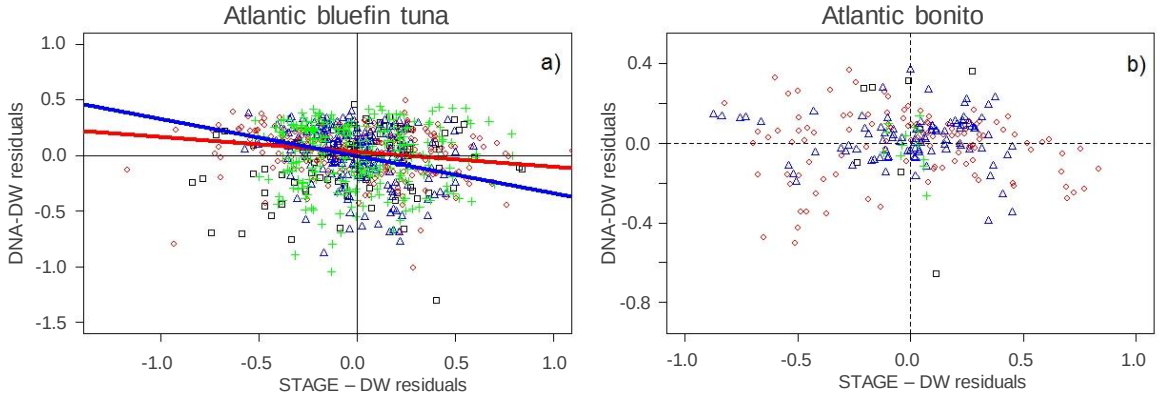


Figure 3.6. Developmental residuals. DNA-DW residuals plotted against STAGE-DW residuals in a) Atlantic bluefin tuna and b) Atlantic bonito larvae. Different stages are shown with different symbols and colors, F0: □, F1: ◊, F2: Δ and F3: +. A significant relationship was found in F1 ($y = 0.031 - (0.133 \cdot x)$, $R^2=0.04$, $p<0.001$, $n=231$) and F2 ($y = -0.008 - (0.333 \cdot x)$, $R^2=0.07$, $p<0.001$, $n=180$) in Atlantic bluefin tuna. In Atlantic bonito, no significant relationship was found.

3.4 Discussion

The nutritional condition of AB and ABFT larvae, measured as the RNA:DNA ratio of the larvae reared at optimal feeding conditions (*ad libitum*) and constant temperature, salinity and light, is proportional to larval growth in dry weight. However, the RNA:DNA ratio did not explain the differences among the different developmental stages. In ABFT the DNA:dry weight ratio reflected differences among the developmental stages. At any given larval size, DNA:dry weight ratio showed the worst cell condition in post-flexion larvae, compared to those in flexion in ABFT. In AB, no such relation with the different stages was seen.

In our study we have estimated survival rates of 1% and 4%, for ABFT and AB respectively, for the period between hatching and post-flexion, and survival rates of 28 % from post-flexion to juvenile in ABFT. Survival rates for laboratory-reared Pacific bluefin tuna and yellowfin have been reported between the time from hatching to juvenile, varying between 0.07% and 3% (Margulies *et al.*, 2007, 2016; Sawada *et al.*, 2005; Tanaka *et al.*, 2018), and from post-flexion to the end of the piscivory phase between 30% and 60% (Chapter 5; Blanco *et al.*, 2017; Reglero *et al.*, 2014a; Seoka *et al.*, 2008; Tanaka *et al.*, 2014a). In AB, survival from hatching to juvenile varies between 2.9% and 10% (Chapter 5; Blanco *et al.*, 2017; De la Gándara *et al.*, 2012; Reglero *et al.*, 2014a). These survival data, in accordance with other laboratory data reported, suggest once the larvae reach flexion, the mortality rates decrease significantly. The high mortalities observed during the first days of life in laboratory reared Pacific bluefin tuna or yellowfin have been related to culture techniques that are still in development (Honryo *et al.*, 2016; Nakagawa *et al.*, 2011; Tanaka *et al.*, 2009), malnutrition (De la Gándara *et al.*, 2012; Margulies *et al.*, 2016; Takebe *et al.*, 2012), adhesion to the surface and to the sinking syndrome (Sawada *et al.*, 2005; Takashi *et al.*, 2006; Tanaka *et al.*, 2009). Besides, rotifers are not the natural prey of tuna larvae from the field which can have an effect in larval survival. Copepods are one of their natural prey and larval survival is known to be improved in comparison with rotifers (Llopiz and Hobday, 2015; Ortega, 2015).

The completion of the head for feeding and respiratory functions, the tail for cruising and escape reactions and the full development of the intestine appears to be given priority during the first days of life rather than growth in total body length (Osse *et al.*, 1995). However, we found similar daily specific growth rates in standard length and dry weight in ABFT and AB. The changes in notochord flexion are accompanied by the development of fin rays, changes in body shape, locomotive ability, and feeding techniques (Kendall *et al.*, 1984). Flexion can be used as a proxy for the development of other non-visible changes, such as the apparition of the first gastric glands, the complete development of the stomach with the apparition of the first pyloric caeca and the development of pharyngeal teeth (Yúfera *et al.*, 2014). Size instead of age is a good proxy for morphological development, since, as we found, there is an overlap in the age at which different developmental stages were observed, while there is no significant overlap in the larval length and weight among developmental stages. The vast majority of the studies regarding tuna larval development are mainly focused

on the development of digestive physiology (*e.g.* Buentello *et al.*, 2011; Miyashita *et al.*, 1998), organogenesis (*e.g.* Fujimoto *et al.*, 2008; Yúfera *et al.*, 2014) and the development of morphological structures (*e.g.* Miyashita *et al.*, 2001). Our results from the laboratory indicate first flexion signs from 5.1 ± 0.5 mm and first post-flexion signs from 6.2 ± 0.7 mm, similar to those reported at 5–5.7 mm and 7–7.4 mm in Pacific bluefin tuna at temperatures of 24.5–27.7 °C and 25 ºC, respectively (Kaji *et al.*, 1996; Miyashita *et al.*, 2001).

We found RNA:DNA reflects the variation in somatic growth in ABFT larvae. The higher values observed in more developed larvae are a consequence of the decreasing DNA (DNA:dry weight) and constant RNA (RNA:dry weight) concentrations with age and development. The decreasing trend in the DNA concentration suggests a switch in the growth mechanisms of the larval cells from a higher proportion of hyperplasia to a higher proportion of hypertrophy (Buckley *et al.*, 1999; Malzahn *et al.*, 2003). Hyperplastic growth occurring by proliferation of new cells is characterized by mitotic activity, whereas hypertrophy is the enlargement of the existing cells (Weatherley *et al.*, 1988). A decrease in the DNA concentration is achieved by a higher increment in the body weight of the larvae (DW) in relation with the DNA content (genetic material) per unit dry weight, suggesting a switch to cell enlargement (hypertrophy). Therefore, our results suggest that once the larvae have completed the post-flexion stage and started piscivory, growth by cell enlargement dominates, a trend that has been observed in the juvenile stage of Pacific bluefin tuna (Tanaka *et al.*, 2007). Evident hypertrophy from histochemical analyses has been documented from 29 dph in Pacific bluefin tuna juveniles, indicating hyperplasia persisted over a long period of time (Roy *et al.*, 2012, 2014). In AB larvae, RNA:DNA, DNA and RNA concentrations remained steady with age and development. The dynamic of hyperplasia and hypertrophy has been seen to determine the ultimate somatic size of the fish. In small adult size species hyperplasia ceased early and most of the growth is attributed to hypertrophy, whereas in those attaining large adult sizes hyperplasia continued for a long time in the development (Weatherley *et al.*, 1988). AB adult sizes are much smaller than those of ABFT, which may explain the earlier combination of hyperplasia and hypertrophy in AB than in ABFT larvae.

The higher RNA:DNA ratio values in ABFT than AB may be explained by the high proportion of the head tissue into the biochemical analyses, resulting in a decrease in the total RNA:DNA content. The RNA:DNA ratio in the head of several species is lower than in the muscles due to higher DNA concentrations derived from the presence of a larger number of small cells in the head (Olivar *et al.*, 2009). AB larvae are characterized for having a very big head, representing more than a third of the body size, until reaching the juvenile stage, whereas ABFT's head is also bigger than in other larval fish species, but proportionally smaller than in AB (Rodríguez *et al.*, 2017).

In this study, RNA:DNA values were similar to 2 in AB and lower than 3 in ABFT. Measurements were conducted in 0.13-0.77 mg larvae at temperatures >26 ºC. RNA:DNA ratios in laboratory reared Pacific bluefin tuna are between 2–4 at 25-28 ºC measured in fed larvae between 3 to 18 dph (Tanaka et al., 2008). Rates lower than 1 or 3 measured in the field suggest bad nutritional condition (García et al., 2006; Tanaka et al., 2008). However, one should be cautious comparing the relative measures between laboratory and field sampled individuals. The physical condition of fish larvae from field sampling may be affected until preservation having an effect in the RNA:DNA value (Chícharo, 1997; Theilacker, 1978) and therefore laboratory results may be more accurate.

We found a positive correlation between short-term growth (RNA:DNA) and long-term growth (size-at-age) in our experiments, suggesting good larval condition in ABFT and AB larvae during the five days or more before sampling. Both the RNA:DNA and the DNA:dry weight ratio did not show significant differences among the different larval developmental stages until the post-flexion stage. We found that the biochemical measures of cell size (DNA:dry weight), rather than the protein synthesis capacity (RNA:DNA), was a better indicator of the condition of the different flexion stages in ABFT larvae (Bergeron, 1997). The DNA:dry weight ratio is sensitive to the nutritional status of the larvae because cell weight is decreasing while DNA concentration is kept constant if feeding decreases (Bergeron, 1997). At a given size, the cell condition of the flexion larvae (F1 and F2) was better than those already in post-flexion (F3). We saw the first signs of post-flexion at 10 dph while the larval prey was first offered to the tanks at 13 dph. The timing to switch to piscivory in ABFT is known to determine further larval growth and survival and a delay of 4 or 8 days can increase mortalities (Reglero *et al.*, 2014a). The delay in prey switch in our experiments may have affected the nutritional state of those larvae already in post-flexion at 10 dph (Takebe *et al.*, 2012). This is supported by the decrease in size of the big larvae (CSD) from 12 to 13 dph. The suboptimal feeding of larvae in post-flexion might be hiding an increase in RNA concentration once the notochord flexion is completely finished. The energy gained by suboptimal prey feeding along with the increase in energy demands due to the active swimming of the post-flexion larvae might exceed larval aerobic capacity, which would lead to a decrease in energy efficiency, and consequently, a decrease in larval condition (Billerbeck *et al.*, 2001; Faria *et al.*, 2011; Illing *et al.*, 2018; Lankford *et al.*, 2001; Moyano *et al.*, 2018). Silva *et al.* (2015) in plaice larvae (*Pleuronectes platessa*) concluded that larvae with lower DNA:dry weight had better swimming abilities. No relationship was found in AB larvae, suggesting that at a specific size their cell condition was similar regardless of developmental stage.

Our results clearly indicate that when parameters such as diet and temperature do not vary, cell growth mechanisms are species-specific. Under cultured conditions, as seen in our results, feeding conditions might not be enough to explain developmental differences in ABFT and AB larvae. The explanation of growth differences and developmental rates in those fast-growing fish larval species might be difficult to identify since short time intervals separate different stages and differences in

growth strategies between individuals (Juan-Jordá *et al.*, 2013). The improvement of our understanding during the first days of life of larval fish is of main interest, since it will determine which larvae survive and grow to post-flexion. It is also essential in larviculture to clarify size grading that might lead to further cannibalism. Besides, it also shows the importance of the timing to piscivory, where a slight delay can have an effect on larval cell condition, as previously seen in growth and survival (Reglero *et al.*, 2014a). Therefore, more information is needed, and other aspects such as energy consumption (*e.g.* swimming, specific dynamic action) should be investigated in order to better understand larval species-specific strategies.

CHAPTER 4

- SIZE-SELECTIVE MORTALITY OF LABORATORY-REARED ATLANTIC BLUEFIN TUNA LARVAE: EVIDENCE FROM MICROSTRUCTURE ANALYSIS OF OTOLITHS DURING THE PISCIVOROUS STAGE -

Size-selective mortality of laboratory-reared Atlantic bluefin tuna larvae: evidence from microstructure analysis of otoliths during the piscivorous stage

ABSTRACT

Atlantic bluefin tuna (*Thunnus thynnus*) larvae show strong piscivorous feeding behavior at the very early larval stage and this enables them to grow at high rates. We conducted a laboratory experiment in which Atlantic bluefin tuna larvae were offered larval prey for the first time at different ages to simulate the early onset of piscivory at three treatments: yolk-sac larvae (YSL), delayed onset of piscivory (DYSL) and a solely planktivorous diet (Rotifers). The otolith microstructure was then used to compare the larval size distribution at the onset of the experiment with the estimated previous size-at-age of the survivors at the end of the experiment by back-calculation. Within a cohort, our results show size-selective mortality of the largest larvae independent of the differences in the timing of onset of piscivory and differences in growth patterns. The results also corroborate the rapid response of Atlantic bluefin tuna to piscivory in terms of growth reflected in the otolith increment widths. Being bigger did not infer a survival advantage and mortality rates did not decline with increasing larval size. Smaller size at a given age could under certain conditions and stages of development confer a survival advantage of individual members of a larval cohort when suitable small-sized prey is available.

4.1 Introduction

The highest mortality during the life of a fish occurs during the larval stage (Houde, 2008). The transition period from endogenous feeding, when energy is available in the yolk-sac, to exogenous feeding, when the larva is typically dependent on available planktonic food resources, is considered a "critical period" (Hjort, 1914). However, in large pelagic species such as bluefin tuna, as in other Scombridae species, piscivory is also observed during the larval stage (Kaji *et al.*, 2002; Sawada *et al.*, 2005). This second dietary transition, from planktivory to piscivory feeding, might represent another major bottleneck in larval survival (Reglero *et al.*, 2014a). Mortality during the larval stage has been suggested to be strongly size-dependent (McGurk, 1986) and large size and fast-growing larvae are typically considered to have higher survival rates (*e.g.* Anderson, 1988; Takasuka *et al.*, 2003, 2004) although the opposite is also true (Meekan and Fortier, 1996). Piscivorous and cannibalistic behaviors can increase size differences dramatically among individuals of the same cohort (Hecht and Pienaar, 1993). Our knowledge is scarce regarding how mortality affects the size spectrum of the larvae that survive through piscivory and become part of the juvenile population.

Atlantic bluefin tuna (*Thunnus thynnus*) is a scombrid with fast-growing warm water larvae that exhibit most of the juvenile anatomical characteristics at sizes around 17 mm when reared under *ad libitum* feeding conditions (21 days post hatch at 23–24 °C, Yúfera *et al.*, 2014). Evidence for the onset of piscivorous behavior in Atlantic and Pacific bluefin tuna (*Thunnus orientalis*) has been observed in the laboratory at larval lengths between 7 and 9 mm (Reglero *et al.*, 2014a; Tanaka *et al.*, 2010, 2014b, 2015). In contrast, there is little evidence of piscivory in the field only reported in some individuals captured in the Gulf of Mexico and recently in the Mediterranean Sea (Llopiz *et al.*, 2015; Uriarte *et al.*, 2017), since larvae longer than 7 mm are rarely captured during ichthyoplankton surveys probably due to net avoidance (Tilley *et al.*, 2016). Growth has been well studied in Pacific bluefin tuna and developmental variability among individuals is apparent during the planktivorous stage (Takebe *et al.*, 2012; Tanaka *et al.*, 2006), and particularly after the transition to piscivory feeding behavior (Tanaka *et al.*, 2010, 2014a, 2014b). Since mortality during the larval stage of fish can be size dependent, empirical evidence of the survival size spectrum is particularly important in order to determine the survival strategy of the species and the contribution to the life stages that come afterwards. In Pacific bluefin tuna, large and fast-growing larvae have increased probability of survival to the post-flexion stage, according to results from repeated field-sampled otoliths of planktivorous larvae (Satoh *et al.*, 2013), and by comparing growth trajectories of planktivorous larvae and juveniles captured in the field (Tanaka *et al.*, 2006; Watai *et al.*, 2017). No potential piscivorous larvae (post-flexion developmental stage) were sampled in the previously cited field studies and therefore size and growth-selective mortality during the transition to piscivorous feeding of Pacific bluefin tuna larvae remains unknown. For Atlantic bluefin tuna larvae, no analyses on size- and growth-dependent mortality have ever been undertaken in the field.

Larval rearing in controlled conditions can provide a set-up to study how growth and survival relate to other factors in the field that can have an effect, such as predation, prey density and temperature variability. These factors can be isolated and studied where piscivorous larvae can be easily obtained. To date, only Tanaka *et al.* (2014a, 2018) have studied the effects of size and growth selective mortality during the piscivorous stage of Pacific bluefin tuna larvae. Their studies on hatchery-reared Pacific bluefin tuna larvae suggested that larger and faster-growing larvae could have advantages both prior to and after the onset of piscivory. However, no size and growth selective mortality patterns in Atlantic bluefin tuna larvae have yet been described during either the planktivorous or piscivorous stage. The knowledge of how selective mortality can affect the size distribution of the surviving larvae during the piscivorous stage may influence processes that will affect juvenile populations of Atlantic bluefin tuna due to cumulative mortality during the larval stage. In this study, we examine how manipulating the timing of onset of piscivory might affect the size-dependent mortality of a population of laboratory-reared Atlantic bluefin tuna larvae based on otolith microstructure back-calculation analysis. Otolith size and increment width are representative of size and growth rates during the larval stage and can therefore serve to trace the growth history of individuals (Campana and Jones, 1992). The use of repeated samplings within the same population at an initial point and at later points in time allowed the estimation of which individuals were most likely to survive (size-selective mortality) by comparing the population characteristics at different times (Mosegaard *et al.*, 2002).

4.2 Materials and Methods

4.2.1 Larval rearing

Fertilized eggs of Atlantic bluefin tuna (ABFT) were provided by the private company Caladeros del Mediterráneo S.L. from naturally spawning captive adults maintained in sea cages. The eggs were placed directly in three 1500 L larval rearing cylindrical tanks at a density of 10 eggs per liter, where they hatched one day after transfer. During the larval rearing period, the photoperiod regime was 16 h of light and 8 h of darkness (16L:8D) and water temperature ranged between 22.4 °C and 25.7 °C. Feeding during larval rearing consisted of enriched rotifers (*Brachionus plicatilis*) supplied twice per day to keep a concentration of 10 rotifers mL^{-1} inside the tanks. This guaranteed *ad libitum* feeding conditions during daylight hours. Pseudo green water culture was obtained by adding cultivated microalgae (*Nannochoropsis gaditana*) twice per day during the first rearing days, and later by changing to a paste of concentrated *Chlorella* (Super fresh Chlorella SV-12, Chlorella industry Co., Ltd., Japan) which was added four times a day. ABFT larvae were maintained in the rearing tanks for four days before the experiment began or 15 days post hatch (dph). For further details on rearing procedures, see Reglero *et al.* (2014a).

4.2.2 Experiment setup

At 15 dph or Experimental day (Eday) -4, four days before the onset of the experiment, a total of 1800 larvae were transferred to 15 experimental tanks of 150 L (120 larvae per tank) for acclimation purposes with a light regime of 15L:9D hereafter. Three times a day, enriched rotifers were added to maintain a concentration of 10 rotifers mL^{-1} for *ad libitum* feeding conditions as explained above. At 19 dph, when the larvae size was between 7 and 9 mm in standard length (SL), and anatomical characteristics enabled the larvae to start with piscivory, the experiment started (Eday 0).

In order to see the effect that different timings of onset of piscivory have on ABFT size distribution, we conducted an experiment with three different feeding treatments, 5 tank replicates each. In the first treatment, referred to as Rotifers from now onwards, only enriched rotifers were provided from Eday 0 until the end of the experiment 8 days later (Eday 8). In the second treatment, referred to as DYSL (delayed yolk-sac larvae) from now onwards, after 4 days with only rotifers provided (Eday 4, 23 dph), sea bream (*Sparus aurata*) yolk-sac larvae were added *ad libitum* in the tanks along with the enriched rotifers until Eday 8. In the last treatment, referred to as YSL from now onwards, sea bream yolk-sac larvae were added *ad libitum* from Eday 0 along with enriched rotifers to Eday 8. Up to at least 300 sea bream yolk-sac larvae of 0–2 dph (3.4 ± 0.04 mm) per individual ABFT were provided to ensure to remain in the tanks at any time in the corresponding treatments which is known to represent *ad libitum* conditions in this species (Ortega, 2015; Reglero *et al.*, 2014a). The number of larval preys incorporated into the tank to ensure *ad libitum* conditions was estimated based on hourly observations to ensure there was always prey available in the tank. The experiment lasted for 8 days (from 19 dph to 27 dph). The average water temperature was 24.8 ± 0.8 °C.

On Eday 0, early in the morning and in darkness, 3 larvae per tank were sub-sampled for body size measurements at the start of the experiment. Shortly after, with light, all the remaining larvae in each tank were counted for survival estimates. On average, 25 larvae remained in each tank after acclimation on Eday 0. On Eday 8 all the surviving larvae were counted and sampled for accurate survival estimates (Table 4.1). Daily survival estimates were not possible to obtain by collecting dead larvae from the bottom of the tanks due to their rapid degradation. The larvae were terminally anesthetized using clove oil (Guinama© Spain), individually photographed for morphometric measures (standard length) to the nearest 0.1 mm and individually frozen in cryotubes at -80 °C (see Reglero *et al.* (2014a) for more details). The frozen larvae were then rinsed with distilled water and dried in a 60 °C oven for 24 h, then weighed to the nearest 0.01 mg. At the end of the experiment, a total of 131 larvae had been sampled, 45 larvae from Eday 0, and 86 from Eday 8.

4.2.3 Otolith microstructure analysis

Both sagittal otoliths from each sampled larva were removed to assess size-selective mortality (Table 4.1). The otoliths were extracted using fine needles under a stereo microscope and cleaned. Immediately after, otoliths were individually placed on a glass slide and mounted in a small drop of thermoplastic cement (Buehler®) with the distal side of the sagitta facing upwards. Sagittal otoliths were chosen due to their larger size among the other otolith pairs (Itoh *et al.*, 2000).

Otoliths were analyzed using a microscope with transmitted light at magnification of 100X and 63X. The microscope was connected to a Leica DFC450 video camera and each otolith was photographed at a resolution of 2560 x 1920 pixels. Later, several measurements were made on the otolith image using Image J® software (National Institute of Health, Bethesda, MD). Otolith radii (OR) were measured along the longest possible post-rostrum axis from the core to the outer edge, perpendicular to the widest part of the otolith (Fig. 4.1), following the procedure described in Folkvord *et al.* (2010). Increment widths were obtained as the difference between the distance from the core to two adjacent increments.

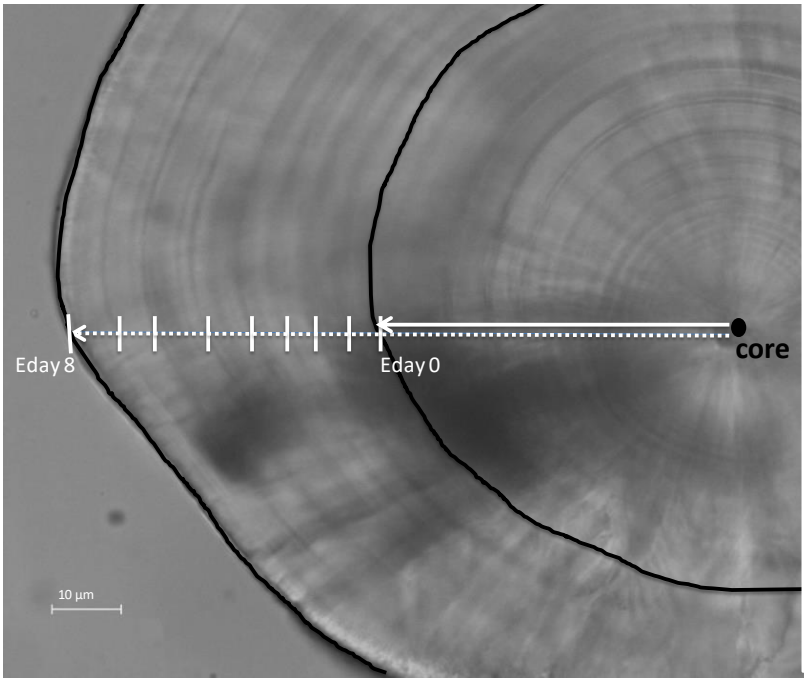


Figure 4.1. Atlantic bluefin tuna sagittal otolith showing the increment measurement methodology utilized. The white dashed line represents the otolith radius-at-age for Experimental day (Eday) 8 and the white solid line represents the otolith radius at Eday 0. Black lines highlight the otolith edge on Eday 8 and the increment that represents back-calculated Eday 0. Vertical white lines along the dashed white line indicate daily increments on completed side of the darker layer. Arrows are offset for clarity (OR=89.4 µm). The otolith core is shown and named. The image was taken using transmitted light.

Back-calculation estimates to Eday 0 were obtained from the Eday 8 otoliths by back-calculating 8 increments from the edge along the OR. These back-calculated OR at Eday 0 were then compared to the initially sampled OR on Eday 0 to obtain estimates of size-selective mortality within each treatment group. Both sagittae were read twice, with one month between reads. In general, no significant age estimate differences have been reported between the left and right sagitta (Campana, 1990). Therefore, the otolith which was easiest to measure was selected for further microstructure analysis.

4.2.4 Statistical analysis

All statistical analyses were carried out using the R statistical software (www.r-project.org). Survival percentages were root-squared and arcsine transformed for normality. Survival during acclimation and during the experiment were compared among treatments using values from replicate tanks in a factorial one-way ANOVA. Instantaneous mortality, M, during the acclimation and the experiments was calculated using the equation:

$$(1)\quad M = 100 \cdot [\mathrm{Ln}\,(N_1) - \mathrm{Ln}\,(N_2) / (t_2 - t_1)]\ (\%\ \mathrm{day}^{-1})$$

where Ln N_2 is the natural logarithm of the number of surviving larvae (N) on Eday 8, and Ln N_1 is the natural logarithm of the initial number of larvae on Eday 0 at time t_2 and t_1 respectively. To study the correlation between final otolith size *vs.* previous back-calculated otolith size within fish in each treatment and overall, Spearman's rank correlation coefficient (SpRC) was calculated. Otolith size distributions were first analyzed for normality and homoscedasticity (Shapiro-Wilk and Levene). Statistical differences in mean larval size and otolith size-at-age between treatments was analyzed by a two-way nested ANOVA with treatment as independent fixed factor and tanks as a random factor nested in treatments. For the size-selective mortality estimation based on otolith size-at-age, a three-way nested ANOVA was used with age of sampling and treatment as independent fixed factors and tanks as a random factor nested. Differences in increments width between days were also analyzed using a three-way nested ANOVA. Significant ANOVAs were followed by a Tukey's honestly significant difference (HSD) multiple comparisons test to determine differences among treatments. All test results were considered significant at a level of 0.05.

Cumulative size distributions and specific growth rate in otolith growth were estimated as described in Folkvord *et al.* (2009). Assuming static ranking of fish sizes within a cohort is unlikely to change much in the short term, cumulative size distributions were used for visualizing variability of sizes within cohorts over time in a single graph. Size-at-age analysis was estimated from cross-sectional data methods by using the mean OR from two different sampling days whereas back-calculation data analysis was done by longitudinal data method with repeated measurements on the same individual (Chambers and Miller, 1995). OR of sampled

larvae from Eday 0 (19 dph) of the three treatments were pooled together since the different treatments had not yet started.

Specific growth rates (SGR) in otolith growth were estimated using the following equation:

$$(2) \qquad SGR = 100 \cdot [\ln(OR_2) - \ln(OR_1) / (t_2 - t_1)] \ (\% \ day^{-1})$$

where Ln OR_2 is the natural logarithm of the final otolith radius (μm) on Eday 8, and Ln OR_1 is the natural logarithm of the OR on Eday 0, of back-calculated Eday 0 or of actual OR observed from Eday 0. We used OR data corresponding to a given percentile of the population (5%, 50% and 95%) on day t_1 and t_2 respectively, obtained from the cumulative size distributions (CSD). The growth of all size classes was estimated by following the size of a given percentile of the population from one sampling to the next (Folkvord *et al.*, 1994).

4.3 Results

Survival during the acclimation time, 4 days prior to the experiment, was similar among the three treatments (one-way ANOVA, $p>0.05$). During the experiment, survival was significantly higher in the YSL treatment than in the Rotifers treatment (Table 4.1, one-way ANOVA, Tukey HSD, $p<0.05$) and similar to the DYSL treatment (Table 4.1, one-way ANOVA, Tukey HSD, $p>0.05$). Instantaneous mortality during the acclimation period was on average 43% day^{-1} and during the experiment it was on average 19% day^{-1} (Table 4.1). In those individuals with both sagittae available there was high correlation among OR measurements at 19 dph (R^2=0.99, $p<0.001$, n=22) and 27 dph larvae (R^2=0.97, $p<0.001$, n=54). There was a high correlation between the first and the second otolith total radius readings using all the otoliths extracted, at 19 dph (R^2=0.99, $p<0.001$, n=64) and 27 dph (R^2=0.99, $p<0.001$, n=137). The standard length of the larvae was linearly correlated to the OR (Fig. 4.2, R^2=0.90, $p<0.001$, n=124), and thus the otolith increment widths were considered proportional to fish growth. Final OR at 27 dph was positively correlated with the previous back-calculated radius within each treatment (Spearman rank correlation, Rotifers: SpRC=0.64, p=0.005, n=18, DYSL: SpRC=0.83, $p<0.001$, n=24, YSL: SpRC=0.80, $p<0.001$, n=40), as it was when treatments were pooled together (Spearman rank correlation, SpRC=0.63, $p<0.005$, n=82).

Table 4.1. Description of the quantities, larval sizes and otoliths analyzed for the 19 days post hatch (dph) and the 27 dph sampling groups for Rotifers, delayed yolk-sac larva (DYSL) and yolk-sac larva (YSL) treatments are provided. One sagittal otolith was used for microstructure analysis however, a subset (n=76) of larvae are represented by two sagittal otoliths. Final mean survival and instantaneous mortality during the acclimation time for the Experiment day (Eday) -4 to 0 and during the Eday 0 to 8 are shown. Different letters (a,b) indicate significantly different treatment means (ANOVA, Tukey HSD, p<0.05).

Sampling	Treatment	Sampled larvae	Otoliths	Standard length (mm)	Dry weight (mg)	Otolith radius (µm)	Mean survival (%)	Instantaneous mortality (% day^{-1})
19 DPH Eday 0	Rotifers	15 (3 per tank)	13	8.2 ± 0.6^{a}	0.63 ± 0.31^{a}	47.9 ± 11.0^{a}	17.5 ± 4.4^{a}	44.2 ± 0.1^{a}
	DYSL	15 (3 per tank)	15	7.9 ± 1.0^{a}	0.60 ± 0.30^{a}	48.8 ± 15.7^{a}	19.7 ± 7.1^{a}	41.8 ± 0.1^{a}
	YSL	15 (3 per tank)	14	8.2 ± 0.8^{a}	0.64 ± 0.24^{a}	49.9 ± 11.7^{a}	18.2 ± 6.5^{a}	43.9 ± 0.1^{a}
27 DPH Eday 8	Rotifers	19 (all survivors)	18	8.7 ± 0.6^{b}	1.06 ± 0.36^{b}	75.5 ± 13.3^{b}	14.4 ± 11.5^{b}	23.6 ± 0.1^{a}
	DYSL	25 (all survivors)	24	10.4 ± 1.5^{b}	2.60 ± 1.50^{b}	93.2 ± 24.0^{b}	21.7 ± 15.3^{a,b}	21.6 ± 0.1^{a,b}
	YSL	42 (all survivors)	40	13.0 ± 2.8^{a}	5.95 ± 4.43^{a}	130.8 ± 40.8^{a}	39.7 ± 16.2^{a}	12.5 ± 0.0^{b}
	TOTAL	**131**	**124**					

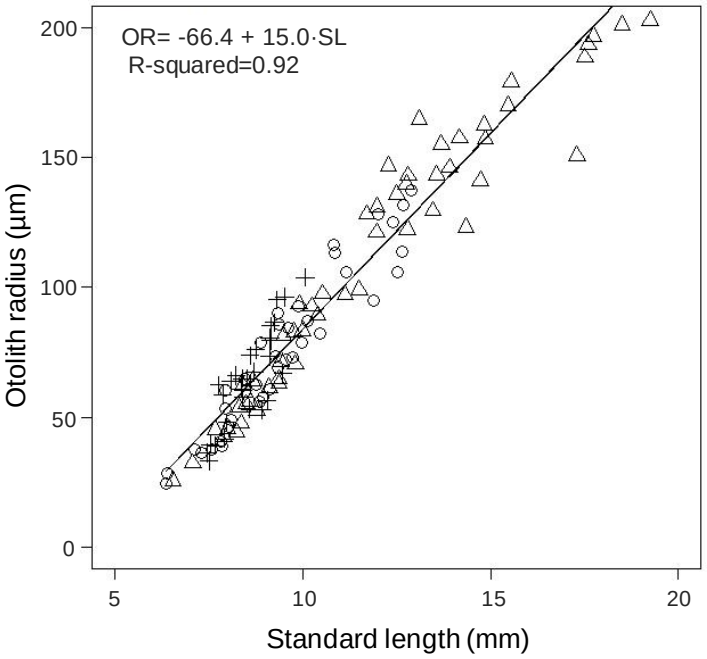


Figure 4.2. The relationship between larval standard length (mm) and otolith radius (µm) for the three treatments combined: Rotifers (+), delayed yolk-sac larvae (O, DYSL) and yolk-sac larvae (Δ, YSL).

The average OR at the onset of the experiment at 19 dph was 48.8 ± 12.8 µm and did not differ among the three experimental treatments (Fig. 4.3, triangle symbols, two-way nested ANOVA, p>0.05). The back-calculated OR at 19 dph of the surviving larvae sampled at 27 dph did not differ significantly between treatments and overall averaged 35.0 ± 8.1 µm in the Rotifers treatment, 40.9 ± 10.6 µm in the DYSL treatment and 37.5 ± 9.7 µm in the YSL treatment. They were significantly smaller than the OR measured at 19 dph at the beginning of the experiment (Fig. 4.3, rectangle and triangle symbols respectively, three-way nested ANOVA, Tukey HSD,

p<0.05). At 27 dph the OR of the surviving larvae were significantly larger in the YSL treatment (average 130.2 ± 40.6 µm) than those in the DYSL and Rotifers treatments (average 93.3 ± 24.0 µm and 75.6 ± 13.3 µm respectively) (Fig. 4.3, circle symbols, two-way nested ANOVA, Tukey HSD, p<0.05), the latter two not being significantly different (two-way nested ANOVA, Tukey HSD, p>0.05).

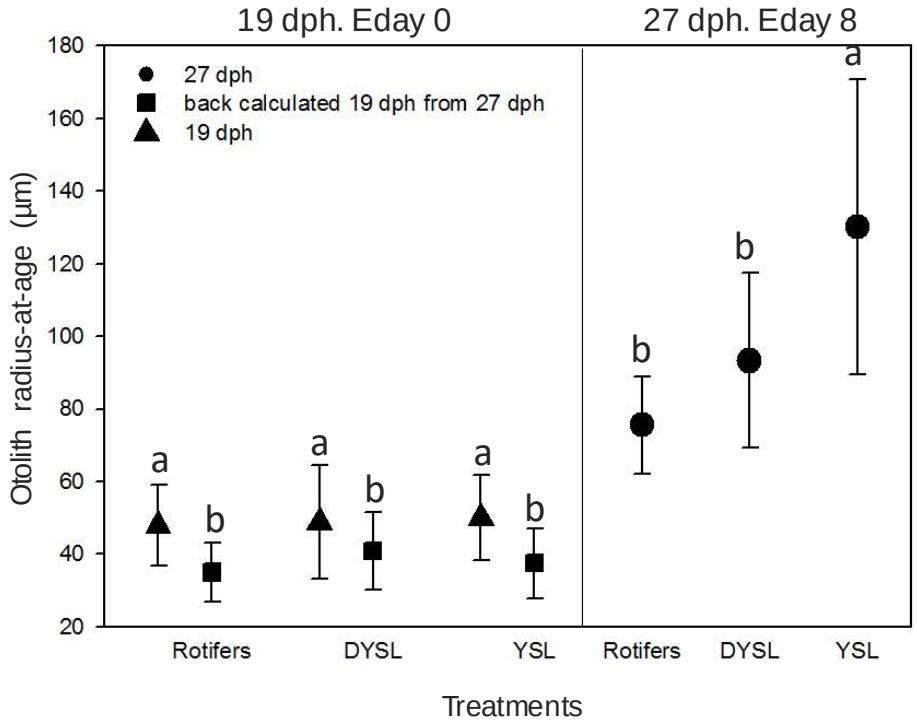


Figure 4.3. Mean otolith radius from treatment groups Rotifers, delayed yolk-sac larvae (DYSL) and yolk-sac larvae (YSL) sampled at different times and showing the data corresponding to Experimental day (Eday) 0 and Eday 8. The circle and triangle symbols represent otolith total radii at 27 dph and 19 dph respectively. Rectangle symbols represent the otolith back-calculated radii to 19 dph from 27 dph sampled otoliths. On Eday 0, different letters at respective groups indicate significantly different otolith sizes-at-age of larvae sampled at different dates (three-way nested ANOVA, Tukey HSD, p<0.05). The two different letters (a,b) indicate differences in otolith sizes among treatments within each day of the experiment (two-way nested ANOVA, Tukey HSD, p<0.05). Whiskers indicate standard deviation.

Due to the similar otolith sizes among treatments of the initial sampling population (19 dph), the otolith sizes were pooled together for the cumulative analysis. The cumulative size distributions between subsequent sampling days (19 dph and 27 dph) were almost parallel in Rotifers and DYSL while in the YSL treatment cumulative size distribution increased with the biggest individuals (Fig. 4.4). Similar cumulative distributions of the back-calculated data were obtained in all treatments.

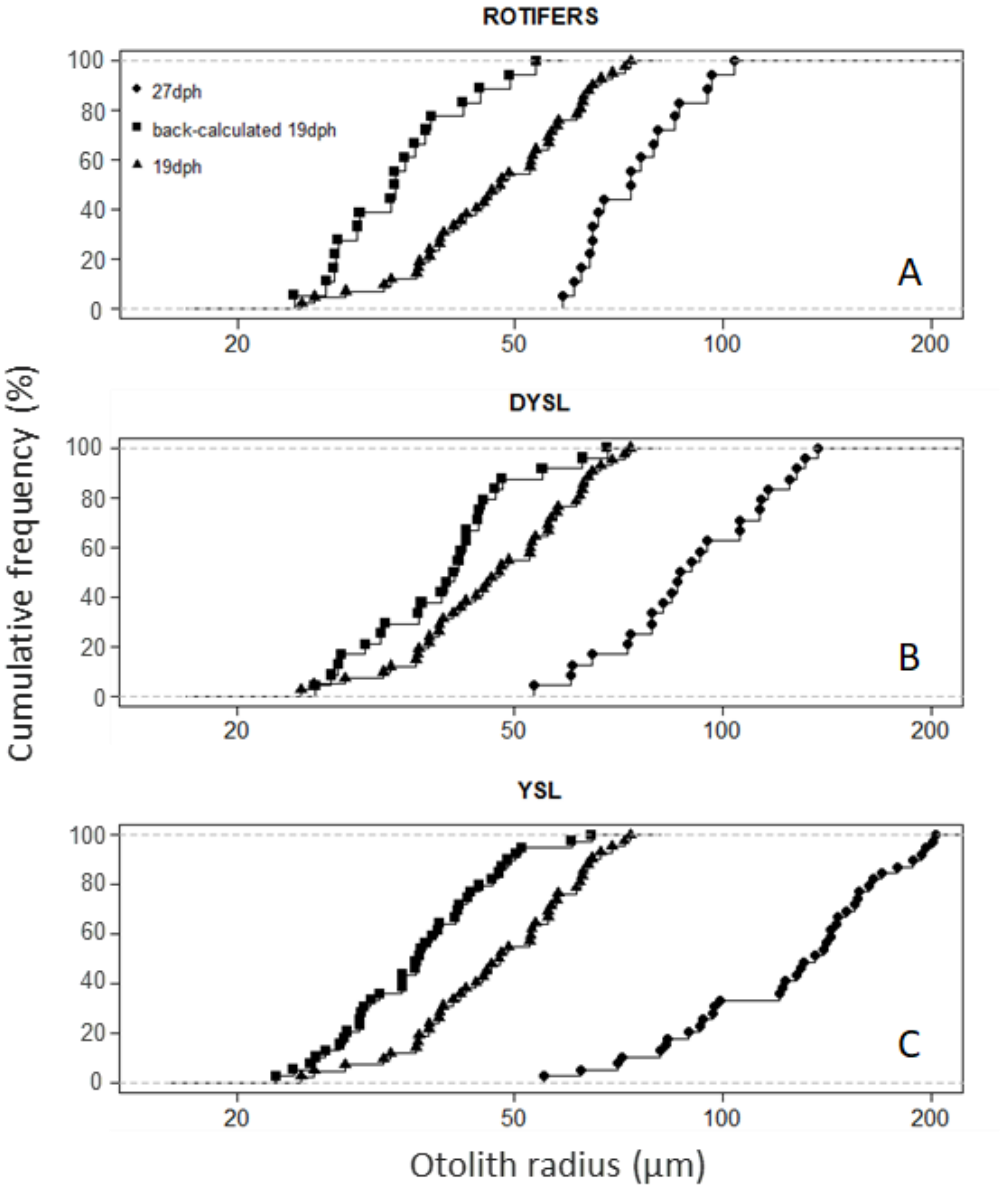


Figure 4.4. Combined cumulative size frequency distributions of otoliths radii at 19 dph (▲), at 27 dph (●) and back-calculated 19 dph radii (■) for the three treatments treatment groups Rotifers, delayed yolk-sac larvae (DYSL) and yolk-sac larvae (YSL). Data from the three treatments at 19 dph are pooled together. Note that the x-axes are log (natural log) transformed, values are untransformed.

The otolith-specific growth rates during the eight experimental days (Eday 0 to 8) of the surviving population based on the back-calculated data were higher than the specific growth rates obtained using initial and final sampled OR data in all the treatments and in all the population percentiles (Table 4.2). The growth rates of the median and large larvae obtained in all the treatments was similar, considering both with and without back-calculation. The higher growth rate of the small larvae in the Rotifer and DYSL treatments differs from the lower growth rate in the small larvae in the YSL treatment (Table 4.2).

Table 4.2. Growth rates in the experiment. The specific otolith growth rate (SGR, µm % day^{-1}) of small (5 percentile), median (50 percentile) and large (95 percentile) larvae are shown for Rotifers, delayed yolk-sac larva (DYSL) and yolk-sac larva (YSL) treatments. The growth rate during the "Experimental time" is shown using otolith back-calculation data from 27 dph sampled larvae (longitudinal data) and without back-calculation analysis using otoliths size-at-age of larvae sampled at 19 dph and 27 dph (cross-sectional data). Also, the growth rate during the "Acclimation time" (four days prior to the start of the experiment) is shown using back-calculated data to 15 dph from larvae sampled at 19 dph (longitudinal data). Data from the different treatments at 19 dph sampled larvae were pooled together, and therefore, the same growth was obtained for the small, median and large larvae.

			SGR Otolith radius (µm % day^{-1})		
			Experimental time		Acclimation time
Larval group	Size	Interval (percentile)	Back-calculated from 27 dph	Size-at-age 19 and 27 dph	Back-calculated from 19 dph
Rotifers	Small	5%	10.4	9.3	8.4
	Median	50%	9.8	5.4	11.5
	Large	95%	8.4	4.3	11.2
DYSL	Small	5%	9.9	9.3	8.4
	Median	50%	9.5	7.7	11.5
	Large	95%	9.4	8.0	11.2
YSL	Small	5%	12.6	11.1	8.4
	Median	50%	16.4	13.0	11.5
	Large	95%	16.6	13.1	11.2

The growth rate of the surviving larvae sampled at 19 dph four days prior to the experiment (Eday -4 to 0) by back-calculation of the otoliths was determined and resulted to be similar among the median and large larvae, and smaller in the small larvae (Table 4.2). The daily increment width increased with age (Fig. 4.5). Four days prior to the experiment, sampled larvae in all the treatments at 19 dph (Eday 0) had relatively constant and similar daily increment growth (three-way nested ANOVA, $p>0.05$). In general, during the experimental period, the narrowest increment widths, on average 3–4 µm, were observed during the first experimental days in all the treatments (Fig. 4.5). An increase in otolith width was already observed one day after the onset of piscivory both in the DYSL and YSL treatments, whereas for the Rotifers treatment otolith increment widths were very similar throughout the entire experiment (Fig. 4.5A). Final otolith increments widths from Eday 26–27 averaged 7.2 ± 2.4 µm, 9.4 ± 3.8 µm and 22.2 ± 8.6 µm for the Rotifers, DYSL and YSL treatments respectively.

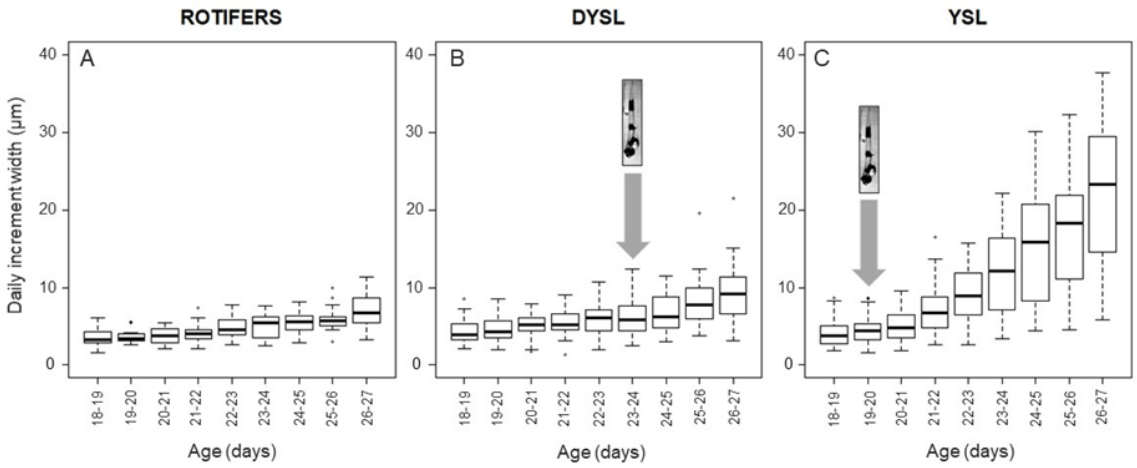


Figure 4.5. Boxplots of daily increment widths back-calculated at 18 to 27 dph for the three sample treatments: A) Rotifers, B) delayed yolk-sac larvae, DYSL and C) yolk-sac larvae, YSL. The line within each box indicates the median daily width (µm), and the whiskers are the 25th and 75th percentiles. A sea bream yolk-sac larvae image is inserted along with a gray arrow in panels b and c showing the days where they were added for the first time to each treatment, at 23 dph in DYSL and at 19 dph in YSL.

4.4 Discussion

The switch from a planktivorous to a piscivorous diet can be considered the second critical window in ABFT and an early transition benefits the survival and growth of the larvae (Reglero *et al.*, 2014a). We have studied the effect that the different timing in the onset of piscivory has on the growth of the population and on the survival of the different size classes of laboratory-reared ABFT using otolith microstructure analysis. Our laboratory experiments show size-selective mortality of the largest larvae at 19 dph independently of the timing of onset of piscivory. This study represents the first attempt to infer size-selective mortality in piscivorous ABFT larvae.

A proportional relationship between otolith and larval size through time has also been reported in the field for smaller ABFT larvae than those considered in our study (Brothers *et al.*, 1983; García *et al.*, 2006). Therefore, we were justified to proceed with larval size back-calculations based on the otolith analyses (Campana, 1990). The first increment formation in the otoliths of laboratory-reared Pacific bluefin tuna correspond with the onset of exogenous feeding and continuous daily periodicity in increment formation has been reported to at least 71 dph (Itoh *et al.*, 2000). In our otoliths all the increments were easy to identify and we concluded that at least one increment was formed per day from 15 dph to 27 dph regardless of diet composition and growth rate.

We found high daily mortalities of around 40% during the acclimation time, probably due to the handling of the larvae during transportation from the rearing tanks to the culture tanks. We think most of the mortality was produced during the first and second acclimation days (personal observation, 60–65% mortality after handling 5–6 mm larvae). The similar growth rates of the surviving larvae during the

experiment after acclimation suggest that the high mortality did not affect size variability within groups.

Our otolith back-calculated results show size-selective mortality of the larger larvae in the three treatments. The survivors at the end of the experiment were the smallest larvae at the beginning of the experiment. This could be linked to the conversion of energy intake into growth, determined by the interaction between the larval rate of feeding, assimilation efficiency and the larval metabolic expenditure (Kiørboe *et al.*, 1987). When large larvae in the Rotifers and DYS treatment fed on rotifers, the net energy gain might be lower than that obtained with optimal diet (yolk-sac larvae) and the larval metabolic expenses will exceed its aerobic capacity leading to energy deficiency, and eventually death (Billerbeck *et al.*, 2001; Lankford *et al.*, 2001). We found high daily mortalities around 20% in both treatments during the experiments that most probably represent these larger larvae. In Pacific bluefin tuna, larvae have very low tolerance to starvation with 100% of the larvae dying after 3–4 days at 25 °C and 2–3 days at 28 °C (Tanaka *et al.*, 2008).

The increase in metabolic expenses related to sub-optimal diet compositions cannot explain the size-selective mortality of the larger larvae in the early switch to piscivory treatment (YSL). These larvae were expected to be preying on large prey items that could be caught avoiding digestion and metabolic limitations. We could not analyze the otoliths of the dead larvae to have information about their previous growth history. The high temperatures in the tanks degraded the larvae in very few hours. Besides, given the short duration of the experiments, we tried to stress and alter the larvae as less as possible avoiding daily cleaning of the tanks that would have caused them additional stress and most probably higher mortality. We suggest a possible tank effect that affects the large larvae of the YSL treatment. Under laboratory conditions, large larvae might not be able to express their behavior fully and grow at their maximal rates. Besides, some individuals may have died due to collisions with the tank walls (personal observation). Also, larger larvae geared towards high growth rates may have high energy demands due to elevated food processing (SDA), and thus be more at risk if food supply is not continuously available (Lankford *et al.*, 2001).

Our results of the lower survival rates of the large and probably faster growing larvae regarding the feeding regime agree with some studies that also show this pattern in the field (*e.g.* Litvak and Leggett, 1992; Meekan and Fortier, 1996; Pepin *et al.*, 1992, 2003), despite the general belief that larger and faster growing larvae have an increased probability of survival (*e.g.* Anderson, 1988; Takasuka *et al.*, 2003, 2004). Under enclosed aquaculture conditions, factors such as predation, transport, environmental variability and prey patch distribution are isolated and larval behavior might be modified compared to the field. Behavior might be especially variable in scombrids with highly variable morphology, physiology and nutritional requirements during their short larval period.

The increased survival of small larvae could be related to the type of food provided to the ABFT larvae during the experiment. Prey type can be particularly important

in fast-growing species, like scombrids, where growth is strongly linked to feeding success (Pepin *et al.*, 2015). Before piscivory, *Thunnus* larvae primarily feed on copepod nauplii, cladocerans and appendicularians (Llopiz *et al.*, 2010). Most species have a particularly narrow diet during their larval stage (Llopiz, 2013; Robert *et al.*, 2014). It could be possible that a rotifer-based diet played against the largest individuals before they initiated piscivory. Rotifers are not natural prey in the field. In other species, the nutritional composition, growth and survival of larvae fed copepods was higher than those fed rotifers (Folkvord *et al.*, 2018). However, we still do not have the technological development necessary to culture ABFT larvae on a copepod-based diet.

Our size-selectivity results evidence the type of mortality acting on ABFT during a specific larval stage, when they are potential piscivorous. No studies of this type have been conducted in other life stages of this species. Different patterns of size-selective mortality may be acting from larval emergence until the point where recruitment is set. During the larval stage when dramatic changes in the morphology occur (Kendall *et al.*, 1984), multiple mortality scenarios can directly affect fish larvae against slow-growing individuals or against fast-growing individuals and is the integration of all selective mortality pressures along the early development that will determine the recruitment. In another piscivorous scombrid, Atlantic mackerel (*Scomber scombrus*), different mortality scenarios during different years have been found with fast larval growth in surviving juveniles associated with both strong and weak recruitment (Robert *et al.*, 2007). In *Thunnus maccoyii* density-dependent reduction of growth rate has been observed when larvae are competing for food, increasing the general high mortality of the larval stage and also increasing the cumulative mortality of slow-growing larvae (Jenkins *et al.*, 1991). Therefore, in the field, with just a few additional days to larval stage duration may result into a massive difference in cumulative survival (Jenkins *et al.*, 1991).

At the beginning of the experiment, probably not all of the larvae were morphologically ready to start piscivory, with large teeth, well-developed stomachs with gastric glands and differentiated pyloric caeca (Yúfera *et al.*, 2014). Consequently, during the YSL treatment, the growth of the small larvae, less developed to prey on fish larvae, was lower than for the median and large larvae, already developed to prey on fish larvae. In contrast, in the Rotifer treatment, the small larvae may be in their optimal size range for rotifer consumption, which explains their higher growth rates compared with the median and large ones that were ready to start piscivory. In the DYSL treatment the median and large larvae may show growth compensation after the onset of piscivory, resulting in similar growth rates to those observed for the small larvae feeding on an optimal diet consisting of rotifers (Hunt von Herbing and Turnbough, 2011). Presenting successive cumulative size distributions is quite useful in revealing growth differences. However, a significant mortality event can have important implications for the interpretation of percentile-based growth estimations, since it can affect cohort size variability (Folkvord *et al.*, 2009).

In our study, we found an increase in growth rates across treatments and also an increase in the daily variability. The early piscivory feeding group (YSL) attained the largest sizes and had the fastest growth rate followed by the group in which piscivory was delayed (DYSL) and the planktivorous group (Rotifers). In the maximum growth treatment, YSL, at 26 dph, 6 days after the start of the piscivory, daily increment width of the 75th percentile was twice that of the 25th percentile found. Such individual differences in growth and the strong growth autocorrelation, generally observed in scombrids, suggests that the cumulative size difference would likely lead to cannibalism in the tank (Pepin *et al.*, 2015). However, we avoided cannibalism by stopping the experiment the day before the start of an aggressive behavior. Little is known about potential factors driving variability in growth under piscivorous diet. In Pacific bluefin tuna larvae, prey utilization could be significantly different among individuals of the same age and reared in the same culture tank, because the first days of the piscivory diet overlaps with a planktivorous diet (Tanaka *et al.*, 2014a). Growth variability during the planktivorous stage can induce large growth variability after the onset of piscivory (Takebe *et al.*, 2012). Several different larval developmental stages have been distinguished at a specific age during the planktivorous stage in ABFT larvae (Chapter 3).

In hatchery-reared piscivorous Pacific bluefin tuna, bigger larvae had advantages in terms of growth-selective survival before and after the start of the piscivory (Tanaka *et al.*, 2014a, 2018). These larvae were reared in big mass-rearing tanks (50 000 L) that may favor larval growth and behavior and diminish the chances of tank collision compared to smaller tanks. We chose to use 150 L tanks because they provided the possibility of having 5 replicates per treatment and it was easy to ensure *ad libitum* conditions (homogeneous prey distribution within the tank and easy recognition of available prey). Larger tanks, of 20 000-50 000 L, are probably better to rear planktivorous and piscivorous larvae, although smaller tanks of 1500 L or 5000 L have also been successfully used for 8-15 mm ABFT (De la Gándara *et al.*, 2012). Given the installations available, there was a trade-off between the number of tanks available for replicates and the volume of the tanks. In general, a smaller number of replicates could be conducted when using larger tanks (note one replicate in Tanaka *et al.* (2014a)), and we selected to have replicates. In the field, several authors have studied size-selective mortality in Pacific bluefin tuna larvae (Satoh *et al.*, 2013; Tanaka *et al.*, 2006; Watai *et al.*, 2017), however, net avoidance by the larger piscivorous larvae, along with the different predation pressure might bias representative sampling (Satoh *et al.*, 2013). Besides, in the field, it is difficult to define a cohort originating from the same environmental conditions.

In summary, our results broaden the previous knowledge in which Reglero *et al.* (2014a) determined that onset of piscivory is crucial for survival to recruitment in ABFT larvae. To our knowledge, this study represents the first study on size-selective mortality in piscivorous ABFT larvae under culture conditions. The results derived suggest that being bigger it is not always the best option to survive and mortality rates do not always decline in culture conditions with increasing larval size. When culturing tuna one of the most important issues faced is when to start

piscivory feeding. The mortality of the large larvae along with the rapid response of piscivory in terms of growth reflects the fact that there is a cost to fast growth. A delay in the onset of piscivory under certain culture conditions might promote the growth of the smaller larvae, as seen in our results. Further research, along the different developmental stages from larvae to juvenile, will allow to determine the mortality causes acting during early development in order to integrate survival data across stages to obtain a more precise estimation of recruitment. Different patterns in size-selective mortality directed against fast-growing or slow-growing ABFT individuals need to be integrated to determine the fraction of the cohort that constitutes the majority of the recruits.

CHAPTER 5

- THE EFFECTS OF LIGHT, DARKNESS AND INTERMITTENT FEEDING ON THE GROWTH AND SURVIVAL OF REARED ATLANTIC BONITO AND ATLANTIC BLUEFIN TUNA LARVAE -

The effects of light, darkness and intermittent feeding on the growth and survival of reared Atlantic bonito and Atlantic bluefin tuna larvae

ABSTRACT

In larval culture, long light photoperiod regimes are used to maximize ingestion rates by increasing the accessibility to prey and therefore enhancing larval growth. Intermittent feeding could provide a viable alternative to the commonly used continuous feeding regimes that aim to improve larval growth and survival. In this study, we investigate the effect of alternating light/darkness regimes with intermittent feeding on the growth and survival of piscivorous larvae of two scombrid species: Atlantic bonito (*Sarda sarda*) (Bloch, 1793) and Atlantic bluefin tuna (*Thunnus thynnus*) (Linnaeus, 1758). First, we tested if the manipulation of a light regime generated intermittent feeding by analysing the larval stomach content. Then, we conducted two laboratory experiments to identify the best alternating light regime that maximized larval growth and survival by comparing the results to those obtained using continuous light regimes. The manipulation of light was optimized to provide intermittent feeding opportunities for the larvae, since we discovered a clear interruption of feeding in darkness. An increase in specific ingestion throughout the day was observed in all experiments, reaching a maximum peak late in the day. Bluefin tuna larval growth rates were similar despite different alternating conditions whereas the bonito larvae grew best when provided with light at three hours interval. In both species, growth under alternating light conditions was similar to the 15 hours continuous light treatment. No differences between the alternating and the continuous light treatments were observed in terms of their survival. Our results suggest that alternating light/feeding periods may have a beneficial effect on ingestion rates; possibly because feeding is less satiation-limited, metabolic costs are lower, or food digestion is more efficient under these conditions. Changes in the light regime, that result in pulse feeding, can thus be an optimal strategy to increase growth at no apparent survival cost in bonito or bluefin tuna larval cultures.

5.1 Introduction

In larviculture, the effect of different light regimes has been studied with the main objective of improving the growth and survival of fish larvae to enhance mass production (*e.g.* Duray and Kohno, 1988; Puvanendran and Brown, 2002; Stuart and Drawbridge, 2012). Most fish larvae are visual feeders: dependent on light to increase their feeding incidence (Hunter, 1980). For this reason, it is common to extend the duration of day light in intensive culture to maximize ingestion rates, along with providing constant high concentrations of prey during these light periods. However, providing food may represent one of the highest economic costs of farming fish and one of the principal factors deciding the profitability of intensive fish farming. Therefore, an appropriate feeding and photoperiod schedule is important to guarantee the most efficient production.

When the results of different photoperiods have been compared in terms of growth in length and weight of larvae, the continuous 24 hours light or the extended 18 hours light and 6 hours darkness have been the most beneficial (Hart *et al.*, 1996; Puvanendran and Brown, 2002; Shi *et al.*, 2010). However, even when survival has positively attributed to growth rate (Duray and Kohno, 1988; Partridge *et al.*, 2011; Shi *et al.*, 2010), the effect of long light regimes on larval survival is uncertain, and there are situations in which no differences were found in growth or survival when comparing long and short light regimes (Cañavate *et al.*, 2006; Fielder *et al.*, 2002; Hart *et al.*, 1996). In some cases, negative effects have even been discovered due to the damage of the metamorphic process generating abnormal development (Cañavate *et al.*, 2006) or manifesting in increasingly aggressive and cannibalistic behavior (Vallés and Estévez, 2013).

In order to improve larval growth and survival, intermittent feeding could be an alternative to the commonly used continuous feeding regimes. As witnessed in previous laboratory studies, intermittent feeding could enhance the growth or survival of fish larvae compared to constantly fed larvae under long light regimes, probably due to the increase of assimilation efficiencies (Brown *et al.*, 1997; Rabe and Brown, 2000). In the sea, fish larvae may not necessarily feed constantly during daylight hours if the food is spatially distributed in patches (Owen, 1989) and may need to overcome periods during the day without food.

Atlantic bluefin tuna (*Thunnus thynnus*) is a large-sized pelagic predator that reproduces in the Mediterranean Sea and the Gulf of Mexico. It is considered a new candidate for aquaculture due to the increase of its global demand that has caused an overexploitation of the wild population (Ottolenghi, 2008). Atlantic bonito (*Sarda sarda*) is a medium-sized pelagic predator that only reproduces in the Mediterranean Sea. The success of the completion of its life cycle in captivity (Ortega *et al.*, 2013), its rapid growth which can reach 1 kg in just a few months (Santamaria *et al.*, 2005) and its capacity to reproduce in the first year of life (Rey *et al.*, 1984), makes bonito an accessible and good model species to improve culture techniques.

The complexity in scombrid larval rearing makes it difficult to mass produce both species (Masuma *et al.*, 2011; Sawada *et al.*, 2005). Our knowledge of the processes that improve larval survival under controlled conditions is very limited and protocols have not yet been described in detail. The larvae of both species are characterized by turning piscivorous after an initial phase of planktivory. Their high growth potential generates a precocious development of the jaw and the digestive system earlier than larvae of other fish species, which allows for this early piscivory behavior (Kaji *et al.*, 1996, 2002).

The development of the digestive system, in general, occurs after the flexion phase of the larvae (Kaji *et al.*, 1996, 1999), and in bonito, this stage occurs earlier than in bluefin tuna (Ortega and De la Gándara, 2009). The transition from their planktivorous to piscivorous diet is critical for the growth and survival of the larvae of both species, as shown in laboratory experiments (Reglero *et al.*, 2014a). Besides, since bluefin tuna larvae inhabit oligotrophic environments, their switch to piscivory behavior may be a key step to sustain the feeding requirements in wild populations (Reglero *et al.*, 2011).

The aim of this work is to investigate the effect alternative feeding regimes have on the growth and survival of bonito and bluefin tuna larvae during the piscivorous feeding phase, one of the most critical and least studied larval stages. We propose that intermittent feeding can be manipulated by alternating the light and darkness regimes due to the visual feeding behavior of the larvae. With that aim in mind, we conducted laboratory experiments to test if the manipulation of light regimes induced intermittent feeding behavior by looking into larval stomach contents. Furthermore, we studied the effect of continuous and various alternating light/feeding regimes on larval growth and survival. The results improved our understanding of the feeding dynamics of the two scombrids with the potential of applying it to the mass production of juveniles.

5.2 Materials and methods

Fertilized eggs of two cohorts of bonito (*Sarda sarda*) and three cohorts of bluefin tuna (*Thunnus thynnus*) were collected from naturally-spawning captive adults (De la Gándara *et al.*, 2011; Ortega *et al.*, 2013). Three experiments conducted in 150 L cylindrical tanks were initiated when the larvae were in the post-flexion stage, being able to develop piscivory (7-8 mm SL and 8 days post hatch (dph) for bonito and 9-10 mm and 21 dph for bluefin tuna). Previously, bonito larvae were reared in 5000 L and bluefin tuna larva in 1500 and 5000 L tanks, both with a photoperiod set at 15L:9D, light intensity of 500 lux, and a planktivorous *ad libitum* diet of enriched rotifers (*Brachionus plicatilis*) (from 2 dph for bonito and 3 dph for bluefin tuna) and enriched *Artemia* nauplia (*Artemia franciscana)* (AF, INVE AQUACULTURE, Belgium) (from 6 dph for bonito and 14 dph for bluefin tuna) (see Reglero *et al.* (2014a) for details). Twice a day the number of prey in the tanks was counted taking three water subsamples to ensure the prey remained in the tank at any time. New

prey was added at 11:00 and 17:00 h to maintain concentrations of 10 rotifers mL^{-1} and 0.5 *Artemia* mL^{-1} constant throughout rearing and the experiments.

Bonito and bluefin tuna larvae were also fed *ad libitum* with sea bream (*Sparus aurata*) yolk-sac larvae of 0-2 dph (3.4 ± 0.04 mm), providing up to 300 preys per individual twice daily from 1-2 days prior to the onset and throughout the experiments. All the larvae were moved to the 150 L tanks one day prior to the onset of the experiments for acclimatization. The light regime was manipulated by covering the 150 L tanks with opaque lids that were periodically slowly removed to match light regimes. The larval behavior was visually checked for several minutes after every light regime change to ensure no mortality due to collisions of the larvae with the walls while removing the lids. Periodical observations of the larvae in the tanks during the course of the day, ensured the lack of cannibalistic behavior during the experiment. Larvae from different cohorts were used in each experiment in order to work with similarly-sized and aged larvae (Fig. 5.1).

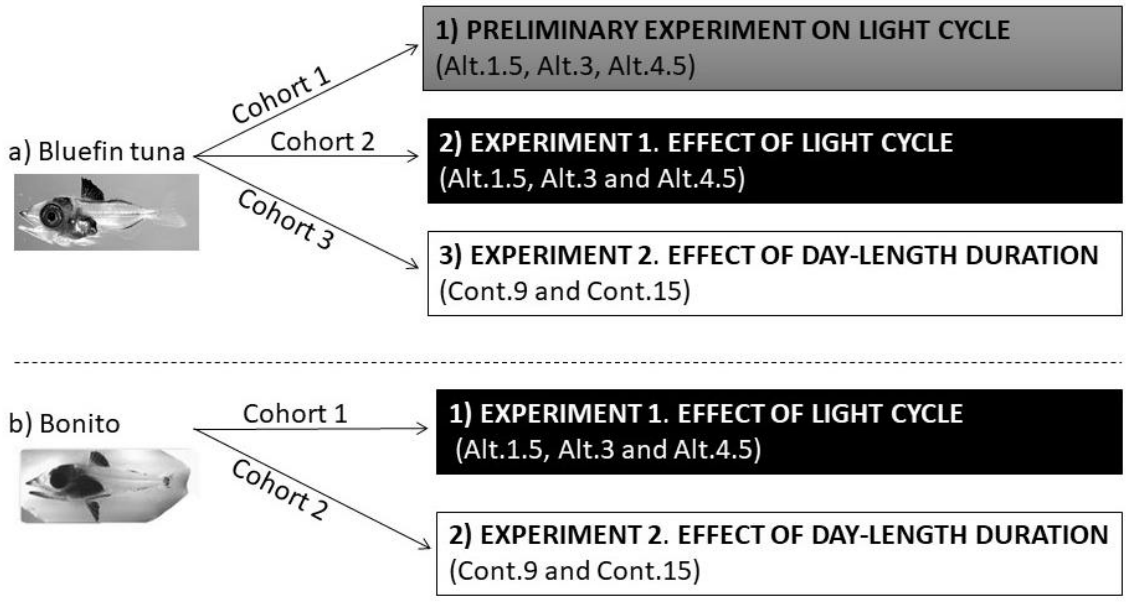


Figure 5.1. Conceptual "time-line" of performed experiments for each species. Three cohorts of bluefin tuna and two cohorts of bonito were used. Cohort 1 of bluefin tuna was used for the preliminary experiment in light cycle. Exp.1 and Exp.2 were conducted using the second and third cohort of bluefin tuna larvae. In bonito, Cohort 1 was first used for Exp.1 and later, Cohort 2 was used for Exp.2. The larval size range used in each experiment was similar.

5.2.1 Preliminary experiment on light cycle

We conducted three experiments, each of one day duration, to test if the manipulation of the photoperiod resulted in pulse feeding. Each consecutive day (from 23-25 dph) around 30 bluefin tuna larvae were transferred from a 5000 L tank to one experimental 150 L tank at 25 ºC (no replication). One day after the first transfer, the photoperiod was manipulated every three hours (alternating 3 hours of light and 3 hours of darkness to complete a total of 9 hours of light and 15 hours of darkness (Alt.3) (Fig. 5.2a)). The day after the second transfer, the photoperiod was manipulated every 4 and a half hours (alternating 4.5 hours of light and 4.5 hours of darkness to complete a total of 9 hours of light and 15 hours of darkness (Alt.4.5) (Fig. 5.2b)). The day after the third transfer, the photoperiod was manipulated every hour and a half, (alternating 1.5 hours of light and 1.5 hours of darkness to complete a total of 9 hours of light and 15 hours of darkness (Alt.1.5) (Fig. 5.2c)). Prior to the onset of the experiment, all test groups were maintained in darkness from around 24:00 until 7:30 when light was introduced. In the three alternating experiments, photoperiod manipulation started at 7:30 in the morning when 3 larvae were sampled, photographed and stored in formaldehyde (3%) every 1.5 hours until 21:00 (Fig. 5.2).

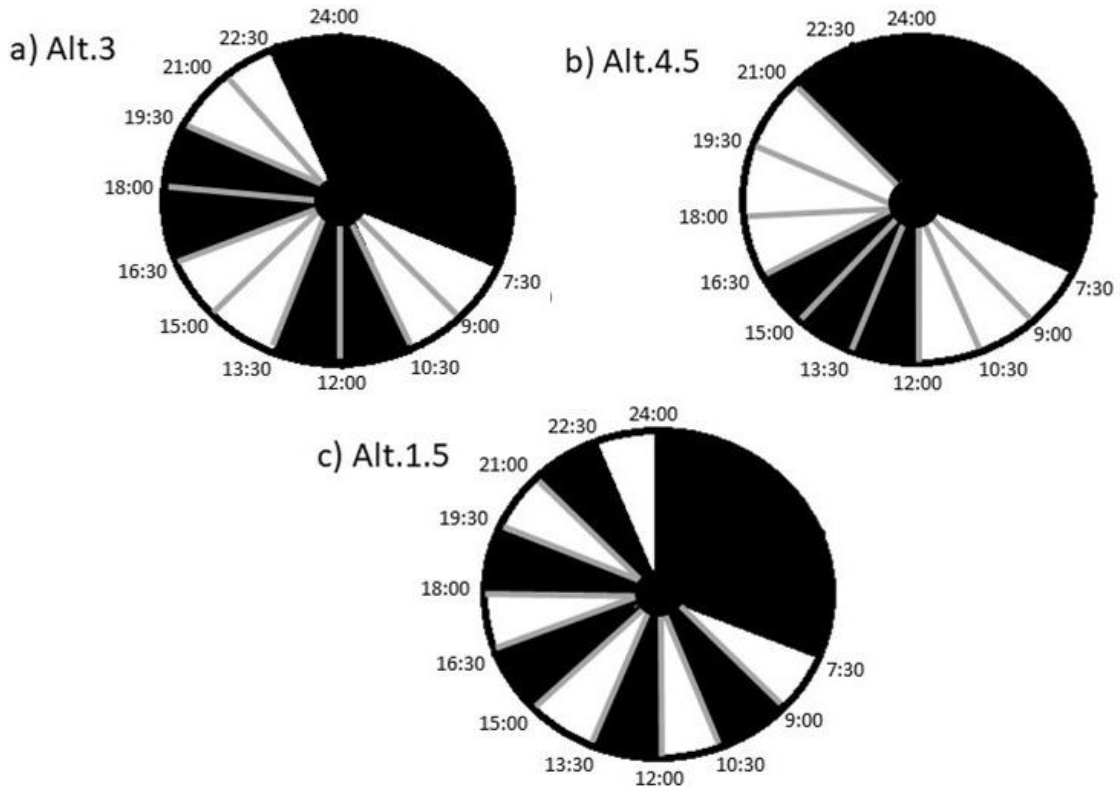


Figure 5.2. Bluefin tuna samplings times (grey lines) for the stomach content analyses are shown over the 24 h cycle for the three different alternating light treatment, a) Alt.3 b) Alt.4.5 and c) Alt.1.5. White color indicates the light period and black color the darkness period. Every 1.5 h, 3 larvae were sampled for stomach content analysis.

Afterwards, each larva was wet weighed, the stomach excised and the content counted and weighed for further stomach content ratio analysis. The initial average size of the larvae at the beginning of the experiments was statistically similar among tanks. We used larvae sizes between 8 and 15 mm lengths, representative of the larvae used in the next experiments.

5.2.2 The effect of light cycle (Exp.1)

In Exp. 1, the effect of three alternating light treatments on the growth and survival of bonito and bluefin tuna larvae was tested. These alternating light treatments were the same as those explained in section 5.2.1: Alt.1.5, Alt.3 and Alt.4.5. Each treatment had three tank replicates. In both species, 63 larvae were added into each of the nine 150 L tanks and the experiment terminated 6 days after. The experiment was conducted at an average temperature of 24.7 ± 0.4 °C for bonito and 25.3 ± 0.7 °C for bluefin tuna (see Table 5.1 for further details).

Table 5.1. Bonito and bluefin tuna average survival in % (± SD) for different treatments (Alt.1.5, Alt.3, Alt.4.5, Cont.9 and Cont.15) estimated at the end of Exp.1 and Exp.2. Number of replicates per treatment is three. All treatments had 9 h of daylight per 24 h except Cont.15 which had 15 light hours. Temperature, initial number of larvae and larval age at the end of the experiments, is indicated for each species.

Final survival (%) in experimental groups								
Species	Exp. 1			Exp. 2		Temp	Larvae	Age
	Alt. 1.5	Alt. 3	Alt. 4.5	Cont. 9	Cont. 15	(°C)	(n)	(DPH)
Bonito	65.6 (± 0.7)	57.4 (± 3.2)	63.9 (± 8.2)	60.4 (± 12.4)	47.5 (± 3.2)	25	63	15
Bluefin tuna	25.0 (± 7.7)	32.8 (± 0.7)	32.9 (± 6.9)	32.8 (± 10.0)	21.7 (± 9.2)	25	63	28

5.2.3 The effect of the day-length duration (Exp.2)

The aim in Exp.2 was to test the effect of day light duration on the growth and survival of piscivorous bonito and bluefin tuna larvae. We considered two light treatments: i) continuous 9 hours of light followed by continuous 15 hours of darkness (Cont.9), and ii) a continuous 15 hours of light followed by a continuous 9 hours of darkness (Cont.15) (see Table 5.1 for details). Each treatment was conducted in three tank replicates. 63 larvae of bonito were added into each six 150 L tanks and kept at an average temperature of 24.7 ± 0.4 °C. The bluefin tuna experiment was conducted at one temperature: 25.3 ± 0.7 °C when 63 larvae were added into each of the 6 tanks (see Table 5.1 for further details). Both experiments in bluefin tuna and bonito ended 6 days later.

5.2.4 Larval sampling in Exp.1 and Exp.2

Early in the morning, in darkness, 3-4 larvae per tank were sampled as the starting point for the first day of experimentation. All survivors were sampled the last day. Immediately following sampling, the larvae were anesthetized using clove oil (Guinama© Spain), individually photographed using an image analysis system connected to a microscope (Leica Microsystems, Inc., Bannockburn, IL), and individually frozen in vials at -20 °C for later dry weight estimations. Once in the laboratory, the larval pictures were measured in standard length (SL) using Image-Pro Plus 6.2 software (Media Cybernetics, Bethesda, MD). The frozen larvae were rinsed with distilled water and dried in a 60 °C oven temperature for 48 hours to later weigh to the nearest 0.01 mg (Seljeset *et al.*, 2010).

3.2.5 Statistics

All the statistical analysis was carried out using R statistical software (www.r-project.org). Dry weight data was transformed (natural log) and survival percentage data was root-squared and arcsin transformed before the statistical analysis to normalize the distributions. Differences among replicates within each treatment were tested using one-way ANOVA, and Bonferroni correction was applied to avoid type I error. Statistical differences among the various treatments for each species were first analyzed using one-way ANOVA following which the means were compared using the Tukey HSD post-hoc test. A significance level of α=0.01 was considered in all test. Stomach content ratio (SCR) was only calculated in the preliminary experiment for all the larvae, individually, every 1.5 hours, using the following formula:

(1) SCR= Stomach content wet weight / (full larval wet weight – stomach content wet weight) (µg µg^{-1})

Daily length increment data was obtained for Exp.1 and Exp.2 using the larvae sampled at the beginning and end of the experiment from different percentiles, obtained from the cumulative size distribution CSD, in which the sizes-at-age between repeated samplings of the same cohort can be compared in a single graph with minimal overlap and crossing of lines (Folkvord *et al.*, 2009). We assume a static ranking of fish sizes within a cohort:

$$(2)\ DLI = (SL_2 - SL_1) / (t_2 - t_1)\ (\text{mm day}^{-1})$$

Where, *DLI* is the daily length increment of a given percentile of the population (5%, 50% and 95%) on day t_1 and t_2. Specific growth rates (SGR) were obtained in a similar manner for dry weight data in the Exp.1 and Exp.2 using the following formula:

$$(3)\ SGR = 100 \cdot [\text{Ln}(DW_2) - \text{Ln}(DW_1) / (t_2 - t_1)]\ (\%\ \text{day}^{-1})$$

Where, Ln *DW* is the natural logarithm of the dry weight increment of a given percentile of the population (5%, 50% and 95%) on day t_1 and t_2 respectively.

5.3 Results

In general, there were no significant differences in the length and weight at the end of the experiments among replicates for each treatment (ANOVA, *p-adj.*>0.01). Therefore, replicates were combined by treatments.

5.3.1 Preliminary experiment on effect of light-cycle on stomach content

There was a clear interruption of feeding in darkness as observed in the experiments of one-day duration under the three alternating light regimes (Fig. 5.3). A clear decrease in the stomach contents was observed 1.5 hours after the start of the darkness period when most larvae had completely or almost completely emptied their stomachs (Fig. 5.3c). Therefore, the manipulation of the photoperiod resulted in pulse feeding. Stomach contents varied from below 2% of the total body weight in the Alt.3 to around 2-6% in the Alt.4.5 and 2-10% in the Alt.1.5 treatment. In all three experiments an important increase in food consumption throughout the day was observed. Stomach content ratios were maintained below 0.05 before 16:30, and later increased to 0.10 at 21:00 (Fig. 5.3).

Once the effect of light manipulation on feeding was studied we conducted two laboratory experiments to identify 1) the alternating light regime (Exp.1) and 2) the continuous light regime that resulted in the best larval growth and survival (Exp.2).

5.3.2 Effect of different light-cycles on growth and survival (Exp.1)

The average survival rate at the end of the experiments were not significantly different for the three alternating light treatments in bonito and bluefin tuna (Table 5.1, ANOVA, p>0.01). Bonito attained the largest body sizes under the Alt.3 treatment (average DW 21.23 mg), followed by Alt.1.5 (average DW 16.75 mg) and Alt.4.5 (average DW 15.91 mg) respectively. In bluefin tuna, similar final weights were obtained across alternating treatments (average DW 12.72 mg). Daily length increments (in mm day^{-1}) in bluefin tuna were almost half those observed in bonito (Table 5.2a). Similar results were obtained in specific growth rates (% day^{-1}) where bonito larva grew around 50-60% day^{-1}, and bluefin tuna larvae 25-35% day^{-1}, half than those of bonito (Table 5.2a). In all cases, DLI and SGR increase with size-at-age (Table 5.2a).

Figure 5.3. Stomach content ratio (white dots) per larva at each sampling time (day-time hour of sampling in the x-axis) for the three bluefin tuna larvae sampled in the a) Alt.3, b) Alt.4.5 and c) Alt.1.5 treatments. On the top of the graphs, white colour rectangles represent the light period, and black colour represent the darkness period.

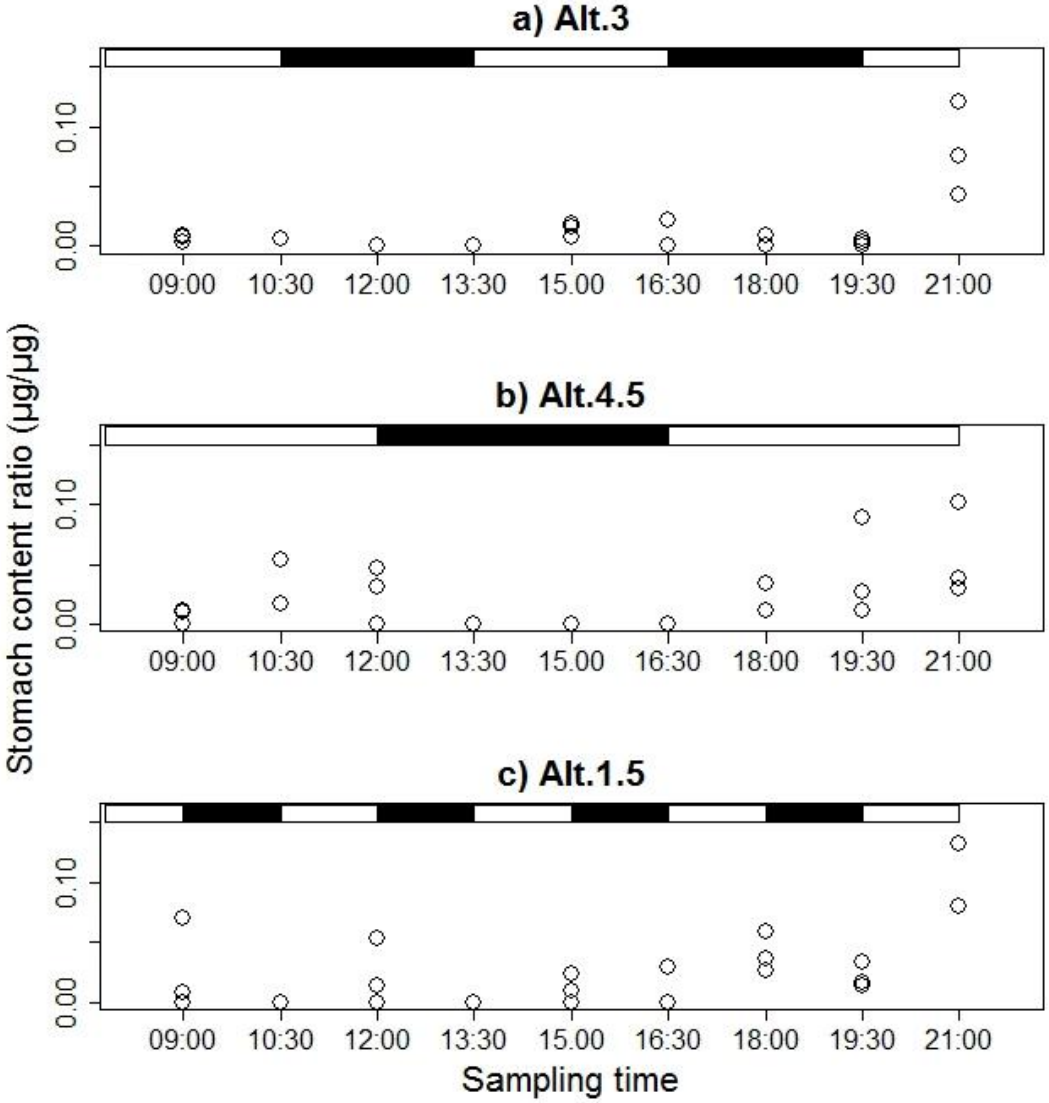


Table 5.2. Daily length increments in standard length (SL, mm day^{-1}) and specific growth rates in dry weight (DW, % day^{-1}) estimated after 6 experimental days for small (5 percentile), medium (50 percentile) and large (95 percentile) bonito and bluefin tuna larvae. Table a) includes growth rates for the alternating treatments in Exp.1 and Table b) results of the continuous treatments in Exp.2.

a) Exp.1

		Alt. 1.5			Alt. 3			Alt. 4.5		
Growth	Species	Small	Medium	Large	Small	Medium	Large	Small	Medium	Large
SL	Bonito	1.41	1.97	2.50	1.64	2.28	2.84	1.23	1.83	2.79
SL	Bluefin tuna	0.68	0.96	1.98	0.70	1.06	1.69	0.46	1.10	1.55
DW	Bonito	51.93	55.03	57.33	54.46	59.06	62.08	48.10	53.25	60.82
DW	Bluefin tuna	31.27	32.74	40.91	29.94	34.67	36.69	22.71	33.67	35.00

b) Exp.2

		Cont.9			Cont.15		
Growth	Species	Small	Medium	Large	Small	Medium	Large
SL	Bonito	0.75	1.34	2.03	2.11	2.52	3.12
SL	Bluefin tuna	0.34	0.62	1.41	0.93	1.31	2.01
DW	Bonito	32.67	43.58	51.68	62.67	62.69	65.32
DW	Bluefin tuna	17.22	25.01	32.94	36.27	39.04	40.64

5.3.3 Effect of different day-length duration on growth and survival (Exp.2)

No survival differences were found between the Cont.9 and Cont.15 treatments for the two species (Table 5.1, ANOVA, p>0.01). Bonito and bluefin tuna larvae attained the largest weight at the end of the experiment in the Cont. 15 treatment (average DW 26.25 mg for bonito and 16.16 mg for bluefin tuna) compared to the Cont. 9 treatment (average DW 8.80 mg and average DW 7.51 mg respectively).

The specific growth rate (% day^{-1}) and daily length increment (mm day^{-1}) were twice as high in bonito compared to bluefin tuna. Also, between Cont. 15 and Cont.9 treatments, growth rates were twice as high in Cont.15 compared to Cont.9 (Table 5.2b). The largest larvae grew the most in length and weight followed by the medium and small-sized larvae, independent of the light treatment in both species (Table 5.2b)

5.4 Comparisons Exp.1 *vs.* Exp.2

In bluefin tuna, the body weight and length in the Cont.15 treatment was similar to any of the other alternating treatments (Fig. 5.4c and d, Table 5.3, ANOVA, p>0.01). In bonito, only the Alt.3 treatment had similar growth to the Cont.15 treatment (ANOVA, p>0.01). Somatic growth rates under the Cont.9 treatment were always the lowest, regardless of the group size, as observed in the cumulative-size distribution graphs (Fig. 5.4) of length (mm) and weight (mg) (Table 5.3). In both species, larvae under any alternating regime grew faster than those under the Cont.9 treatment despite having the same total number of light hours (Fig. 5.4).

Table 5.3. Tukey HSD post-hoc results comparing larval weight and length (DW, SL) at the end of the experiments among all treatments for bonito (top) and bluefin tuna (bottom). Treatments within each species and size measure with non-overlapping letters are significantly different (Tukey HSD, $p<0.01$), with "a" assigned to the treatment with largest mean.

		DW (mg) Tukey HSD	SL (mm) Tukey HSD
Bonito	Alt. 1.5	bc	b
	Alt. 3	ab	a
	Alt. 4.5	c	b
	Cont. 9	d	c
	Cont. 15	a	a
Bluefin tuna	Alt. 1.5	a	a
	Alt. 3	a	a
	Alt. 4.5	a	a
	Cont. 9	b	b
	Cont. 15	a	a

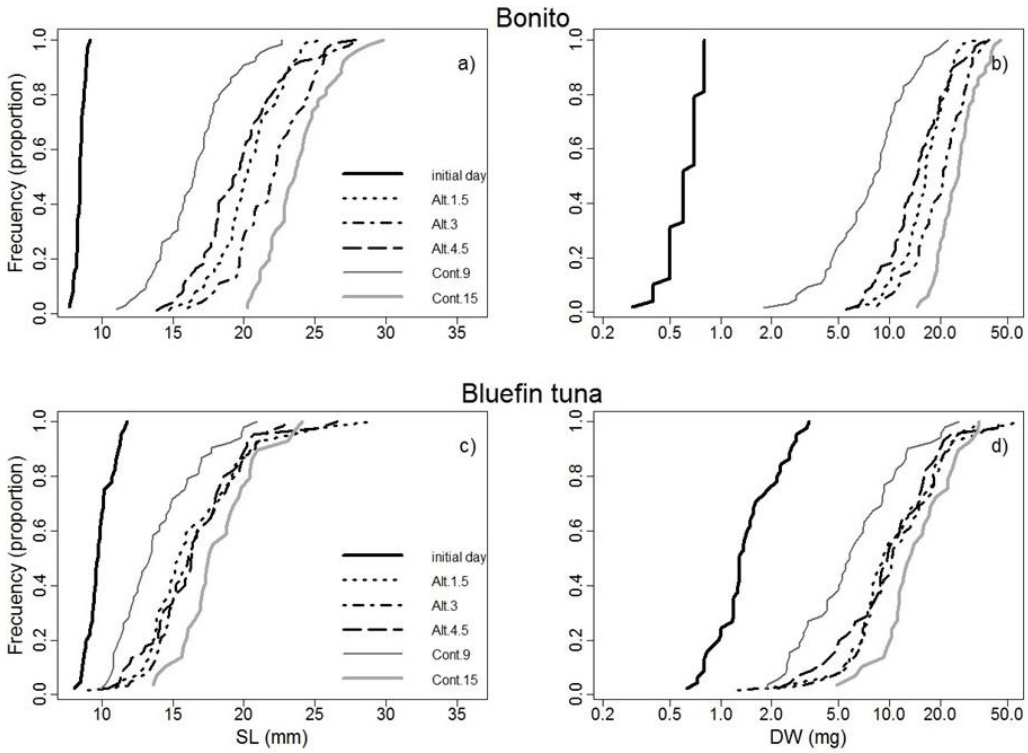


Figure 5.4. Cumulative size distributions on standard length (SL, mm) and dry weight (DW, mg) from Exp.1. and Exp.2 for a) bonito SL, b) bonito DW, c) bluefin tuna SL and d) bluefin tuna DW. Cumulative size distributions at the end of the experiment are shown for the different treatments: Alt.1.5 (⋯), Alt.3 (- - -), Alt.4.5 (- - -) Cont.9 (grey –) and Cont.15 (grey -). Note: The x-axis is natural-log transformed for DW in panels b) and d).

5.4 Discussion

Our laboratory experiments demonstrate that in piscivorous larva, alternating feeding regimes can enhance larval growth and maintain or improve survival rates, compared to continuous feeding. The analyses of stomach content revealed that the larvae only fed during the light hours and stopped feeding during darkness. Therefore, it was appropriate to use alternating light regimes to provide intermittent feeding for the larvae.

5.4.1 Feeding-ingestion

Piscivorous bluefin tuna larvae feed continuously in light, as observed from the cooccurrence of newly-ingested and digested larval prey in their stomachs. Stomach analyses from field-captured planktivorous tuna larvae show active feeding during day light hours in most specimens (Catalán *et al.*, 2011; Morote *et al.*, 2008; Uotani *et al.*, 1990; Young and Davis, 1990). However, apart from the Scomberomorus species, for which piscivory is observed already at first-feeding (Shoji *et al.*, 2001), continuous piscivory had never previously been tested.

During the dark phase on the one-day duration experiments, bluefin tuna larvae had completely or almost completely emptied their stomachs after 1.5 hours of darkness, independent of the light regime. Such evacuation time (~1.5 - 2 hours) is shorter than the 3-4 hours reported from field observations on similar planktivores (Llopiz *et al.*, 2010; Young and Davis, 1990). Our results may be caused by the high digestion capacity, when the stomach is completely formed (Rønnestad *et al.*, 2013), an event that in tuna matches the start of piscivory (Yúfera *et al.*, 2014). The larvae may also show more rapid energy absorption in the stomach and gastric evacuation when feeding on yolk-sac fish larvae than when feeding on other planktonic prey. More than that, the digestibility of sea bream yolk-sac larvae should be higher than the digestibility of *Artemia*, as it was suggested by Seoka *et al.* (2007).

The continuous feeding behavior and the increase of ingested prey throughout the day may have been caused by the lack of satiation-regulating hormones in the larval stage, and an endocrine system that is not completely functional (Rønnestad *et al.*, 2013). In scombrid larvae fed *ad libitum*, the limit for maximum food ingestion may be determined by the maximum expansion of the stomach cavity and the sum of the cost of prey capture, ingestion, digestion and assimilation, rather than satiation.

The increase of prey in the stomach cavity during light time, from the morning to late in the day, may be explained by an improvement in the skills of this species in capturing prey within these hours, regardless of the photoperiod, or may be caused by a circadian rhythm that prevails from the previous rearing period, signalling a starvation period at night. The same trend has been reported in the stomach content of laboratory reared Japanese Spanish mackerel (*Scomberomorus niphonius*) (Shoji *et al.*, 2001). Stomach content ratios were lower in the Alt.3 experiment where the larvae were the youngest, 23 dph, compared to larvae in the Alt.4.5 and the Alt.1.5 experiments that were 24 and 25 dph respectively. Increasing feeding rates with age

was also evident in the amount of sea bream yolk-sac prey that was necessary to add daily to the tanks in order to ensure food *ad libitum*.

5.4.2 Growth-survival

The positive effect of increasing the number of continuous light hours on growth has been extensively recorded in numerous fish larval species and stages (*e.g.* Fielder *et al.*, 2002; Hart *et al.*, 1996; Puvanendran and Brown, 2002) including two scombrids, yellowfin tuna (*Thunnus albacares*) (Papandroulakis *et al.*, 2010; Partridge *et al.*, 2011) and bluefin tuna (*Thunnus thynnus*) (Ortega *et al.*, 2014). Growth enhancement has been related to longer foraging times and subsequently greater food intake (Ortega *et al.*, 2014; Partridge *et al.*, 2011). In this study, best growths rates have been obtained in bonito and bluefin tuna larvae under the Cont.15 treatment closely followed by larvae in the alternating treatments.

Resting periods during darkness may enhance growth in the alternating light regimes. In darkness, fish larvae mostly use their energy source to grow, digest and absorb food, instead of swimming, as when they are in a light environment. Bonito and bluefin tuna larvae grew larger in size, from the alternating light treatments (Alt.1.5, Alt.3 and Alt.4.5) than those from the Cont.9 treatment despite the fact that all were exposed to 9 hours of daily light feeding hours. In both species, growth under alternating treatments was always similar to the Cont.15. However, larval behavior in darkness needs to be recorded to document resting behavior. The increase of the expression of the growth hormone during darkness (Adachi *et al.*, 2008), may stimulate the appetite (Johnsson and Björnsson, 1994) and promote lipid mobilization and protein deposition that might be reflected in an increase in somatic growth (Björnsson *et al.*, 2002). Further biochemical analyses are needed to verify if digestion efficiencies and energy utilization are the main reasons for these differences.

Our survival data show no significant differences across the five different alternating/continuous regimes for each species. This result may be caused by the high fluctuations in survival rates between tanks. Therefore, even though they are not significant (ANOVA, $p\text{-}adj.>0.01$), survival seems to be affected by the light regimes, as it was lower under Cont.15 than under Cont.9 treatment, both for bonito and bluefin tuna. These differences might decrease if looking into survival results at a specific size instead of specific age. The high survival results obtained for bonito are in accordance with previous results (Reglero *et al.*, 2014a) but in bluefin tuna, we obtained slightly lower survival rates than in previous piscivorous studies (Reglero *et al.*, 2014a; Seoka *et al.*, 2007). Larval stress may be diminished by slowly removing/covering the lids of the tanks to generate gradual light attenuation. The sudden on/off of the lights produce a short excitement in the larvae which could increase collision to the tank walls, though this was not considered the case in our experiments. Our survival data have not been affected by cannibalism. We were able to overcome cannibalism by using homogenous size batches (or by diminishing variability in size), planning experiments of short duration (1 and 6 days) and by

feeding with enough amount of sea bream yolk-sac larvae (Masuma *et al.*, 2011; Sawada *et al.*, 2005).

Alternating light regimes may be a good alternative in intensive cultures; reducing rearing cost due to the reduction of the timing of feeding and lighting, especially when rearing piscivorous species whose diet in that critical developmental stage is not easy to obtain due a dependency on available fish larvae (Sawada *et al.*, 2005). However, specific optimum light regimes may be species-stage dependent. Manipulation of the photoperiod can affect feed-conversion ratios or efficiencies, demonstrating the importance of investigating the effect of photoperiod on feeding efficiency (Rabe and Brown, 2000). The effect of endocrine factors on the appetite and growth of fish larvae should also be studied. Our optimal growth results support the idea that larvae do not need to feed constantly to maximize growth rates. The option to feed at certain time intervals may prevent them from becoming satiated and then increase their total ingestion, per unit time of active foraging. This leads to more efficient use of the available food. In addition, our results suggest that larvae may survive short periods of starvation in the field if food is distributed in patches. Our experimental food-deprived larvae could reduce their swimming in darkness, which also could improve the growth rate. However, in the field they may continue swimming due to the daylight present. These differences in energy expenditure should be considered in further research before applying the results to the field.

CHAPTER 6

- DIFFERENCES IN THE METABOLIC RATES IN LIGHT AND DARKNESS CONDITIONS DURING THE PISCIVOROUS STAGE OF ATLANTIC BLUEFIN TUNA LARVAE -

Differences in the metabolic rates in light and darkness conditions during the piscivorous stage of Atlantic bluefin tuna larvae

ABSTRACT

Measuring metabolism can help us to understand how individuals adapt their physiology in terms of energy to face variability in intrinsic and extrinsic factors. The mechanisms of how fish larvae balance growth and activity are of great interest since metabolic costs are very high compared to juveniles and adults. Fish larvae acquire most morphological and physiological characteristics of the adult stage in a short time during the first weeks of life and oxygen is primordial to support these changes and their growth rates. In darkness, fish larvae may reduce their swimming and feeding activity and as a consequence metabolic rates decrease. Atlantic bluefin tuna, as other Scombridae species, is characterized by an early switch from planktivory to piscivory in the larval stage that yields growth and survival advantages. However, there is a lack of information about the metabolic cost in scombrid fish larvae. In this study, we: i) measure metabolism during light and darkness situations, ii) estimate the relationship between the metabolic rate and the larval dry weight (mass scaling exponent), iii) estimate the relationship between the nutritional status and the larval dry weight and iv) investigate the correlation between the nutritional status of the larvae based on the RNA:DNA, DNA:dry weight and the metabolic expenses. Our results show the same oxygen consumption for Atlantic bluefin tuna larvae of different sizes, ranging from 0.6 to 23 mg, during closed respirometry in light and darkness. The relationship between metabolism and size showed an isometric relationship (slope, b=1) higher than the average relationship in other species. A possible regulation (decrease) of their swimming activity with the consequent decrease in the oxygen consumption in light situations is discussed. The nutritional condition, measured as the RNA:DNA ratio, was proportional to the larval size in dry weight but did not explain differences between light and darkness measurements. The more sensitive ratio DNA:dry weight showed a decrease in the condition of the larvae in darkness. No relationship was found between the oxygen consumption and fish larval nutritional condition after accounting for the size effect. This study confirms the importance of body size in the metabolism of this species.

6.1 Introduction

Metabolism is the sum of the processes by which energy and materials are transformed into various living structures and activities (Gillooly *et al.*, 2001). Metabolism supports not only cellular and tissue maintenance, but also additional vital activities, including growth, reproduction, locomotion and thermo-regulation and indicates how organisms partition energy resources to activities that allow them survive, grow and reproduces (Post and Lee, 1996). The energy in an organism can be obtained by aerobic or anaerobic pathways, depending if the oxygen is used or not in the metabolic reactions. The anaerobic pathway is used in high-energy demands situations, such as the burst swimming in hunting or to escape from predators. The aerobic pathway is used to fuel the development and the rest of activities. The metabolic rate is the rate at which organism transform energy and materials (Gillooly *et al.*, 2001), and is proportional to the oxygen consumption. Many factors can influence energetics in fish individuals, such as body size, availability of prey and food assimilation efficiency, amount of light, and temperature, with increasing the oxygen consumption as the temperature increase (Auer *et al.*, 2015; Glazier, 2015; Killen, 2014). Metabolic studies can help us to understand physiological adaptations in terms of energy.

Estimates of metabolic rates (MO_2) in fish larvae are commonly determined in closed respirometry chambers measuring the oxygen uptake of the organism before and after a time interval and by using the differential as the measure of metabolic energy expenditure during that time (Nelson, 2016). There is different terminology in the literature to define oxygen consumption rates that can be measured in fish. The most common oxygen measurement is the routine metabolic rate (RMR) which is the sum of the minimum maintenance metabolic rate and the metabolic cost of swimming activity in an individual (Brett and Groves, 1979; Houde and Schekter, 1983; Peck and Buckley, 2008). The energy used for the ingestion, digestion, absorption, and assimilation of a meal is known as the specific dynamic action (SDA) (Secor, 2009). The standard metabolic rate (SMR), is defined as the minimum maintenance metabolic rate of a non-feeding and inactive fish with no anaerobic activity debt (Chabot *et al.*, 2016). At the other end of the metabolic scale, maximum metabolic rate (MMR) provides the upper boundary for aerobic energy metabolism and is conventionally measured at the point of exhaustion (Nilsson *et al.*, 2007; Norin and Clark, 2016).

The difference between the standard and the maximum metabolism is the aerobic scope, being a useful framework to define metabolic limits. In fish larvae, the aerobic scope is known to be very narrow, and therefore, the energy demanding processes of growth and activity may be in conflict (Killen *et al.*, 2007; Wieser *et al.*, 1988). When energy expenses are low compared to the aerobic scope then the surplus of energy is allocated to growth (Priede, 1985). Larval fish may have high metabolic requirements being vulnerable to starvation due to their small size and rapid growth to enhance survival (Nilsson *et al.*, 2007). Understanding how fish larvae balance the processes of growth and activity (*e.g.* foraging effort required to support rapid

growth against the high cost of swimming) is of great interest since the metabolic costs (mass-specific O_2 consumption) are very high compared to larger juveniles and adults. There is a lack of information about fish larval aerobic scope due to the difficulty of measuring metabolism mainly due to their small size and handling difficulty (Peck and Moyano, 2016).

Metabolic rates are known to vary with the body size and the rate of oxygen consumption per body mass is reported as the mass-specific metabolic rates (VO_2) (Post and Lee, 1996). The value for the scaling component (slope, b) varies among species in response different lifestyle and habitat (Killen *et al.*, 2010). It is also being influenced by the techniques and protocols used to perform the measurements (Peck and Moyano, 2016). Several estimates of the metabolic exponent suggest isometric relationships (b=1) where oxygen uptake increases in direct proportion to body weight although allometric relationships (b≠1) have also been reported when metabolic rates also increases with the body weight but not in direct proportion (*e.g.* allometry, Finn *et al.*, 2002; isometry, Giguère *et al.*, 1988). There is still a lack of information on how different metabolic levels (*e.g.* SMR, RMR, MMR) change with larval size (Peck and Moyano, 2016). Among other factors, their small size, high sensitivity to stress from handling and confinement, and the different methodologies used, have likely contributed to the relatively low number of metabolic studies on marine fish larvae and the high variability in the results obtained to date (Peck and Moyano, 2016). Estimates of how metabolic rates vary with body size are particularly important in tropical and warm water species larvae (such as scombrids) where growth rates are normally above 30% day^{-1} of their body size (Houde, 1989). This is the case of Atlantic bluefin tuna larvae (*Thunnus thynnus*) that before the third week of life have already developed an adult type digestive system and most juvenile morphological characteristics (Reglero *et al.*, 2014a; Yúfera *et al.*, 2014). Tuna adult individuals (as the rest of scombrids), are ram-ventilators and they must swim continuously to maintain water flow across the gills and prevent sinking, which consequently prevents them from ever achieving the necessary state of complete rest that is required to fulfill the definition of SMR (Graham and Laurs, 1982; Wegner *et al.*, 2013). However, as larvae, they are able to actively obtain oxygen, a capacity that seem to be lost from 2 cm (Wegner *et al.*, 2013, personal observation). They are impressive athletes that can undertake extensive vertical and horizontal migrations throughout the oceans of the world (Block *et al.*, 2001, 2005). Such behavior along with the rapid growth demands high rates of oxygen consumption to fuel metabolic reactions.

This study is the first respiration study in Atlantic bluefin tuna larvae. To date, a metabolic rate in this species has never been measured in adults, juveniles and larvae. In other bluefin tuna species only one study has examined ontogenetic changes in oxygen consumption by respirometry. Miyashita *et al.* (1999) found that oxygen consumption and metabolic activity rapidly increased in the period between hatching and juvenile metamorphosis in Pacific bluefin tuna larvae.

Tuna larvae, as most marine fish larvae are visual feeders (Margulies, 1997). Although light is known to influence activity, the effect of light on larval fish respiration is not well known, and its effect on Atlantic bluefin tuna larvae has not been studied previously. Studies using other species of fish larvae have found that light increases the oxygen consumption (Porter, 2001; Finn *et al.*, 1995, 2002). With light fish larvae become more active in feeding and searching, and swimming is energetically costly for fish and in the dark, the activity of fish larvae is assumed to be reduced.

The four main objectives of our study are to i) measure metabolic rates by oxygen consumption comparing light and darkness situations, ii) estimate the relationship between the metabolic rate and the larval dry weight (mass scaling exponent), iii) estimate the relationship between the nutritional status and the larval dry weight and iv) investigate the correlation between the nutritional status of the larvae based on the RNA:DNA, DNA:dry weight and the metabolic expenses. We used two different indices to determine fish larval nutritional condition: the RNA:DNA and the DNA:dry weight ratios. The RNA:DNA ratio is an index of cell metabolic intensity and it is used as an approach for recent growth and recent nutritional condition of fish larvae (Clemmesen, 1994; Folkvord *et al.*, 1996). The amount of DNA is stable under changing environmental situations reflecting the number of cells of an individual, whereas the amount RNA is directly proportional to the protein synthesized in the cell which is highly dependent to food quantity/quality (Buckley *et al.*, 1999). Therefore, well-fed larvae, metabolically more active, grow better and have relatively higher RNA:DNA ratio compared to poor-fed larvae with less active metabolism (Clemmesen, 1987, 1994). On the other hand, the DNA:dry weight ratio show larval cell condition, increasing when condition decreases, because less weight is attributed per cell number.

6.2 Materials and methods

6.2.1 Larval rearing

Fertilized Atlantic bluefin tuna (ABFT) eggs were obtained from naturally spawning captive adults in the farming facilities at El Gorguel, Cartagena (SE Spain), owned by Caladeros del Mediterráneo S.L. The eggs were collected and transported to the Spanish Institute of Oceanography (IEO) facilities in Mazarrón (SE Spain) where the experiment was performed. Approximately 50 000 eggs were incubated in a 5000 L tank where the larvae hatched and remained until 26 days post hatching (dph). At 26 dph all the larvae were removed from the tank, 203 in total, and the smallest 100 larvae (10.1 ± 2.8 mg) were transferred to a new 1500 L rearing tank and the rest (22.8 ± 4.7 mg) were removed for weaning proposes and not used in the rest of the experiment. Every day, from 17 to 26 dph, groups of larvae were randomly sampled from the 5000 L tank and from 26 to 30 dph from the 1500 L tank for oxygen measurements. During the larval rearing period, the photoperiod regime was 14 hours of light and 10 hours of darkness, 14L:10D. Temperature during the rearing period was the natural from the sea ranging from 24 to 26 °C and salinity was kept

constant at 38. The feeding schedule consisted of enriched rotifers (*Brachionus plicatilis*) from 4 to 16 dph, enriched *Artemia* nauplii (*Artemia franciscana* instar II) (AF, INVE AQUACULTURE, Belgium) from 11 to 23 dph and sea bream (*Sparus aurata*) yolk-sac larvae (YSL) from 17 to 30 dph. *Artemia* and sea bream YSL were added twice per day in adequate amount to ensure availability of preys during all the rearing experiment. Rotifers were completed to 5 rotifers mL^{-1} twice per day. In addition, cultivated microalgae (*Nannochlororopsis gaditana*) were added two times per day until 16 dph and 0.8 g dry weight of paste of concentrated *Chlorella* (Super fresh Chlorella SV-12, Chlorella Industry Co., Ltd., Japan) per m^3 three times a day until the end of the experiment.

6.2.2 Oxygen consumption measurements

The larval oxygen consumption was measured by closed (static) respirometry, using the equipment and the software of PyroScience company (www.Pyroscience.com). The FireStingO_2 oxygen meter instrument uses fiber-optical contactless oxygen sensors (optodes) and is based on red light excitation and detection in the near infrared using unique luminiscence oxygen indicators caused by collisions between oxygen molecules (REDFLASH technology). The system had four analog channels and an external submersible temperature sensor in order to compensate automatically the oxygen sensor signals for temperature variations in the setup (an increase of temperature decreases the oxygen solubility). Glass chambers of 20 mL of the PyroScience company were used until the larvae reach maximum sizes of 8.8 mm and 1.5 mg (from 17 to 19 dph, n=24) and common Winkler bottles of 100 mL adapted with optodes were used for the bigger larvae with sizes up to 22.4 mm and 31.5 mg (from 17 to 30 dph, n=136). Respiration chambers were large relative to the size of the fish larvae as recommended by Peck and Moyano (2016). The water volume to larval volume ratio in the 20 mL chambers of around 10 000:1 and in the 100 mL chambers from 3000:1 to 200 000:1.

Every day, before the start of respiration measures, the oxygen sensors were calibrated at the experimental temperature both in oxygen free water (0% O_2 sat calibration) achieved by adding 30 g L^{-1} of sodium sulphite (Na_2SO_3), and fully aerated water (100% O_2 sat calibration) obtained by bubbling the water with air minutes before the calibration. Room temperature was maintained at 19-20 °C in order to avoid ambient humidity and the atmospheric pressure was assumed that of normal condition 1013 mbar. The outside noise that could affect larval behavior and sensors sensitivity was avoided by measuring in an isolated room. A water bath was used in order to maintain stable temperature at 26 ± 0.1 °C of variation and walls were wrapped in green plastic paper to simulate similar ambient conditions as in the larval rearing tanks.

Every day ABFT larvae were fasted during night time, 8-9 hours, allowing sufficient time for food to be cleared from the gut (Chapter 5; Blanco *et al.*, 2017) in order to remove cost of the specific dynamic action (SDA), in the metabolic measurement. Respirometry measurements were conducted in light and darkness. Measures in light were considered as RMR whereas in darkness considered a proxy for SMR,

assuming low larval activity during darkness. Temperature in the rearing tanks were similar to the experimental temperature therefore, the larvae did not need to be acclimated. From 7:00-9:00 in the morning, measures in darkness conditions were carried out and from 9:00-11:00 when the lights were switch on, measures were done in light conditions. During darkness measurement, the water bath was completely covered to prevent any light entering from the outside. Several runs of four simultaneous measurements were done. Chambers were filled saturated filtered seawater (10 μm) at experimental temperature and overturned and separated from each other to avoid the larvae to see each other. Daily, a control blank (no larva) in light and in darkness was measured. The blank was done to control for bacterial and microbial respiration "background respiration" (chemical and biological oxygen demand) and to correct larval oxygen values. In order to avoid possible water stratification and for a good correction for background respiration, a small piece of glass was introduced to the blank chamber, and gently manually shaken before the stop of the measure for water mixing (Rodgers *et al.*, 2016). One to two drops of water from the larval water tanks was introduced to the blank chamber in order to compensate for the possible bacterial respiration associated with the larvae. Except for the blank, one larva was loaded in each chamber.

Measures between 30 minutes and a maximum of 55 minutes of the rate of oxygen consumption (μmol O_2 L^{-1}) were made, with the first 10 minutes used as an adaptation period to avoid stress caused by handling. As Peck and Moyano (2016) suggested, larval activity was visually checked at the beginning and at the end of each measure to guarantee optimal larval swimming activity during darkness (using a red-light lantern) and light conditions. Immediately after the oxygen measurement, the larvae were euthanized using clove oil (Guinama© Spain), rinsed in distilled water, individually photographed for morphometric measures (standard length) and individually frozen in cryotubes at -80 °C for later lyophilized dry weight and nucleic acid estimations (accurate to ± 0.01 mg). Under the binocular individual larval stomach was visually inspected to ensure empty guts and if any prey were found, the data from that larvae were removed for further statistical analysis.

Larvae were excluded from further analysis (~ 25%) when died during handling, showed continuous burst activity or collisions with the chamber walls were observed. If the oxygen partial pressure decreased 80% of saturation the measurement was stopped and the larva was removed from the chamber. A total of optimal 160 larval measures were conducted and 31 blanks. 82 measures were done to the larvae in light and to 78 in darkness (Table 6.1). Of those larvae, only 13 were in flexion stage, while the rest were in post-flexion (Kendall *et al.*, 1984). Measures in the respirometer were done in μmol O_2 L^{-1}. Oxygen consumption rates in the controls were always <20% (typically 1–5%) of those in chambers with a larva.

Table 6.1. Summary of the daily data of the Atlantic bluefin tuna larvae sampled during the experimental time from 17 to 30 dph. Oxygen consumption measurements were done at 26 °C. The average standard length (SL) and dry weight (DW) with the corresponding standard deviation of the data (± SD) of all the larvae samples is shown every day. Daily total number of larvae sampled (n) in dark and light conditions is shown. Fish larvae were feeding *Artemia* at the beginning of the experiment until 23 dph and started with sea bream yolk-sac larvae feeding at 17 dph to the end of the experiment.

dph	SL (mm)	DW (mg)	Light (n)	Darkness (n)	Feeding regime	
17	7.8 ± 0.8	0.93 ± 0.31	5	6		
18	7.3 ± 0.2	0.70 ± 0.15	6	7		
19	8.2 ± 0.5	1.15 ± 0.30	6	4		
20	8.7 ± 0.5	1.44 ± 0.40	11	9	*Artemia*	
21	9.4 ± 0.8	2.14 ± 0.65	3	4		
22	8.8 ± 0.5	1.40 ± 0.30	7	7		Sea
23	8.7 ± 0.8	1.40 ± 0.54	4	6		bream
24	12.6 ± 0.9	5.61 ± 1.20	0	4		yolk-sac
25	11.9 ± 1.9	6.51 ± 3.56	7	4		larvae
26	18.1 ± 1.7	17.40 ± 5.50	9	7		
27	14.6 ± 1.8	8.60 ± 3.55	7	7		
28	15.1 ± 1.1	9.20 ± 2.40	7	3		
29	16.8 ± 1.4	13.05 ± 3.30	7	7		
30	18.7 ± 1.9	17.20 ± 5.92	3	3		
		Total	***82***	***78***		

6.2.3 Nutritional condition: nucleic acid analyses

The protocol to analyze nucleid acids using a spectrofluorometer followed the protocol by ICES (2004). Individual measures of RNA and DNA content were carried out using the whole larval body. Sodium lauryl sulfate (SDS) was used to break cell membranes (plasmatic and nuclear) and release the nucleic acids and the fluorophore Ethidium bromide (EB) to measure total nucleic acids. RNase was added to differentiate RNA from DNA. The difference between the total acid nucleic fluorescence and the DNA fluorescence (obtained by RNase) determine the RNA content, assuming insignificant residual fluorescence. RNA and DNA data were standardized as suggested by Caldarone *et al.* (2006) assuming for DNA, a ratio of 2.4 RNA concentrations. DNA standard was constructed from genomic ultrapure calf thymus (Sigma Aldrich) and RNA from baker yeast (Sigma Aldrich). The fluorescence was measured in runs of 20 samples in order to avoid the EB degradation. The analysis was completed within 4-5 months after sampling. For more details, see Chapter 1.

6.2.4 Data analysis and statistics

The exact water volume contained in each respirometer chamber was estimated gravimetrically. Firstly, the chambers, clean and dry, and the associated closures were weighted. Then, they were filled with distilled water at room temperature and weighted again. The volume of each chamber was expressed at the standard temperature of 20 °C (at normal laboratory conditions) following the $NSOP_2$

procedure of Go-Ship Hydrographic Manual and calculated to 26 °C (Hydes *et al.*, 2010). Each larval displaced volume was assumed to be negligible in the high respirometer-to-fish volume ratio.

Larval individual respiration was calculated as the decline in oxygen concentration during the measure time (slope) and normalized by the chamber volume. It was corrected for the oxygen consumption rate measured within the blank chamber at a specific time (slope) also normalized by the chamber volume (µmol O_2 individual^{-1} h^{-1}). The metabolic exponent (slope, b) is defined as the changes of respiration rate per unit body mass and it is estimated by the exponent of the equation that relates metabolic rate with size ($MO_2 = a\ W^b$) or the slope of the linear regression when the equation is (natural log) ln-transformed ($\ln MO_2 = \ln a + b \cdot \ln W$). It shows if the metabolic rate increases in proportion to size (b=1, isometric relationship), increases faster than size (b>1, allometric relationship) or increases more slowly than size (b<1, allometric relationship).

All statistical analyses were carried out using the R statistical software (www.r-project.org). Mass-specific oxygen consumption (µmol mg^{-1} h^{-1}) and condition metric ratios (RNA:DNA and DNA:dry weight) data were log-transformed prior to statistical analyses. A Kolmogorov-Smirnov test was used to assess normality and homogeneity of variances was tested using Levene-test. The effect of vial volume (20 mL and 100 mL) and larval stage (pre-flexion and in post-flexion) during 17 to 20 dph was analyzed by a three-way ANOVA with treatment (light and darkness) also as factor, in the oxygen consumption (µmol mg^{-1} h^{-1}) and condition metrics (RNA:DNA and DNA:dry weight). The significance level was accepted at $p<0.05$.

A linear regression was performed on ln-transformed oxygen consumption (µmol O_2 individual^{-1} h^{-1}) and ln-transformed dry weight data on larvae in light and in darkness, and in ln-transformed RNA:DNA and DNA:dry weight data. Analysis of covariance (ANCOVA) was run to test for differences in the variables between light and darkness treatments.

Inter-individual variations in the oxygen consumption and nutritional condition ratios were compared using residuals of predictive regressions in order to explore the relationship without size-effect. Residuals from the respiration-dry weight (respiration *versus* dry weight) were compared to the RNA-DNA, DNA-dry weight and dry weight-dph (size-at-age) residuals using linear regression. Condition metrics residuals, RNA-DNA and DNA-dry weight were also compared to the dry weight-dph (size-at-age) residuals by a linear regression.

6.3 Results

There were no significant differences in the oxygen consumption (mass-specific) between the larvae placed in the 20 mL and the 100 mL chambers and between different larval stages (pre-flexion and post-flexion) nor between light and darkness interaction (three-way ANOVA, $p>0.05$).

There were no significant differences in the oxygen consumption between larvae in light and darkness (ANCOVA, $p>0.05$). Oxygen consumption increased isometrically with dry weight, the mass scaling exponent being 1.0 (Fig. 6.1) and the larval weight range between 0.5 and 31 mg (age 17 to 30 dph). On average, from 17 to 30 dph mass-specific oxygen consumption of the larvae was 0.424 ± 0.163 µmol O2 mg^{-1} h^{-1}.

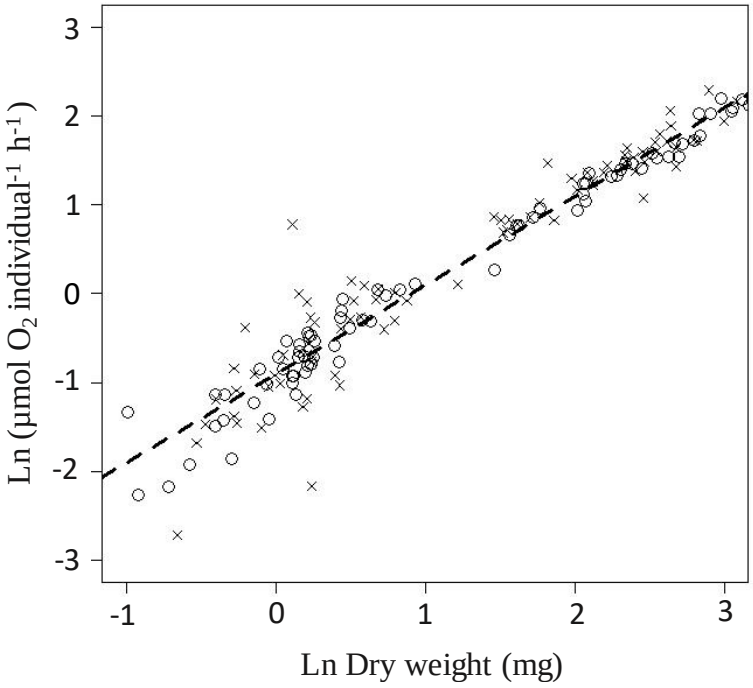


Figure 6.1. Ln-transformed metabolic rates (µmol O_2 $individual^{-1}$ h^{-1}) and larval dry weight (mg) of Atlantic bluefin tuna larva from 17 to 30 days post hatch. Oxygen consumption data measured in darkness is shown with a circle (o) and with light is shown with a (x). No differences were found between light and darkness results, thus only one linear regression is calculated. Regression equations are shown in Table 6.2.

There were no significant differences in the RNA:DNA ratio between larvae maintained in light and darkness (ANCOVA, $p>0.05$). We found a significant allometric (b=0.34) increasing trend in the RNA:DNA ratio with the larval dry weight (R^2=0.50, *Res.St.Error*=0.42, $p<0.05$, n=160) (Fig. 6.2a, Table 6.2). Significant differences were found in the DNA:dry weight ratio between light and darkness (ANCOVA, $p<0.05$). The DNA:dry weight ratio decreased with larval dry weight allometrically with a scaling component (b) of -0.28 during light significantly higher than the -0.21 obtained in darkness (Table 6.2). With light, small larvae had higher DNA:dry weight ratio, while the tendency switches with increasing larval dry weight (Fig. 6.2b).

Table 6.2. Regressions and the parameters estimated of metabolic rate (MO_2) and nucleic acids (RNA:DNA and DNA:dry weight) to dry weight (DW) for Atlantic bluefin tuna larva, during the piscivorous phase from 17 to 30 dph at 26 °C.

Measurement		Equation	b (slope)	R^2	Res.st.error.	n
O_2	Darkness+Light	Ln O_2 = -0.91 (±0.06 SE) + 1.0 (±0.04 SE)·Ln DW	1.00	0.93	0.30	160
Nucleic acids	Darkness+Light	Ln RD = 0.51 (±0.08 SE) + 0.34 (±0.04 SE)·Ln DW	0.34	0.49	0.42	160
	Darkness	Ln DNA:DW = 3.08 (±0.04 SE) - 0.21 (±0.02 SE)·Ln DW	-0.21	0.66	0.20	160
	Light	Ln DNA:DW = 3.10 (±0.06 SE) - 0.28 (±0.04 SE)·Ln DW	-0.28	0.70	0.21	160

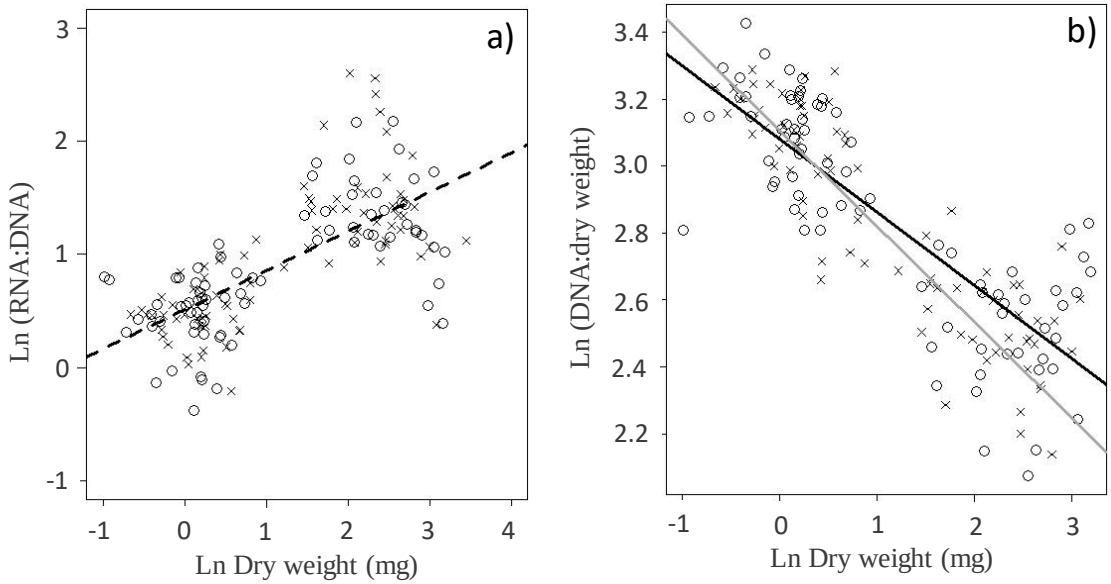


Figure 6.2. Relationship between log transformed Atlantic bluefin tuna larval nutritional condition as a) RNA:DNA ratio and b) DNA:dry weight ratio and individual dry weight (mg). Oxygen consumption in darkness is shown with a circle (o) and in light is shown with a (x). In the a) graph, no differences were found between light and darkness, thus only one linear regression is calculated. In the b) graph, differences were found between light and darkness, therefore, the regression of the light conditions is represented by the grey line and darkness with the black line. Regression equations are shown in Table 6.2.

Inter-individual differences per any given size of larvae in the oxygen consumption and condition metrics (RNA:DNA and DNA:dry weight) were not correlated (Fig. 6.3a and b, p>0.05). Oxygen consumption was not correlated with larval size-at-age (Fig. 6.3c, p>0.05). We found no significant correlations between the condition metrics and the size-at-age data (Fig. 6.3d and e, p>0.05).

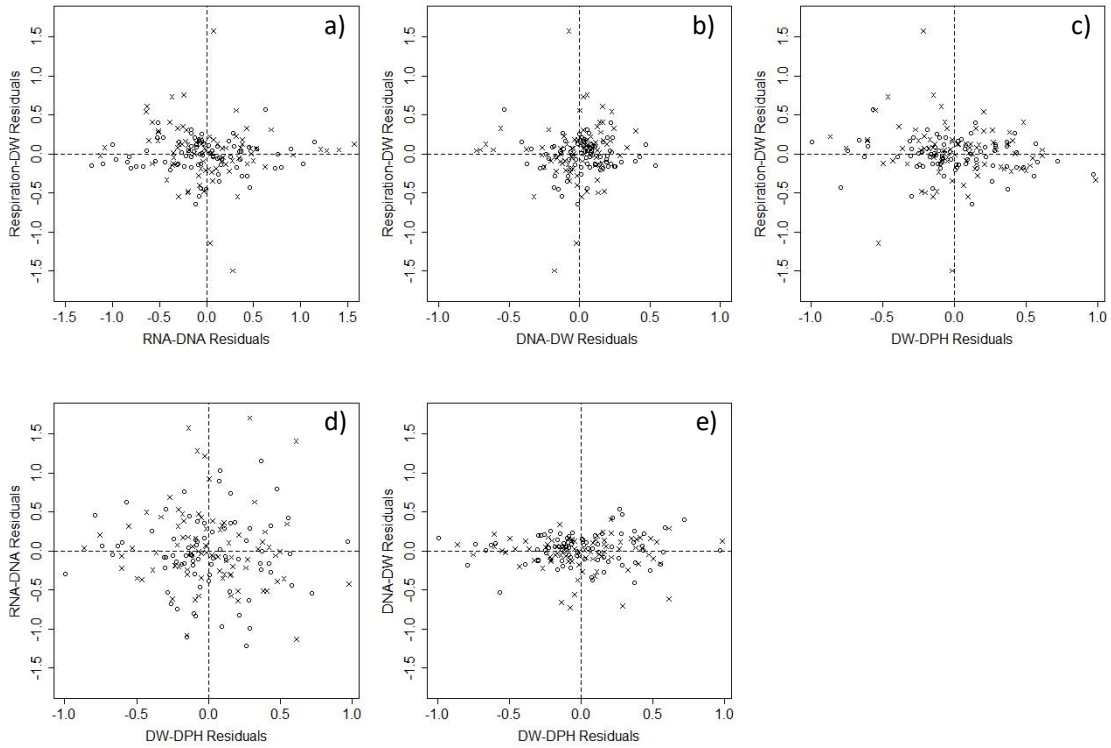


Figure 6.3. Inter-individual relationship between the residuals of a) Respiration-DW *vs.* RNA-DNA, b) Respiration-DW *vs.* DNA-DW, c) Respiration-DW *vs.* DW-DPH, d) RNA-DNA *vs.* DW-DPH and e) DNA-DW *vs.* DW-DPH. Oxygen consumption in darkness is shown with a circle (o) and in light is shown with a (x). No significant relationship was found in any of the graphs.

6.4 Discussion

Body size, and light are two important factors that can affect the respiration rate of fish larvae. The relationship between the metabolic rate and size in ABFT larvae showed an isometric relationship from 0.6 to 23 mg, higher than the average relationship in other species which is probably caused by a regulation (decrease) of the swimming activity with the consequent decrease in the oxygen consumption in light. The nutritional condition as RNA:DNA ratio was proportional to the larval size in dry weight but did not explain differences between light and darkness measurements. However, the more sensitive ratio DNA:dry weight, showed a decrease in the condition of the larvae in darkness. No relationship was found between the oxygen consumption and fish larval nutritional condition after correcting for the size effect, suggesting the importance of the size in the response of this species.

In this study, population growth from 17 to 30 dph shows a no linear pattern. The larvae, at 17 dph started with the piscivorous diet, which is known to increase growth rates to 60% of the dry weight daily (Reglero *et al.*, 2014a). Those growth are not reflected in our sampled larval growth pattern. Sampling in a 5000 L tank is a very difficult task that inevitably selects the smaller larvae since the large ones will be harder to capture. For this reason, similar sized individuals were sampled from

17 to 23 dph. Once the *Artemia* preys were stopped adding, probably part of the smallest larvae that still depend on it, died which coincides with the bigger survivals individuals captured from 24 dph. It is known that after only one day of starvation, more than the 50% of the individuals die (Tanaka *et al.*, 2008). The decrease in the sizes from 27 dph represent those selected smaller larvae that were separated to smaller tank in order to facilitate sampling and remove the biggest ones for weaning purposes.

6.4.1 Metabolic rate during light and darkness

The use of anaesthetics to induce a resting state been the most common method used to date to measure SMR in fish larvae (De Silva *et al.*, 1986; Kiørboe *et al.*, 1987; Moyano *et al.*, 2014) and adult individuals (Brill, 1979, 1987). However, anaesthetics are not recommended since there may be bias due to the use of different types and do to a possible depression of the metabolism (Javahery *et al.*, 2012; Peck and Moyano, 2016). In general, anesthetized larvae consumed 30% of the oxygen consumed by non-anaesthetized larvae (*e.g.* Davenport and Lönning, 1980; De Silva *et al.*, 1986; Houlihan *et al.*, 1995). Our experience in ABFT is that larvae do not survive after the use of anesthetics (personal observation). Another method to estimate SMR is the extrapolating curves of oxygen consumption and swimming speed back to zero velocity (Dewar and Graham, 1994; Gooding *et al.*, 1981; Graham and Laurs, 1982). The limitation for this method is the need of specific equipments to measure the oxygen consumption at controlled swimming speeds that were not available when doing this study. Therefore, measuring oxygen consumption in darkness was considered an appropriate approach to estimate the minimal functional activity, SMR, assuming during darkness the larval activity is reduced (Rønnestad *et al.*, 1994; Ruzicka and Gallager, 2006a; Yamashita and Bailey, 1989). The cost of routine movements has been suggested to be 1.5 times higher than those of darkness (or the cost of activity, in light being 70-80% of the total metabolic rate) (Peck and Moyano, 2016; Porter, 2001; Ruzicka and Gallager, 2006a). However, those measures of light and darkness oxygen consumption could have been affected by the pool of different aged larval oxygen consumption together which could be influenced by the size effect (Ruzicka and Gallager, 2006a). Also, by the measure of several larvae in the same chamber (Porter, 2001; Yamashita and Bailey, 1989) and by any metabolic expenses of larval circadian cycle because mostly, measures do not correspond to night-time and day-time hours and probably hunger which eventually can lead to increasing activity (foraging).

The differences in oxygen uptake between light (RMR, more active larvae) and darkness (SMR, less active larvae) could represent the cost of minimal swimming activity. However, several studies considered the differences in oxygen uptake between light and darkness as different levels of RMR activity, since even in darkness several spontaneous movements are seen in fish larvae (Finn *et al.*, 1995, 2002; Porter, 2001; Ruzicka and Gallager, 2006a). This relation RMR (active):RMR (inactive), tends to be higher than the RMR:SMR, when the definition of SMR is fulfil. The similarity in the metabolic rates measured in our study during light and

darkness suggests a possible similar swimming behaviors and therefore oxygen expenses in ABFT larvae. We suggest that larval oxygen consumption in light condition could have been affected by a diminishment of the activity inside a small closed chamber. Swimming activity within a respirometry chamber is likely to be suppressed relative to freely swimming larvae (Ruzicka and Gallager, 2006a). Ruzicka and Gallager (2006a) have compared the swimming behavior of larval cod in large tank (250 L) and in respiration chambers, and found that activity levels in the tanks were about 3.8 times higher in absence of prey and 4.9 times with prey than in small respirometry chambers. With light but in absence of preys that stimulate the foraging activity, ABFT larvae might have changed their behavior in order to balance the activity consumption with the energy needed to grow (Wieser *et al.*, 1988). They might have decreased the swimming activity and oxygen consumption to reduce the cost of activity to find a balance between the lack of energy consumption and activity expenses that result in similar metabolic rates during light and darkness. A reduction in swimming activity and the consequent oxygen consumption has been simulated (IBM) to improve herring larval survival compared to individuals without a behavioral modification (Illing *et al.*, 2018).

A possible stress caused by handling could explain the lack of differences between light and darkness. However, we removed the first 10 minutes of oxygen measurements considered an acclimation period. Intermittent-flow respirometry is considered a better alternative to static repirometry since allows longer measurement periods that are very useful if diel differences in metabolic rates are to be explored and in order to obtain a more reliable estimates (Peck and Moyano, 2016). However, it is technically challenging when used with tuna and bonito larvae (personal observation). Only two studies have used it with fish larvae in the last decade (McKenzie *et al.* 2008; McLeod *et al.* 2013).

Besides, in our study, measures in light were done few hour later than the usual lights on and subsequent star of feeding of the larvae which could have led to a hunger effect in the larvae. A decrease in the respiration measurements in herring larvae were found after several days without preys suggesting a metabolic down-regulation in their SMR (Illing *et al.*, 2018; Kiørboe *et al.*, 1987; Moyano *et al.*, 2018). With hunger, larval swimming activity and behavior is also known to be suppressed (Munk, 1995; Ruzicka and Gallager, 2006b). However, in our study, the effect on swimming performance was not recorded. A trade-off between growth and locomotion in well fed larvae has also been described where the amount of energy available for swimming decreases and might be devoted to growth and feeding (feeding, digestion and food conversion) (Moyano *et al.*, 2016).

6.4.2 SDA as a possible cause of differences between SMR and RMR

The effect of light and darkness in fish larvae is known to affect feeding incidence since ABFT, as most fish larvae are visual feeders, they fed with light and stop in darkness (Margulies, 1997). In larviculture, continuous feeding regimes during day light have been commonly used in order to increase feeding incidence along with growth and survival (Duray and Kohno, 1988; Partridge *et al.*, 2011; Shi *et al.*, 2010).

However, intermittent feeding during daylight has been found to be an alternative that enhance the growth or survival (Brown *et al.*, 1997; Rabe and Brown, 2000). Recently, Blanco *et al.* (2017) (Chapter 5), studied the effect of intermittent feeding by alternating every 1.5, 3 and 4.5 hours the light/feeding regime in ABFT, allowing them to digest preys in darkness, within a final total photoperiod of 9L:15D. They found similar growth and survival when compared with larvae reared with a photoperiod of 15L:9D and continuous feeding under *ad libitum* feeding conditions in tanks where active swimming was possible. They suggested that fish larval metabolic expenses (swimming) might be lower and digestion efficiency improved increasing the energy available to growth when food digestion is done in darkness.

The similar swimming expenses between light and darkness obtained in this study and if we assume similar behavior in the respirometry chamber and in the tanks, we could suggest that the efficiency by which ingested food is biochemically processed (digested and assimilated) might be a plausible explanation behind the beneficial growth results of alternating light/feeding regimes seen in Blanco *et al.* (2017) (Chapter 5). Specially, since ABFT larval swimming behavior it has been quantified to be similar under different feeding situations (just fed, 4 hours after feeding and late in the evening) (Reglero *et al.*, 2015b).

When feeding ceases in darkness, the costs of SDA might decrease in comparison with a continuous feeding where digestive activity continues. When factors such as temperature or prey availability are constant, ingestion and activity are important in the balance between growth and metabolism. However, to date, no information exists on the important influence of light on the metabolic rate of the early stage of ABFT and the only study done to date in the closely related Pacific bluefin tuna, is on the oxygen consumption in light conditions (Miyashita *et al.*, 1999).

There is still a lack of knowledge about the cost of SDA (of biochemically processing food) in fish larvae (Peck and Moyano, 2016). In larvae, direct measurements of SDA have rarely been made and sometimes, SDA has been defined based on the increase in oxygen consumption after feeding by inactive fishes whereas, in other cases, measurements of SDA also included the costs of routine foraging. Most often, oxygen consumption rates are measured with light for larvae fed an *ab libitum* prey ration and compared with those of larvae unfed for some period of time. The increase in oxygen consumption rates observed in fed larvae have been attributed to SDA (*e.g.* 24 h, Torres *et al.*, 1996). Several studies have estimated to be a factor of 0.11 of the growth (Kiørboe *et al.*, 1987), whereas in other studies, factors of 0.36–0.49 have been suggested (Wieser and Medgyesy, 1990a, 1990b). The reported SDA values are extremely variable (8–60% of consumed food energy) which, in all likelihood, is attributable to both biological differences and methodological issues. Big discrepancies exist in the SDA data, may be due to the use of anaesthetized larvae and non-simultaneous measurements of ingestion, growth, and metabolism in the Kiørboe *et al.* (1987) study. Further biochemical analyses are needed to verify if digestion efficiencies are the main reason of the differences in growth that they found.

6.4.3 The link of nutritional condition and oxygen consumption

The RNA:DNA ratio was used as an approach for recent growth condition (quick response time within a time frame of days prior to sampling) (Clemmesen, 1994; Folkvord *et al.*, 1996). The RNA:DNA ratio is susceptible to changes in the environment, which may affect the physiology of the organism; for example, low prey availability (Chícharo and Chícharo 1995). We found a positive correlation between RNA:DNA and larval dry weight, both in light and darkness, which suggests that the larvae we used were in good nutritional conditions during the experimental time. A rapid decrease in the nucleic acid concentration over a period of hours is knonw to reflect starvation conditions of hours in Pacific bluefin tuna larva (Tanaka *et al.*, 2008). Even though a clear sampling selection of the smallest larvae, the *ad libitum* feeding conditions and optimal tank settings had a positive impact on the good nutritional condition of all the larvae sampled. This sampling biases do not affect our analysis since residuals of size-at-age were used indirectly as somatic growth assuming a linear relationship between dry weight and dph.

Diel changes in the RNA:DNA ratio have been previously found in several larval studies assuming the existence of an endogenous rhythm that raises the RNA concentrations during the night time (Chícharo *et al.*, 1998, 2001; Rooker and Holt, 1996). In this study, the lack of differences between light and darkness in the RNA:DNA ratio support a possible change in the endogenous rhythm of the larvae.

We found significant differences in the cell condition (DNA:dry weight) between light and darkness with a switch in the tendency with larval dry weight. With light, small larvae had higher DNA:dry weight ratio, while the tendency switches with increasing larval dry weight. Fish larvae that were measured in light, had a longer starvation period (2-3 hours more) than those larvae measured in darkness. This starving period, might have affected more to the small larvae rather than to the biggest larvae since, energy reserves and starvation resistance is probably bigger in bigger larvae (Schultz and Conover, 1999). Bergeron (1997) already suggested that the DNA:dry weight appears to be a more stable and sensitive index in fish larvae than the common RNA:DNA where we did not find any differences. As seen in this study, the DNA:dry weight, might have a faster response time to a change in the nutritional condition than the RNA:DNA. Besides, in a previous study in ABFT and Atlantic bonito larvae (Chapter 3), the DNA:dry weight ratio was a better indicator than the RNA:DNA of the larval condition in ABFT larvae because it was sensitive enough to differ among different stages during the first days of life. However, diel differences in the DNA:dry weight ratio have not been determined jet.

As seen in our residual analyses there was no correlation between the oxygen consumption and nutritional condition of the larvae when extracting the size effect. These results are in accordance with those of other species, like herring, where they also found that the differences in SMR were not explained by the nutritional condition (RNA:DNA) (Moyano *et al.*, 2018). We also found no relationship in the residual between the oxygen consumption and nutritional condition (RNA:DNA and DNA:dry weight) with the larval size-at-age. These results emphasize the fact that

it is the size of the larvae that determines changes in the oxygen consumption and condition, as seen in the high correlations with larval size. In a previous study in ABFT larva, Chapter 3, a positive correlation between the residuals of the nutritional condition and the larval size-at-age during their planktivorous stage. Both analyses (this and Chapter 3) where done using the same methodology and run by the same person which remove the methodology effect in the results. Several reasons may explain these observed differences. Firstly, this study was done in piscivorous rather than in planktivorous larvae. Those two phases are very different from each other and characterized by different gastrointestinal and morphological development (Kaji, 2003; Kendall *et al.*, 1984; Yúfera *et al.*, 2014). Besides, the initiation of piscivory is characterized by the generation of marked growth variations, higher than those observed in larvae in the planktivorous stage (Takebe *et al.*, 2012; Tanaka *et al.*, 2014a). Thus, different results are apparent during different ABFT larval phases. And secondly, the piscivorous phase is also characterized by the increase in the growth rate of the larvae, due to the fact that using fish larvae as a prey is nutritionally more beneficial than having a planktivorous diet (Reglero *et al.*, 2014a, Seoka *et al.*, 2008). Thus, those high growth rates, might have generated a variability in the inter-individual oxygen uptake and nucleic acid content as shown by the lack of correlation between these measures. We found high variability among individuals in our larvae, with residuals standard error being from 30-40% in both measurement (respiration and nutritional condition). In fish larvae, it is very common to obtain high variability in nutritional condition influenced by larval ontogenetics (Foley *et al.*, 2016; Meyer *et al.*, 2012), because different growth and development is known to occur (Chapter 3).

6.4.4 Metabolic exponent (mass scaling exponent)

The relationship between body mass and metabolism is described by a potential equation (exponential relationship) where the slope indicates the metabolic exponent. The metabolic exponent represents the change in the metabolic rate of an organism as it gains mass. The large majority of the work that has empirically examined the metabolic rate *vs.* body mass relationship has used standard or routine metabolic rates (SMR or RMR). In our study we found an isometrical metabolic exponent (slope equal to 1) for ABFT larvae. There is not a universal metabolic exponent for fish larvae and values vary between 0.60 and 1.20 (review considering 32 species of marine fish larvae by Peck and Moyano, 2016). Which means that individual oxygen consumption is changing a power of 0.6 - 1.20 times the body mass. However, variability in the metabolic exponent across studies might be caused by methodological differences. The use of different types of anaesthetics to induce the larvae to rest (*e.g.* propanidid, MS-222, benzoxaine, metomidate) (Kiørboe *et al.*, 1987; De Silva and Tytler, 1973; Houlihan *et al.*, 1995; Moyano *et al.*, 2014) and the use of different techniques (calorimetry, manometers, electrode, polarographics or optodes), different respirometers (plastics and glass) and set-ups used to measure oxygen consumption might cause discrepancies when comparing the different results in the literature. In Atlantic herring, for example, different mass scaling exponents were found between North Sea Autumn Spawning individuals (1.07) and Western

Baltic Spring Spawning individuals (0.83) at 7 ˚C and measured with different sensors, electrode and needle optode respectively (Moyano *et al.*, 2017).

Our isometrical result (b=1.0) in pisicivorous post-flexion larvae (0.6 - 23 mg dry weight, 17-30 dph) at 26 ºC coincides with the value in light measures obtained by Miyashita *et al.* (1999) in piscivorous Pacific bluefin tuna larvae at 25 ˚C (from 0.09 - 50 mg dry weight, 17-30 dph). Even though oxygen consumption increases when the temperature becomes warmer (Blank *et al.*, 2007; Finn *et al.*, 2002; Peck and Buckley, 2008), there is no clear effect of temperature on body mass scaling (Giguère *et al.*, 1988; Moyano *et al.*, 2018), which agrees with the similarity between our study and Miyashita *et al.* (1999). Miyashita *et al.* (1999) found changes of metabolic scaling exponent related to different ontogenetic stages: b=0.10 in larva from 0 to 5 dph, b=1.38 from 6-17 dph, b=1.01 from 17-30 dph and b=0.66 from 30-126 dph. Those results show the high oxygen consumption demand before piscivory, related with the development of an adult type digestive system and external characteristics (Kaji, 2003; Yúfera *et al.*, 2014) that improves once they start piscivory. In the relative species Atlantic mackerel, *Scomber scombrus*, larvae, Giguère *et al.* (1988) also found an isometric exponent with increasing body mass (0.05 - 4.56 mg) (0.97-1.0). The general tent assumes that metabolic rate scales monotonically with body size, however, as seen in tunas, metabolic scaling often shows marked shifts during ontogeny. The oxygen consumption rapidly increases during early ontogeny and then from juvenile decreases (Miyashita *et al.*, 1999).

Apart from the ontogenetic study in Pacific bluefin tuna larvae (Miyashita *et al.*, 1999), and the optimal oxygen ranges described for *Thunnus albacares* larvae (Wexler *et al.*, 2011) the rest of the oxygen related studies done in several tuna species have been done in adult individuals. In adults, the relationship between fish size and SMR suggest an allometric relationship with b=0.5-0.6 (*e.g.* Brill 1979, 1987; Dewar and Graham, 1994; Sepulveda and Dickson, 2000) and for RMR it is 1.18-1.19 (*e.g.* Gooding *et al.*, 1981; Graham and Laurs, 1982). Tunas adults appear to have lower standard metabolic exponent compared with the general exponent of 0.8 proposed in juvenile and adult stages of fishes (Clarke and Johnston, 1999; Wieser, 1995) and the exponent generally accepted in the animal kingdom (0.75) (Savage *et al.*, 2004). This indicates that mass-specific SMR decreases relatively rapidly as body mass increases (Brill, 1987). However, the reason the reasons of this are difficult to interpret. The high routine metabolic scaling rate of adults compared with the general trend, might be mainly contributed by the cost of endothermy and the extreme swimming capability which result in greater locomotion cost (ram-ventilation and swim power) in comparison with other fishes (Graham and Dickson, 2004). Adult bluefin tuna demand a higher oxygen concentration that other marine fishes (Korsmeyer and Dewar, 2001).

In our study, an average of 0.424 μmol mg^{-1} h^{-1} of oxygen was consumed by piscivorous 0.6 to 23 mg dry weigh (26 ºC, 17-30 dph) ABFT larva individuals. This consumption, is inside the range 0.272-0.509 μmol mg^{-1} h^{-1} obtained in light manometrically in a respirometer in the scombrid Pacific mackerel (*Scomber*

japonicus) of much smaller sizes 0.025-12.740 mg dry weight (18-22 ºC, 3-5 dph) (Hunter and Kimbrell, 1980). In bay anchovy *Anchoa mitchilli* also similar oxygen consumption was obtained in light using a polarographic oxygen electrode, 0.367 µmol mg^{-1} h^{-1} in also smaller larvae 0.424 mg individuals (26 ºC, 12 dph) (Houde and Schekter, 1983). Due to the discrepancies concerning the type of methodology, it is difficult to estimate if they are higher than in other fish larva (see review Peck and Moyano, 2016). However, the high scaling component (b=1) obtained in our study, could give us an idea that at least during the piscivorous stage, ABFT larval oxygen consumption might be bigger than in other fish larvae. During the piscivorous stage (25 ºC, 19-27 dph), growth rates of 1.03 mm day^{-1} and 54% dry weight day^{-1}, have been reported in ABFT larvae (Reglero *et al.*, 2014a), and during the plankitivorous stage (27 ºC, 8-13 dph), 0.48 mm day^{-1} and 35% dry weight day^{-1} (Chapter 3). The growth rates during the planktivorous stage are similar to those seen in similar sized European sardine (*Sardina pilchardus*) at 15-16 ºC, 0.32-0.41 mm day^{-1} (Moyano *et al.*, 2014; Ramírez *et al.*, 2001). However, the growth rates during the piscivorous stage might be responsible of the high oxygen consumption in ABFT larvae, even if a possible regulation of the oxygen consumption is assumed.

The similar oxygen consumption results between light and darkness obtained in this study, might represent a suppression of the swimming activity within a respirometry chamber. Therefore, careful used of the data should be done when applying to the field. However, the data give us an idea of the high oxygen consumption during the piscivorous stage of this species, unknown until this work. The present study represents the first metabolic data of ABFT larvae.

CHAPTER 7

- VERTICAL DISTRIBUTION OF ATLANTIC BLUEFIN TUNA (Thunnus thynnus) AND BONITO (Sarda sarda) LARVAE IS RELATED TO TEMPERATURE PREFERENCE –

Vertical distribution of Atlantic bluefin tuna (*Thunnus thynnus*) and bonito (*Sarda sarda*) larvae is related to temperature preference

ABSTRACT

Temperature ranges are important in explaining the worldwide distribution of tuna and bonito larval habitats. Less is known about how the thermal tolerance of these species' larvae restricts their vertical distribution. Here we combined field and laboratory data to explore the role of temperature on the vertical distribution of Atlantic bluefin tuna (*Thunnus thynnus*) and Atlantic bonito (*Sarda sarda*) larvae. First, we related the vertical structure of several environmental variables to larval vertical distribution in a recognized tuna spawning area in the Mediterranean. The field data indicated temperature-dependent behavior both in bluefin tuna and albacore larvae, with a clear preference for the higher temperatures found in upper water layers in strong thermal-gradient environments. No Atlantic bonito larvae were caught in the field samples. Second, we confirmed such behavior under controlled conditions, observing fed larvae of bluefin tuna and bonito in experimental columns with temperature gradients similar to those experienced in the NW Mediterranean (22–25.6 °C for bluefin, 18–23 °C for bonito) and with no temperature gradients (24.4 °C for bluefin, 23 °C for bonito). The larvae were distributed significantly shallower in the stratified than in the isothermal experimental water columns in both light and dark conditions. These results suggest that the vertical distribution of tuna and bonito larvae is spatially constrained by larval temperature tolerance. In comparing our results to other geographical areas, we found that the vertical habitat of tuna larvae that spawn in regions with strong thermal gradients is smaller than in regions with weaker thermal gradients.

7.1 Introduction

Variability in water column properties may influence the growth, survival and dispersal of larval fish and thus their distribution and abundance. Larval fish can modify their behavior in response to their environment by moving into more favorable conditions that increase survival. Diel vertical migrations illustrate behavior that could balance trade-offs between feeding and predation risk within the water column (Fiksen *et al.*, 2007).

The vertical position of the larvae also influences their horizontal advection from spawning to optimal nursery areas, since currents are vertically stratified in the water column (Leis, 2006). Whatever the behavioral response to biological and physical conditions, the overall vertical distribution range will be limited by the organism's physiological tolerance to temperature imposed by either their maximum or minimum thermal constraints. When large changes in water temperatures occur over short vertical distances, as in strong thermoclines, the prevalence of an organism in a depth range may be related to its temperature preferences. This effect may be stronger in larval fish that are very sensitive to temperature variability, particularly in species whose early life stages have a relative narrow thermal tolerance range (Pörtner and Peck, 2010; Vollset *et al.*, 2013).

All climate change projections for the Mediterranean Sea indicate substantial warming and an increase in the frequency and intensity of heat waves (Lionello *et al.*, 2012). This semi-enclosed sea offers an analogy to explore the resiliency of top predators in pelagic oceanic systems (Lejeusne *et al.*, 2010). This type of study is of particular importance for the large pelagic top predatory species that migrate annually to their summer spawning grounds in the NW Mediterranean, such as tuna and bonito. These species share the common trait that their larvae inhabit only warm waters at sea temperatures above 20–22 °C (Boyce *et al.*, 2008; Muhling *et al.*, 2017; Reglero *et al.*, 2014b).

Thus, high temperatures seem to be a general characteristic of tuna and bonito larval habitats, although the role of temperature as an environmental cue for the vertical distribution of larvae in the water column and its importance in relation to other environmental variables is not yet clear. In the NW Mediterranean, where climate warming is expected to strongly increase the stratification of the water column (Coma *et al.*, 2009), it is important to know how temperature stratification triggers larval distribution.

Bluefin tunas have the most spatially and temporally constrained larval habitats of all tuna species (Reglero *et al.*, 2014b). Atlantic bluefin tuna (*Thunnus thynnus*), 1 of the 3 bluefin tuna species (the other 2 being Pacific bluefin tuna *T.orientalis* and southern bluefin tuna *T.maccoyii*), reproduces in the NW Mediterranean Sea. Albacore (*T.alalunga*) and bullet tuna (*Auxis rochei*) larvae co-occur with Atlantic bluefin tuna larvae in the NW Mediterranean (Alemany *et al.*, 2010; Reglero *et al.*, 2012). Aside from these species, larvae of the small tropical tunas little tunny (*Euthynnus alleteratus*) and skipjack tuna (*Katsuwonus pelamis*) are also caught in

this area (Alemany *et al.*, 2010; Torres *et al.*, 2011). These species usually have a wider spawning area and a more extended spawning period than bluefin tunas (Reglero *et al.*, 2014b) and can be found both in tropical areas, where water temperatures show only a slight temperature gradient over the upper 100 m, and in temperate areas, where water temperatures show a strong vertical gradient (Llopiz and Hobday, 2015). Atlantic bonito (*Sarda sarda*) share the Atlantic bluefin tuna spawning grounds in the NW Mediterranean, where water temperatures are above 18 °C (Sabatés and Recasens, 2001; Torres *et al.*, 2011). Since most tuna species, including Atlantic bluefin tuna and Atlantic bonito, are already strongly piscivorous during their larval stage and can prey on each other (Llopiz and Hobday, 2015; Reglero *et al.*, 2014a), it is important to understand the mechanisms that may increase spatial overlap in the water column among these species.

The thermal preferences of the larvae of the 3 bluefin tuna species have been relatively well studied. When reared in captivity, temperatures above 19–20 °C are necessary for the larvae to hatch, and larvae have been successfully reared at temperatures above 22 °C (Gordoa and Carreras, 2014; Miyashita *et al.*, 2000; Reglero *et al.*, 2014a; Tanaka *et al.*, 2008; Wexler *et al.*, 2011; Woolley *et al.*, 2009), suggesting a minimum thermal constraint at 19 °C. Although it is generally accepted that bluefin tuna larvae are associated with warm temperatures, it is difficult to disentangle the effect of temperature on the vertical distribution of these species. Southern bluefin tuna larvae in the Indian and Pacific Oceans, and Atlantic bluefin tuna larvae in the Gulf of Mexico, though most abundant in the first 20 m, have been caught down to depths of 50–60 m, a depth range where temperatures are above 20 °C during the spawning season (Boehlert and Mundy, 1994; Davis *et al.*, 1990; Habtes *et al.*, 2014). More restricted vertical distributions, down to only 20 m, have been described for Pacific bluefin tuna, a pattern that has been related to variables other than temperature, in particular to the existence of pycnoclines (Satoh, 2010). Atlantic bluefin tuna, Atlantic bonito and striped bonito *S. orientalis* have been successfully reared in captivity at temperatures above 19 °C (Chapter 5; Blanco *et al.*, 2017; McFarlane *et al.*, 2000; Ortega, 2015; Ortega and Mourente, 2010; Reglero *et al.*, 2014a). There are no descriptions of the vertical distribution of these and companion species of Atlantic bluefin tuna in the NW Mediterranean. The vertical distribution of bullet tuna has been described as restricted to the top 20 m of the water column, although temperature profiles were not analyzed (Morote *et al.*, 2008). In other areas, skipjack tuna larvae have been caught down to 50 m (Davis *et al.*, 1990; Habtes *et al.*, 2014; Matsumoto, 1958) and even at 80 m depth (Boehlert and Mundy, 1994; Llopiz *et al.*, 2010), while albacore larvae have been caught down to 40 m (Davis *et al.*, 1990; Habtes *et al.*, 2014) and little tunny down to 100 m depth (Habtes *et al.*, 2014; Llopiz *et al.*, 2010). These different distribution patterns among bluefin and other tuna species indicate the need for studies at the local scale to ensure that vertical distributions are well described.

Analyzing the effect of vertical temperature gradients on vertical larval distribution from field studies alone can be complex due to the many confounding variables besides temperature, such as light absorption, the existence of pycnoclines or the

occurrence of prey items. Observing the behavior of larvae in relation to experimental vertical temperature gradients can help to understand the response to temperature gradients in the field (Vollset *et al.*, 2009). The comparison between experimental and field studies will improve our understanding of the larval response of tuna and bonito to temperature gradients. We expect the vertical distribution of larvae of tuna and bonito species, particularly Atlantic bluefin tuna and Atlantic bonito larvae, for which a minimum thermal constraint has been identified in the laboratory, to be limited by their thermal tolerance in the sharp thermal gradients observed in the NW Mediterranean. We hypothesized that the vertical distribution of Atlantic bluefin tuna larvae in the NW Mediterranean, where the thermal structure of the water column during the summer, coinciding with the spawning season, presents sharp vertical gradients (Torres *et al.*, 2014), may be a response to bluefin tuna larvae thermal preferences. Therefore, the depth distribution range for bluefin tuna larvae is dependent on the window of physiologically favorable temperatures.

Our aim in this study was to evaluate whether temperature may be a dominant cue influencing the vertical distribution of tuna and bonito larvae in temperate areas using a combination of field and laboratory experiments. Field data on the vertical distribution of scombrid larvae were obtained from 2 surveys conducted in the Balearic Sea (NW Mediterranean) during summer 2011 and 2012 and then correlated with temperature, salinity and chlorophyll measurements. The behavior of Atlantic bluefin tuna and Atlantic bonito larvae in response to vertical environmental temperature gradients similar to those observed in nature was investigated through the use of experimental thermoclines. Knowledge of the ecology of the larvae of Atlantic bluefin tuna and other co-occuring scombrids, has improved our understanding of the environmental requirements for Atlantic bluefin tuna spawning grounds and stock management (Ingram *et al.*, 2017; Muhling *et al.*, 2017). Improving our understanding of the mechanisms that explain the role of temperature in driving the vertical distribution of large pelagic fish is necessary to assess these animals' vulnerability to climate warming and enhanced stratification and to improve their conservation and management (Horodysky *et al.*, 2016).

7.2 Materials and methods

7.2.1 Field surveys

Two multidisciplinary research surveys were conducted off the Balearic Islands, western Mediterranean Sea: one in 2011, onboard the fishing vessel 'Tio Gel II' (Grup Balfegó SL), and the other in 2012, onboard the RV 'Ramon Margalef.' Within these cruises, a series of ichthyoplankton samplings were carried out by means of oblique tows using a bongo net of 90 cm mouth diameter with a mesh size of 500 µm, performed over a systematic 10 × 10 nautical mile grid, to localize patches with high densities of tuna larvae (see Alemany *et al.* (2010) for a detailed description of the systematic grid). Once a high-density bluefin tuna larval patch was identified, which in both years occurred off the Cabrera archipelago, its position was tracked by a

Lagrangian iridium buoy fixed in the mixed layer with a sock drogue deployed between 8 and 15 m depth. While following the tuna larval patches, we conducted 14 stratified vertical sampling tows between 20 and 22 June 2011 and 9 tows between 9 and 11 July 2012 (Fig. 7.1).

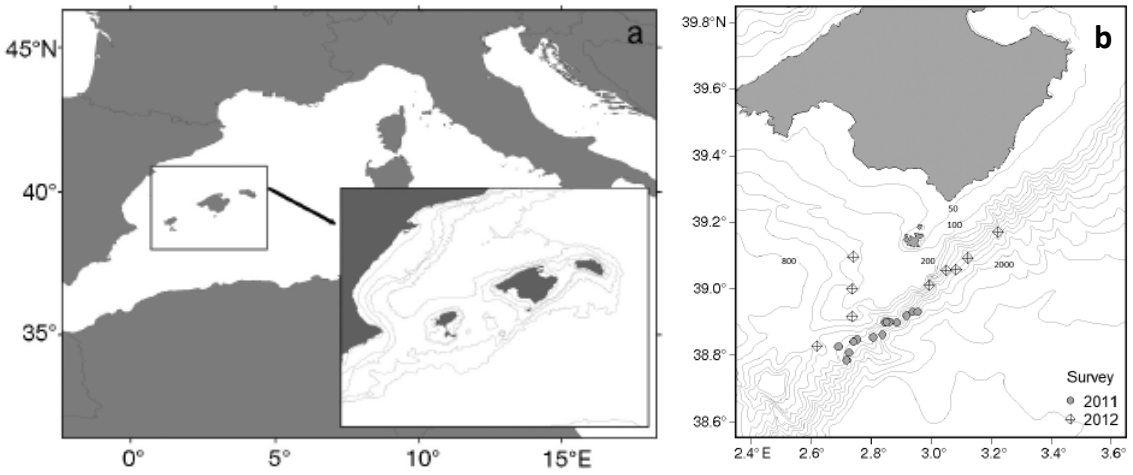


Figure 7.1. a) Study region off the Balearic Islands in the NW Mediterranean Sea, a primary spawning area for tuna species. b) Stratified vertical sampling locations with tuna larvae sampled in 2011 (14 operations, grey circles) and 2012 (9 operations, crossed rhomboids). Light grey lines represent the bathymetric lines with depth (m). Only those stations where vertical resolution sampling was conducted are shown.

In 2011, we used a HYDRO-BIOS multi-net, sampling 5 depth strata (0–5, 5–10, 10–15, 15–20, 20–30 m), whereas in 2012 we used a Multiple Opening Closing Net and Environmental Sensing System (MOCNESS), sampling 6 depth strata (0–10, 10–20, 20–30, 30–40, 40–50, 50–60 m). The net mouth openings were 0.25 and 1 m^2, respectively, and the mesh size was 333 µm for both nets. Both devices were repeatedly towed at ~2 knots during day and night. All samples were preserved immediately after collection in ~4% borax buffered formaldehyde, prepared using seawater. Both in 2011 and 2012, vertical profiles of temperature, salinity and fluorescence were determined using a CTD (SBE 911). Once in the laboratory, plankton samples were sorted for fish larvae, which were individually identified to species level (Alemany, 1997). Tuna species were identified, photographed and their standard length (SL, mm) measured using an image analysis system equipped with Image-Pro Plus 6.2 software (Media Cybernetics). Lengths of the preserved larvae were corrected for shrinkage using the algorithm developed for *Thunnus thynnus* by Reglero *et al.* (2013). The number of larvae caught in each depth range was standardized to number of larvae 100 m^{-3}.

7.2.2 Larval rearing

Rearing of Atlantic bonito and Atlantic bluefin tuna was conducted in the summers of 2013 and 2014, respectively. These are the only bonito and tuna species reproducing in the Mediterranean Sea for which laboratory culture methods have been developed (Ortega, 2015; Reglero *et al.*, 2014a). Bonito eggs were obtained from captive broodstocks at the Spanish Institute of Oceanography (IEO) rearing facilities at Mazarrón (Spain), where all experiments were conducted. Batches of fertilized bluefin tuna eggs were obtained from naturally spawning captive adult tuna in farming facilities at El Gorguel (Caladeros del Mediterráneo S.L., Spain) and transported to the experimental facilities at Mazarrón. Bonito and bluefin tuna eggs were incubated separately, and the larvae were fed a planktivorous diet following the protocol described by Reglero *et al.* (2014a). The experimental treatments were conducted on larvae of (mean ± SD) 8.3 ± 0.83 mm SL for tuna and 7.5 ± 0.96 mm for bonito, corresponding to the flexion and post-flexion developmental stage. Small individuals in the yolk-sac and the pre-flexion developmental stage were not included in the analyses due to potential errors associated with limitations on the visual identification of the larvae in the experimental columns.

7.2.3 Experimental set-up

The experimental set-up was modified from that described by Vollset *et al.* (2009). It consisted of 6 cylindrical methacrylate experimental columns 102 cm long and 20 cm diameter with the capacity for approximately 30 L of water. The columns were illuminated from behind, uniformly over the whole water column, using fluorescent lamps. A mark was drawn with a permanent pen dividing each column every 17 cm (ca. 5 L in each segment).

During the evening, each column was filled with 25 L of oxygen-saturated marine water, leaving the upper 17 cm of the water column empty. Two column stratification regimes were used, *e.g.* stratified and isothermal. To create the thermocline, 3 submersible aquarium heaters were placed in each of the columns at depths between 17 and 34 cm from the top of the column to warm the water in the surface up to 23 °C for the experiments with Atlantic bonito and to 25.5 °C for the experiments with Atlantic bluefin. The room temperature was kept at 19 and 22 °C for bonito and bluefin, respectively, by means of an air conditioner, to create a gradient with temperatures ranging from 23 to 18 °C for bonito and 25.6 to 22 °C for bluefin tuna (Fig. 7.2). For the isothermal treatment, the air-conditioning of the room was set so that overall the experimental column temperatures were (mean ± SD) 23.1 °C ± 0.08 °C and 24.4 °C ± 0.1 °C for bonito and bluefin, respectively (Fig. 7.2), maintaining the heaters inside the column but not in operation. Differences in the temperature range for both species were set according to their temperature range in captive conditions (Ortega, 2015).

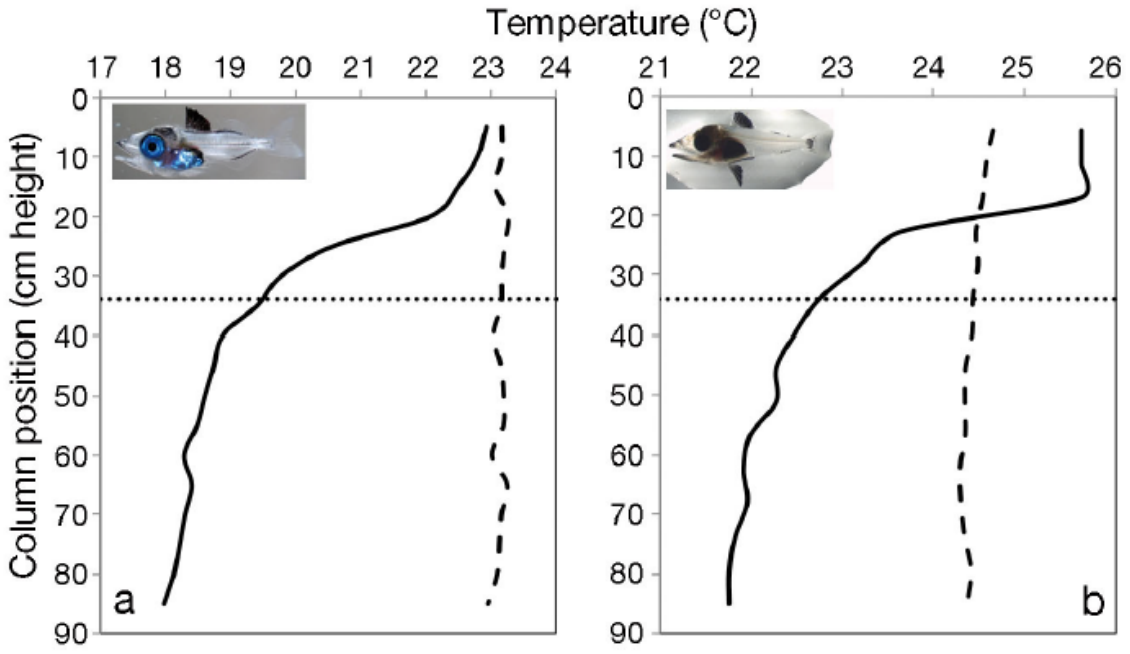


Figure 7.2. Average temperatures in the experimental vertical columns for a) Atlantic bonito (*Sarda sarda*) and b) Atlantic bluefin tuna (*Thunnus thynnus*). The continuous line represents the temperature regime for the thermocline treatment and the dashed line for the isothermal treatment. The dotted line indicates the separation between the upper and lower sections in the water column. Larvae were counted in the upper part of the water column (upper 34 cm).

For bonito, we simultaneously used 3 experimental columns for the thermocline treatment and 3 for the isothermal treatment each day. For bluefin, 6 experimental columns were used daily either for the thermocline or the isothermal treatment. The water in the columns was mixed before the onset of the isothermal trials. No aeration was provided in any of the columns since oxygen levels were always closed to saturation for the duration of the experiments. The vertical temperature distribution in each water column was measured before and after the completion of the experiment using 15 HOBO™ (Onset) data loggers homogenously separated along depth intervals in each water column to ensure that the temperature was stable during the experiment. The HOBOs were removed before the larvae were placed into the water columns. The columns were emptied and cleaned immediately every day after the end of the treatment.

7.2.4 Treatments

The larvae were kept in a 1500 L tank without feeding during the night prior to the start of the experiment. Every morning, we switched on the light in the 1500 L tank and the larvae were fed to satiation for 3 h. Afterwards, 60 larvae were removed from the 1500 L tank, and 10 larvae were placed in each of the experimental columns from above while the light was on. The larvae acclimated rapidly to the column, swimming around calmly within a few minutes. After 15 min of acclimation, the larvae in each experimental column were monitored 4 times daily by 2 observers who

independently counted the number of larvae in the upper 34 cm of the water column, corresponding to the upper part of the thermocline in the thermocline treatments. To count the larvae, the observers wore dark clothing and during darkness used a lantern with red light at low intensity to avoid disturbing the larvae. The larvae were counted 30 min ($I1_{bft}$ for isothermal, $T1_{bft}$ for thermocline) and 60 min ($I2_{bft}$, $T2_{bft}$) after the onset of the experiment in Atlantic bluefin tuna and 60 min ($I1_{bon}$ for isothermal, $T1_{bon}$ for thermocline) and 120 min ($I2_{bon}$, $T2_{bon}$) after the onset of the experiment in Atlantic bonito while the light was on. The light was completely switched off, the larvae acclimated to darkness during 15 min, and the larvae were monitored again 105 min ($I3_{bft}$, $T3_{bft}$) and 135 min ($I4_{bft}$, $T4_{bft}$) after the onset of the experiment for Atlantic bluefin tuna and after 195 min ($I3_{bon}$, $T3_{bon}$) and 255 min ($I4_{bon}$, $T4_{bon}$) for Atlantic bonito. After the last measurement (135 min for bluefin tuna and 255 min for bonito), the larvae were moved to another tank and kept alive while a subsample was photographed, measured (SL) and frozen.

The experiment with bonito lasted for 8 d, and a total of 470 bonito larvae were used (60 larvae d^{-1}, 10 in each experimental column except 1 that was not used due to technical problems). For bluefin tuna, the experiment lasted for 7 d (the thermocline treatment was conducted on experimental days 1, 3, 4, 6 and 7, and the isothermal treatment was conducted on experimental days 2 and 5), and a total of 380 bluefin tuna larvae were used (60 larvae d^{-1}, 10 in each experimental column except 4 experimental columns that were not used due to technical problems).

7.2.5 Data analysis

7.2.5.1 Field data

We used a generalized additive model (GAM), a nonparametric approach that allows non-linear relationships between response and explanatory variables that follows the general equation:

$$(1)\quad N_{larvae} = g^{-1}\left(\beta_0 + \mathit{offset}(\mathrm{Ln}(vol)) + \text{factor (day or night)} + \Sigma\, S\,(\text{environmental variable})\right)$$

where g represents the link function, β_0 is the model intercept, and S is a smoothing function with no *a priori* assumption of linearity for each explanatory environmental variable. We used a Poisson distribution, suitable for count data, to model the number of larvae (N_{larvae}) in relation to temperature, salinity and fluorescence, using a natural-log (Ln) link function. The volume of water filtered (vol) was included as an offset after natural log transformation to account for the effort used in catching the sample. Time of sampling as day or night was included as a factor in the model to account for daily variability in larval abundance. The variable selection criteria were based on the confidence region for the smoothing effect, the percentage of deviance explained (increasing after adding significant covariates) and the UBRE score (decreasing after adding significant covariates) (Reglero *et al.*, 2012; Wood, 2006). GAMs were fitted using the 'mgcv' library in R statistical software (https://cran.r-project.org/web/packages/mgcv).

7.2.5.2 Experimental data

The effect of the thermocline on the vertical distribution of the larvae during light was tested separately for bluefin tuna and bonito using a generalized linear model (GLM). Unlike for the statistical analysis on field data, we were not detecting non-linear effects of the dependent variables on the response variable and therefore used GLMs instead of nonparametric GAMs. The GLM modeled the number of larvae counted in the upper 34 cm of the column including isothermal replicates 1 and 2 ($I1_{bft}$ and $I2_{bft}$ for bluefin tuna and $I1_{bon}$ and $I2_{bon}$ for bonito) and thermocline replicates 1 and 2 ($T1_{bft}$ and $T2_{bft}$; $T1_{bon}$ and $T2_{bon}$) during the light period. The same procedure was used to test the effect of the thermocline on the vertical distribution of the larvae during darkness but including isothermal replicates 3 and 4 ($I3_{bft}$ and $I4_{bft}$; $I3_{bon}$ and $I4_{bon}$) and thermocline replicates 3 and 4 ($T3_{bft}$ and $T4_{bft}$; $T3_{bon}$ and $T4_{bon}$) during the dark period. We then tested differences in the vertical position of the larvae between light and darkness in the thermocline including thermocline replicates during the light period ($T1_{bft}$ and $T2_{bft}$; $T1_{bon}$ and $T2_{bon}$) and during darkness ($T3_{bft}$ and $T4_{bft}$; $T3_{bon}$ and $T4_{bon}$). The same procedure was used to test differences in the vertical position of the larvae during the isothermal treatment between light ($I1_{bft}$ and $I2_{bft}$; $I1_{bon}$ and $I2_{bon}$) and darkness ($I3_{bft}$ and $I4_{bft}$; $I3_{bon}$ and $I4_{bon}$). All comparisons were done separately for bonito and bluefin tuna. We applied a Bonferroni correction to adjust probability values for replicated trials and to avoid type I error (*p-adj.*, defined as the probability divided by the number of tests). All data analyses were conducted using R (www.r-project. org).

7.3 Results

7.3.1 Field larvae

The vertical distribution of the hydrographical variables, *e.g.* temperature, salinity and fluorescence, indicates that environmental conditions along the water column were very similar in 2011 and 2012 (Fig. 7.3). The water column was characterized in both years by a marked thermocline located around 20 m depth (Fig. 7.3a, b), with no clear halocline (Fig. 7.3c, d) and chlorophyll increasing towards maximum values at 60 m depth (Fig. 7.3e, f), though particularly in 2012 chlorophyll values were very low and constant over the water column (Fig. 7.3f). There was no correlation between the thermocline and the other environmental variables (chlorophyll and salinity).

Larvae of Atlantic bluefin tuna were caught in high abundances in both years, and larvae of albacore and bullet tuna, although less abundant than bluefin, were also caught both years (Table 7.1). Other tuna species, *e.g.* 2 larvae of little tunny (*Euthynnus alletaratus)* (3.1 mm SL) and 1 larva of skipjack (*Katsuwonus pelamis)* (4.3 mm SL), were caught only in 2012. Atlantic bluefin, albacore and bullet tuna larvae were caught in the first 20 m depth, whereas the little tunny and skipjack larvae were caught in the first 10 m depth.

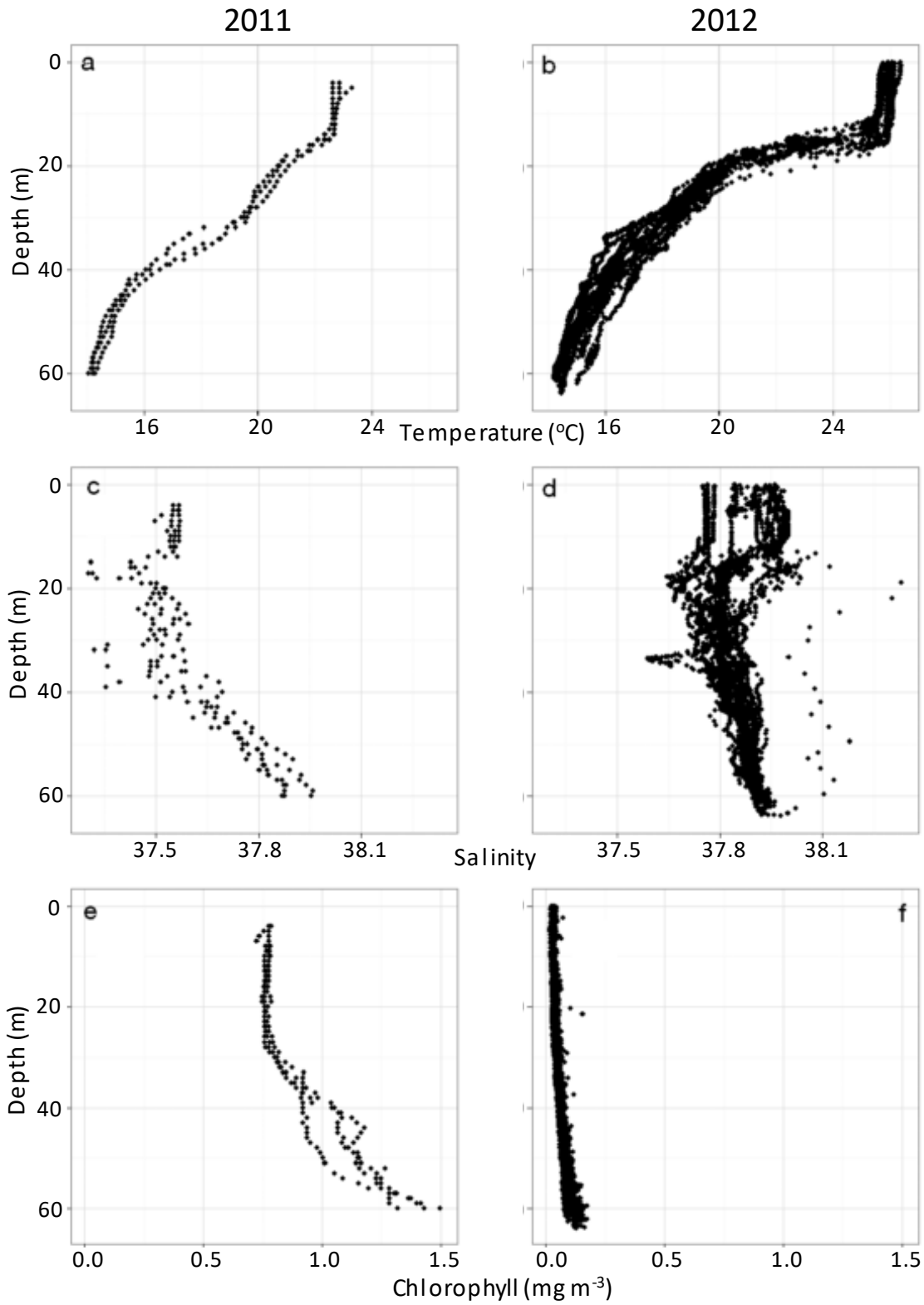


Figure 7.3. Vertical profiles of hydrographical variables (temperature, salinity, chlorophyll concentration) measured during the 2011 and 2012 research cruises.

Table 7.1. Total number (N) of larvae of Atlantic bluefin tuna *Thunnus thynnus*, albacore *T.alalunga* and bullet tuna *Auxis rochei*, caught in 2011 and 2012. For each species, mean ± SD standard length (SL) after shrinkage correction is shown.

Species	2011		2012	
	N	SL (mm)	N	SL (mm)
Thunnus thynnus	333	4.2 ± 0.6	1629	4.7 ± 0.8
Thunnus alalunga	13	3.9 ± 0.6	9	4.3 ± 0.9
Auxis rochei	5	3.9 ± 0.3	1	4

Temperature alone significantly explained most of the variance both in bluefin tuna and albacore (Table S1 in the Supplement, 41% in 2011 both species and 71 and 51% for bluefin tuna and albacore, respectively, in 2012), with increasing larval abundances as temperature increased (Fig. 7.4). Salinity was also significant, although it explained less variance than temperature (Table S1 in the Supplement). The significant relationship with salinity was not related to its vertical profile but indicated that more larvae were caught in stations with slightly less saline waters in the upper layers. No significant differences in the day and night patterns were observed for bluefin tuna and albacore, the most abundant tuna species (Fig. 7.5; factor [day or night] in Table S1) except in the case of bluefin tuna in 2012 when more larvae were caught at night than during the day; in all other cases, factor (day or night) was not significant (Table S1, Fig. 7.5). The data for the other species were too limited to compare day *vs*. night distributions. The average size of the bluefin tuna and albacore larvae was very homogeneous (Table 7.1), so no ontogenetic variation in the vertical distribution could be analyzed.

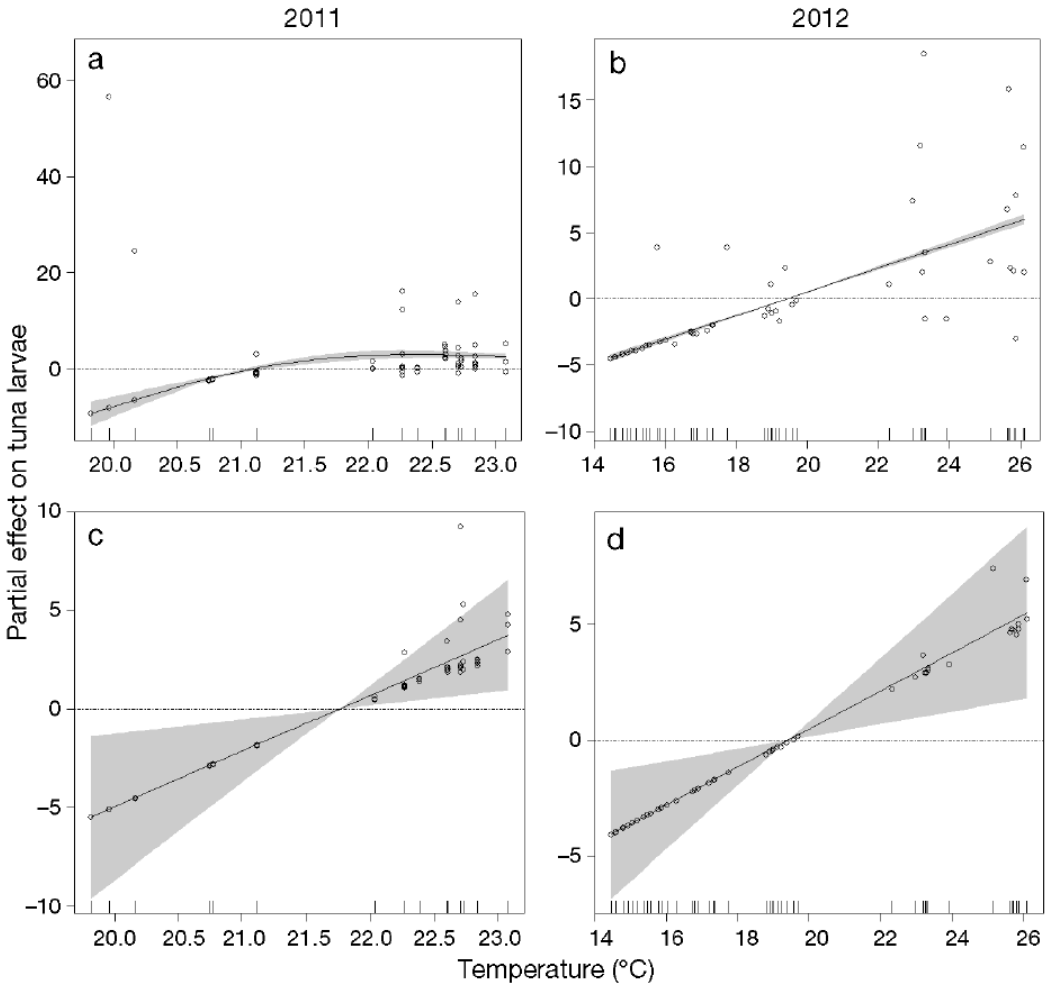


Figure 7.4. Model results of the significant partial effect of temperature on larval abundance of a) bluefin tuna collected from cruises in 2011 and b) 2012, and c) albacore in 2011 and d) 2012; y-axes: values below (above) 0 indicate a negative (positive) effect of the variable on the larval abundance. Fitted lines, 95% confidence intervals (grey shaded areas) and partial residuals (dots) are shown. Whiskers on x-axes: field observations for that covariate.

7.3.2 Experiments

Thermal stratified and isothermal conditions were successfully established and documented with vertical temperature profiles (Fig. 7.2). The number of larvae located above the thermocline in the water column during the thermocline treatment was significantly higher compared to the isothermal treatment, both during light and dark periods in both species (Table S2 in the Supplement, *p-adj.*< 0.01, Fig. 7.6). There was no significant difference in the position of the larvae between light and dark in the thermocline or the isothermal experiment in both species (Table S2, *p-adj.*>0.01, Fig. 7.6).

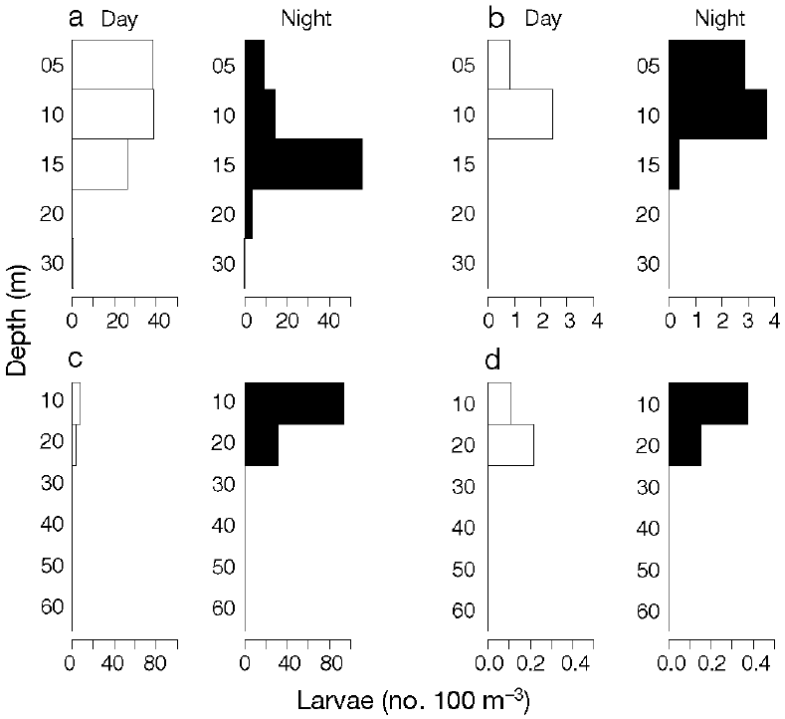


Figure 7.5. Vertical distribution of (a, c) *Thunnus thynnus* and (b,d) *T.alalunga* in (a, b) 2011 and (c, d) 2012 during daytime and night time. Note that the different axes for depth correspond to the different depth strata sampled in 2011 and 2012.

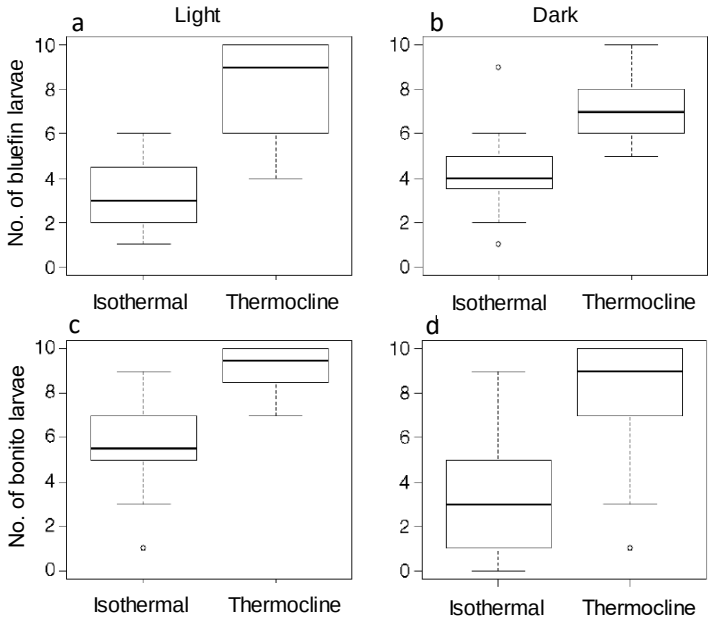


Figure 7.6. Number of larvae counted in the upper part of the isothermal and thermocline water experimental columns (top 34 cm of the water column) for a) light treatment in bluefin tuna, b) dark treatment in bluefin tuna, c) light treatment in bonito and d) dark treatment in bonito larvae. The black line is the median, the top and the bottom of the box represent the 75th and 25th percentiles, respectively, and the whiskers represent the maximum and minimum values. Outliers are shown by open circles.

7.4 Discussion

Tuna larvae showed a significant preference for the higher water temperatures found above the thermocline. Field data and laboratory experiments on Atlantic bluefin tuna and laboratory experiments on Atlantic bonito confirmed larval preference for warm waters above the thermocline, suggesting that temperature is a significant cue for the vertical distribution of these species during their early life. In the Balearic Islands, tunas spawn during summer when temperatures in the upper layers of the water column reach the minimum required for spawning. We observed that at 20–25 m depth, temperatures decreased below 20 °C, the minimum temperature reported to date for the presence of tuna larvae (Reglero *et al.*, 2014b). Atlantic bonito larvae have been reported at sea surface temperatures of 25.4 °C (± 0.58 °C SD) in coastal areas (Sabatés and Recasens, 2001) and between 24 and 26 °C in the open sea (Torres *et al.*, 2011).

The temperature-dependent behavior suggested by the field data for all tuna species is supported by the results obtained from our experiments, where Atlantic bluefin tuna and Atlantic bonito larvae were distributed significantly shallower in the stratified than in the isothermal experimental water columns both during light and dark periods. Therefore, the lowest thermal range for these species sets a natural boundary that confines the optimal vertical habitat for tuna and bonito larvae to the upper part of the water column of the Mediterranean Sea. The temperature-related behavior found in our study for Atlantic bluefin tuna has not been so clearly indicated in previous studies that have used samples taken in other geographic regions or other bluefin tuna species. Similar to our results for Atlantic blue fin tuna in the NW Mediterranean, the depth distribution of larvae of Pacific bluefin tuna in the NW Pacific Ocean ranges from 0 to 20 m, a distribution that has been related to the presence of a pycnocline (Satoh, 2010). Atlantic bluefin tuna larvae in the Gulf of Mexico are caught mostly in the first 20 m of the water column, although larvae have been found down to 50 m (Habtes *et al.*, 2014). The fact that Atlantic blue fin tuna larvae are sometimes found deeper than 20 m in the Gulf of Mexico but not in the other areas could be due to the sharper thermal gradients observed in the Mediterranean Sea and the Pacific Ocean than in the Gulf of Mexico (Llopiz and Hobday, 2015).

Larvae of all tuna species have been found to be distributed in the mixed layer and usually near the surface, being most abundant in the first 30 m of the water column (Boehlert and Mundy 1994; Davis *et al.*, 1990; Habtes *et al.*, 2014; Matsumoto, 1958; Satoh, 2010; Strasburg, 1960; Ueyanagi, 1969). Skipjack has been described as the tuna species with the deepest distribution, with larvae caught at 50 m (Davis *et al.*, 1990; Habtes *et al.*, 2014; Matsumoto, 1958), and even down to 80 m (Boehlert and Mundy 1994; Llopiz *et al.*, 2010). Little tunny has been found at 40 m and even 100 m depth in the Gulf of Mexico and waters off the coast of Florida (Habtes *et al.*, 2014; Llopiz *et al.*, 2010). In our study, we only caught 1 larva of skipjack tuna and 2 of little tunny, both in the first meters of the water column. These 2 species of tropical tuna are increasing in abundance in the NW Mediterranean, where they have been

periodically spawning in recent years (Saber *et al.*, 2015), and further studies are needed before definitive conclusions can be made regarding their larval vertical distribution. Albacore larvae in our study were clearly distributed in the upper 20 m of the water column. These 3 species, *e.g.* albacore, skipjack and little tunny, share tropical and temperate larval habitats (Reglero *et al.*, 2014b). Therefore, their vertical distribution may vary from being more widely distributed in areas with slight thermal gradients to being more narrowly distributed and restricted to shallower layers in areas with strong thermal gradients.

There is no common agreement on the diel vertical migration patterns of tuna larvae. Southern bluefin tuna and albacore in the East Indian Ocean show a shallower distribution during daytime than during the night (Davis *et al.*, 1990), but no evidence of daily migrations has been reported for albacore in the Indo-Pacific (Ueyanagi, 1969) or for Pacific bluefin tuna (Satoh, 2010). On the other hand, ontogenetic patterns in the vertical distribution of larvae of tuna species have never been reported. Pacific bluefin tuna larvae in the laboratory are able to change their density by inflating their swim bladder at night and deflating it during the day. The density of the larvae also increases with age (Takashi *et al.*, 2006). Night inflation of the swim bladder can prevent the larvae from sinking during the night while not swimming, thereby conserving energy (Hunter and Sanchez, 1976; Takashi *et al.*, 2006). In Atlantic bluefin tuna and southern bluefin tuna, the swim bladder is completely developed around 10 d after hatching (Woolley *et al.*, 2013; Yúfera *et al.*, 2014).

Our results regarding larval responses to temperature are strengthened by our use of both field and laboratory studies and the comparison of the results from both. Our design of the experimental thermocline used to test the effect of temperature gradients on the vertical distribution of tuna and bonito larvae is based on designs used in previous studies on cod and herring (Catalán *et al.*, 2011; Vollset *et al.*, 2009). The experimental work was designed so that only gradients of temperature were modified while all other variables (internal and external) were controlled. The thermal range that we used in the laboratory was adapted to that experienced by each species in the field and included the thermal tolerances for the 2 species. One substantial modification in relation to work by Vollset *et al.* (2009) was that the water columns were illuminated from behind instead of from above so that light levels were uniform in the water column. Due to obvious technical reasons, thermal gradients are by necessity steeper in the laboratory than in the field. Therefore, we have not compared the speed or actual distance covered by the larvae in the laboratory and the field. Our main result is that larvae control their vertical position to stay in the warmer upper water layers both in the experimental water column and in the pelagic environment. The larvae in the experimental columns were located significantly shallower in the stratified than in the isothermal experimental water columns, a result that matches results obtained from the field.

Temperature may act as a physiological barrier for the vertical distribution of larvae when gradients in the field are sharp, whereas larvae move to deeper depths in areas

where temperatures are homogeneously distributed, although always within the thermal tolerance range of the species. The vertical position within the depth range limited by the physio logical tolerance of the larvae to temperature involves trade-offs that could be explained by a thermo regulatory strategy whereby metabolism is reduced at cold temperatures and growth rates are increased at high temperatures (Urtizberea, 2009). In our experiments, all larvae were fed before the onset of the experiment, so results were independent of stomach fullness (Vollset *et al.*, 2013). However, the feeding state could also have an effect on the vertical temperature preference of larvae in the dark. Atlantic bluefin tuna and bonito larvae are visual predators that only feed during daylight and digest their food very fast (Chapter 5; Blanco *et al.*, 2017). Once the larvae empty their stomachs they could move down in the water column to colder temperatures during darkness to lower their metabolism until feeding recommences at daylight. However, we did not find any clear evidence of diel vertical migration in the field data. In the experimental columns, no significant differences were found in average larval positioning during periods of light (30–60 and 60–120 min after the beginning of the experiment in bluefin tuna and bonito, respectively) and darkness (105–135 and 195–255 min after the beginning of the experiment, respectively). On the other hand, laboratory experiments on larval Pacific bluefin tuna, Atlantic bluefin tuna and yellowfin tuna have shown increasing growth rates with increasing temperature (tested up to 28 °C) when food was provided *ad libitum* (Kimura *et al.*, 2010; Reglero *et al.*, 2018b; Wexler *et al.*, 2011). The same pattern is observed when analyzing data from different tuna species together (Reglero *et al.*, 2011). Therefore, by staying in the upper warmer layers, tuna larvae may grow faster compared to those located in the colder water temperatures, increasing survival if it relates to faster growth.

A global increase in temperature and major stratification of the water column is expected in the Mediterranean Sea with future climate changes (Coma *et al.*, 2009). The highest temperatures in the Mediterranean Sea measured during periods of spawning have not exceeded 28 °C, although higher temperatures, up to 30 °C, could be reached in the future if climate change follows expected trends. We have identified the minimum thermal constraint that influences the vertical range of tuna and bonito larvae, but less is known regarding maximum thermal constraints. In general, tuna species may have a higher tolerance for warmer waters and a lower tolerance for colder waters (Boyce *et al.*, 2008). The occurrence of tuna larvae in water temperatures up to 30 °C worldwide (Reglero *et al.*, 2014b) suggests that these species may adapt to temperatures higher than those observed at present in the Mediterranean Sea.

Larval fish behavioral responses observed under experimental conditions cannot be directly extrapolated to the field, but it is worth noting that they match well with the findings regarding tuna and bonito larval response to thermal gradients in their natural larval habitat. We found no correlation between the thermocline and the other environmental variables. In the Balearic Sea, variations in salinity are linked to the water mass defining frontal structures due to the confluence of recent Atlantic water and resident Atlantic water (Balbín *et al.*, 2014). However, our results showed

that variability in salinity over the water column was not important. We used chlorophyll as an indicator for food abundance. We found no correlation between food abundance and the thermo cline since most potential preys are located near the deep chlorophyll maximum, which occurs in this region and season between 50 and 70 m depth, far below the thermocline (Torres *et al.*, 2014). However, chlorophyll is not a good proxy for food availability for tuna larvae (Llopiz and Hobday, 2015), and therefore prey fields should be further investigated to include micro- and mesozooplankton.

We could not analyze the ontogenetic effect in the vertical distribution of the larvae, either in the field or in the laboratory, since the average length of the larvae caught in the field was very similar across species in both years. Smaller larvae could not be used in the experiments because they are difficult to detect visually, whereas large larvae were stressed when placed in the experimental water columns. The homogeneity of larval sizes in the field is attributable to our sampling strategy, since to ensure representativeness of samples, both night and day, we carried out an intensive sampling on single high-density larval patches, resulting from the spawning activity of adult schools in a given location over a short period of time, and hence most larvae were within restricted age/length ranges. Moreover, we expected little change to occur in the average body length over the sampling period, since the larvae could only be tracked over a few days each year. Aside from Atlantic bluefin tuna larvae, we found few larvae of other tuna species, and no Atlantic bonito larvae were caught. In general, few bonito larvae are caught in ichthyoplankton surveys in the region (Sabatés and Recasens, 2001; Torres *et al.*, 2011), despite the fact that *Sarda sarda* is one of the most abundant scombrid species in the Mediterranean Sea, with a spawning season occurring mainly in later spring and early summer (Macías *et al.*, 2005), a period which coincides with our surveys. Most likely, the low abundance of bonito larvae in the NW Mediterranean is in part due to their fast development, since bonito larvae reach the post-flexion stage only 8 d after hatching, at 21°C (Reglero *et al.*, 2014a, 2015a) and hence are only available for plankton net samplings during a very short time period each year.

Describing the distribution of critical habitats for the larval stage within the water column in relation to water temperature can provide essential information for introducing larval behavior into dispersal models and for understanding predator–prey interactions and the coexistence of fish species. Our results show that the vertical habitat of tuna and bonito larvae that spawn in temperate regions with strong thermal gradients is reduced to the first meters of the water column. Therefore, we expect that interactions between tuna species and their prey may be stronger in the first meters of the water column in the Mediterranean than in tropical areas with wider mixed layers due to physiological limitations forced by temperature. The dispersal of larvae towards nursery areas is expected to be strongly affected by processes at the surface of the water column in temperate areas.

The following supplement accompanies the chapter

Table S1. Model results of the relationship between the larvae of Atlantic bluefin tuna and albacore captured during ichthyoplankton surveys conducted in 2011 and 2012 with respect to temperature, salinity and chlorophyll. For each variable we included the probability (p), the estimated degrees of freedom (indicative of departure from linearity), the % of deviance explained and the unbiased risk estimator (UBRE score) of a model that contains the described variable in addition to all those preceding it in the table. The n.s. means no significant effect of the model variable in the tuna distribution.

Year	Species	Model variable	df	p	Dev explained cumulative	UBRE score
2011	Bluefin tuna	Factor(day:night)		n.s.		
		Temperature	2	<0.001	41.2	10.2
		Salinity	2	<0.001	45.8	9.4
		Chlorophyll		n.s.		
	Albacore	Factor(day:night)		n.s.		
		Temperature	1	0.007	41.6	-0.3
		Salinity		n.s.		
		Chlorophyll		n.s.		
2012	Bluefin tuna	Factor(day:night)		<0.001		
		Temperature	1	<0.001	71.1	37.7
		Salinity	2	<0.001	91.9	10
		Chlorophyll		n.s.		
	Albacore	Factor(day:night)		n.s.		
		Temperature	1	0.003	51.2	-0.5
		Salinity	1	0.003	78	-0.7
		Chlorophyll		n.s.		

Table S2. Generalized additive model results testing the effect of the thermocline and light:darkness treatments on the vertical distribution of bluefin tuna and bonito larvae in the experimental columns. For replicates description see material and methods. We applied a Bonferroni correction to adjust probability values for replicated trials and avoid type I error (*p-adj*, defined as probability divided by the number of test).

Species	Effect	Treatment	Replicate	Probability	*p-adj*
Bluefin tuna	Thermocline effect	Light	$I2_{bft}$	0.4	0.1
			$T1_{bft}$	<0.001	<0.001
			$T2_{bft}$	<0.001	<0.001
		Darkness	$I3_{bft}$	0.2	0.05
			$T3_{bft}$	0.002	0.0005
			$T4_{bft}$	0.004	0.001
	day:night	Thermocline	$T2_{bft}$	0.3	0.075
			$T3_{bft}$	0.5	0.125
			$T4_{bft}$	0.4	0.1
		Isocline	$I2_{bft}$	0.4	0.1
			$I3_{bft}$	0.02	0.005
			$I4_{bft}$	0.3	0.0750
Bonito	Thermocline effect	Light	$I2_{bon}$	0.2	0.05
			$T1_{bon}$	0.005	0.00125
			$T2_{bon}$	0.07	0.0175
		Darkness	$I3_{bon}$	0.5	0.125
			$T3_{bon}$	<0.001	<0.001
			$T4_{bon}$	<0.001	<0.001
	day:night	Thermocline	$T2_{bon}$	0.3	0.075
			$T3_{bon}$	0.2	0.05
			$T4_{bon}$	0.1	0.025
		Isocline	$I2_{bon}$	0.2	0.05
			$I3_{bon}$	0.002	0.0005
			$I4_{bon}$	<0.001	<0.001

CHAPTER 8

- GENERAL DISUSSION -

GENERAL DISCUSSION

8.1 Synthesis

Understanding the complexity of the processes that regulate growth and survival in larval fish is crucial. Field surveys provide key information to understand the relationship between the abundance and distribution of fish larvae and habitat variables. In addition, physiological and behavioral experiments under cultured controlled conditions, can provide knowledge on the mechanisms driving larval growth and survival, therefore broadening our view to understand the ecology, fisheries biology and aquaculture for the species.

This thesis throughout the different chapters (from Chapter 3 to 7) is a significant advance in the mechanistic knowledge of the effect that nutritional condition, diet, light, and temperature have in the growth and survival of Atlantic bluefin tuna and Atlantic bonito larvae using an experimental approach. The results are of value for aquaculture and stock advice. The relationship between the nutritional condition was analysed in relation to the flexion of the notochord to explain how larvae from the same cohort reach flexion at different timings despite being cultured under the same conditions (Chapter 3). The effect of the diet was examined looking at the size spectrum of the larvae that will be more affected by a manipulation of the timing of onset of piscivory (Chapter 4) and also looking at the effect that a change in the feeding regime once piscivory was well set, from continuous to intermittent feeding, have in the larval growth and survival (Chapter 5). The effect of light was analysed in Chapter 6 estimating the metabolic cost (oxygen consumption) of Atlantic bluefin tuna larvae during light and darkness. Finally, the effect of temperature as major drive of the fish larval vertical distribution was analysed in Chapter 7.

Piscivory, when eating larval prey, is widespread during the larval stage within Scombridae (Chapter 4, 5 and 6; Kaji *et al.*, 2002; Ortega, 2015; Sawada *et al.*, 2005). In the field, it has been hypothesized that it may reduce starvation improving the survival of the larvae in oligotrophic ocean areas although it may also be a substantial mortality component depending on the availability of alternative prey (Reglero *et al.*, 2011). In this thesis it is shown that under cultured conditions, piscivory increases fish larval growth and survival (Chapter 4; Reglero *et al.*, 2014a; Tanaka *et al.*, 2014a). In Atlantic bluefin tuna and in Atlantic bonito, the start of piscivory coincides with the flexion of the notochord (Chapter 3; De la Gándara *et al.*, 2012; Ortega, 2015; Reglero *et al.*, 2014a). However, before the reach of the piscivory or complete flexion stage, the larvae must suffer a high rate of changes during the stage where mortalities are size-dependent (Kendall *et al.*, 1984; McGurk, 1986). Until the complete flexion, the larvae can be classified into four stages of life according to morphological aspects and length intervals: yolk-sac larvae (up to 3 mm), larvae in notochord pre-flexion, first caudal fin formation, flexion and post-flexion (De la Gándara *et al.*, 2013; Kaji *et al.*, 1996; Kendall *et al.*, 1984). Reaching the flexion as soon as possible can be considered beneficial for the growth and survival of the larvae, since improves swimming capacity and feeding techniques,

and would probably determine which larvae reach the next crucial point, piscivory. Growth rate, physiology, and foraging capabilities often change with the shift from a planktivorous to a piscivorous diet (Kaji *et al.*, 2002; Tanaka et al., 2014a, 2014b). The nutritional condition might be though to be a possible explanation since it reflects the energy reserves available for maintenance, growth and activity and the individual responses to variability in food supply and feeding success are ultimately related to survival and growth. In Chapter 3, nutritional condition of fish larvae was examined by the RNA:DNA and the DNA:dry weight ratios. The nutritional condition as RNA:DNA ratio of Atlantic bluefin tuna and Atlantic bonito larvae, reared at optimal feeding conditions (*ad libitum*) and constant temperature, salinity and light, was proportional to differences in their growth in dry weight. However, RNA:DNA ratio did not explain differences among the different developmental stages (pre-flexion to post-flexion). In Atlantic bluefin tuna the DNA:dry weight ratio reflected differences among the developmental stages. At any given larval size, DNA:dry weight ratio showed the worst cell condition in post-flexion larvae, compared to those in flexion in Atlantic bluefin tuna. In Atlantic bonito, no such relation with the different stages was seen. Under cultured conditions, as seen in our results, feeding condition might not be enough to explain developmental differences in Atlantic bluefin tuna and Atlantic bonito larvae. The explanation of growth differences and developmental rates in those fast-growing fish larval species might be a difficult to identify since short time intervals separate different stages and differences in growth strategies between individuals (Juan-Jordá *et al.*, 2013).

The accomplishment of the complete notochord flexion and the fact of being ready (morphological and physiologically) will be considered beneficial for the growth and survival of the larvae as long as there is availability of the pisicivorous preys. The overlap of larvae and their prey is considered the most important factor affecting whether fish would have a strong or weak year class (Cushing, 1990). When culturing tuna, one of the most important issues faced is when to start piscivory feeding. In Atlantic bluefin tuna and in Atlantic bonito larvae, the switch from a planktivorous to a piscivorous diet it has been suggested as a second critical window, with an early transition benefiting larval growth and survival (Reglero *et al.*, 2014a). Since mortality during the larval stage of fish can be size dependent (McGurk, 1986), empirical evidence of the survival size spectrum is particularly important in order to determine the survival strategy of the species and the contribution to the life stages afterwards. In Chapter 4 the effect that the different timing in the onset of piscivory has on the growth of the population and on the survival of the different size classes of laboratory-reared Atlantic bluefin tuna using otolith microstructure analysis was analyzed. The results show size-selective mortality of the largest larvae independently of the timing of onset of piscivory. The selective mortality operated against the largest individuals in the original population and as a result, the population of survivors was primarily composed of the smallest individuals that were present in the original population. Therefore, being bigger it is not always the best option to survive and mortality rates do not always decline in culture conditions with increasing larval size. The mortality of the large larvae along with the rapid response of piscivory in terms of growth reflects the fact that there is a cost to fast growth. A

delay in the onset of piscivory under certain culture conditions might promote the growth of the smaller larvae, as seen in our results.

In the field, it has been recently proved that Atlantic bluefin tuna adults spawning phenology is driven to matching offspring with ocean productivity and the prey peak (Reglero *et al.*, 2018b). However, in the field, preys may be difficult to encounter because it is assumed they are patchy distributed (Owen, 1989). Predators might not have always the patch to feed, which might not be a big problem for their growth and survival in the sea (Cushing, 1990). Nevertheless, in culture conditions fishes are fed *ad libitum* with preys (always enough preys in the tanks) and long light photoperiod regimes are used to maximize ingestion rates and therefore growth (*e.g.* Hart *et al.*, 1996; Puvanendran and Brown, 2002, Shi *et al.*, 2010) because most fish larvae are visual feeders and dependent on light to increase their feeding (Hunter, 1980). Continuous feeding is especially expensive during the pisicivorous stage of the larvae since auxiliary adult breeding stock is need in order to produce enough yolk-sacs to use as preys (De la Gándara *et al.*, 2016; Sawada *et al.*, 2005). From the patchy field feeding thinking point of view, in Chapter 5 it was tested if intermittent feeding could provide a viable alternative to the commonly used continuous feeding regime in Atlantic bluefin tuna and Atlantic bonito aquaculture. The results showed that alternating feeding regimes can enhance larval growth and maintain or improve survival rates, compared to continuous feeding. Besides, stomach content analysis revealed that the larvae only fed during the light hours and stopped feeding during darkness. Therefore, alternating light regimes may be a good alternative in intensive cultures; reducing rearing cost due to the reduction of the timing of feeding and lighting, especially when rearing piscivorous species whose diet in that critical developmental stage is not easy to obtain due a dependency on available fish larvae (Sawada *et al.*, 2005). The option to feed at certain time intervals may prevent them from becoming satiated and then increase their total ingestion, per unit time of active foraging. This leads to more efficient use of the available food. In addition, our results suggest that larvae may survive to short periods of starvation in the field if food is distributed in patches. Our optimal growth results support the idea that larvae do not need to feed constantly to maximize growth rates.

In darkness fish larvae are assumed to reduce their swimming and stop feeding (visual feeders) and as a consequence, fish larval metabolic expenses might be lowered. However, to date, no information exists on the important influence of light on the metabolic rate of the early stage of Atlantic bluefin tuna. In Chapter 6 the first respiration data in Atlantic bluefin tuna piscivorous larvae was obtained by taking measurements in light and darkness conditions. The oxygen consumption of piscivorous Atlantic bluefin tuna larvae from 17 to 30 dph was similar in light and darkness, which suggests similar swimming behavior and oxygen expenses. Inside a close chamber, with light conditions but without preys and foraging behavior, the larvae might have regulated (decrease) their swimming behaviour and oxygen consumption to reduce the cost of activity to find a balance between the lack of energy consumption and activity expenses which result in similar metabolic rates during light and darkness. Oxygen consumption increased isometrically with dry weight,

being the mass scaling exponent 1.0 in larvae weighted between 0.6 to 23 mg. The relationship is higher than the average of other species which is probably caused by the high oxygen consumption demand caused by the fast growth of the species adapted at high temperature (26 ºC) and under a piscivorous diet. The nutritional condition as RNA:DNA ratio was proportional to the larval growth in dry weight but did not explain differences between light and darkness measurements. However, the more sensitive ration DNA:dry weight, showed a correlation between larvae sampled in light and darkness and a decrease in the condition of the larvae in darkness. No relationship was found between the oxygen consumption and fish larval nutritional condition after deleting the size effect, suggesting the importance of the size in the response of this species.

Describing the distribution of critical habitats for the larval stage within the water column in relation to water temperature can provide essential information for introducing larval behavior into dispersal models and for understanding predator-prey interactions and the coexistence of fish species. Tuna larvae showed a significant preference for the higher water temperatures found above the thermocline (Chapter 7). Field data and laboratory experiments on Atlantic bluefin tuna and laboratory experiments on Atlantic bonito confirmed larval preference for warm waters above the thermocline, suggesting that temperature is a significant cue for the vertical distribution of these species during their early life. In the Balearic Islands, tunas spawn during summer when temperatures in the upper layers of the water column reach the minimum required for spawning. It was observed that at 20–25 m depth, temperatures decreased below 20 °C, the minimum temperature reported to date for the presence of tuna larvae (Reglero *et al.*, 2014a). Atlantic bonito larvae have been reported at sea surface temperatures of 25.4 ± 0.58 °C in coastal areas (Sabatés and Recasens, 2001) and between 24 and 26 °C in the open sea (Torres *et al.*, 2011).

8.2 Contribution of this thesis to the state of the art on the biology and aquaculture of these species

The results of the present thesis demonstrate the importance of understanding the complexity of the processes that regulate growth and survival during the larval stage of these species, which to date were not well known. Behavioral and physiological responses of fish larvae to variability in environmental conditions are central to determine demographic processes at population level. Although field surveys provide key information to understand the relationship between the abundance and distribution of fish larvae and habitat variables, they are less suitable to provide mechanistic explanations (Horodysky *et al.*, 2015Margulies). Conclusions based only on survey data could lead to biased inferences on habitat requirements changes in stock size occurring over time if mechanisms underlying distributional and abundance dynamics are not explicitly understood.

Despite recent advances, we still had limited knowledge of the early life stages of both Atlantic bonito and Atlantic bluefin tuna. Only few Atlantic bonito larvae have

ever been captured in the field and large Atlantic bluefin tuna larval sizes are usually under-represented due to net avoidance. On the other hand, field-captured larvae may be difficult to identify to species level and the capacity to conduct physiological measurements onboard is very limited. One example of uncompleted information from the field is the lack of evidence of piscivory in Atlantic bluefin tuna captured in the Mediterranean Sea. Only recently, piscivory has been first identified in Atlantic bluefin tuna larvae (Uriarte *et al.*, 2017) whereas piscivory is long recognized a major process influencing larval growth and survival in laboratory studies (Reglero *et al.*, 2011, 2014a; Tanaka *et al.*, 2014a, 2014b).

This thesis contributes to the state of the art of field environment, experimental and aquaculture of both species (Figure 8.1). This thesis is an advance in the experimental and field science since it provides mechanistic knowledge that describe growth as a function of temperature and diet (Chapters 3-5); mortality, as a function of larval size and diet (Chapter 4); feeding patterns throughout the day with evidence of visual feeding (Chapter 5) and metabolism in relation to larval size, measured for the first time in Atlantic bluefin tuna (Chapter 6). Particular examples of direct applications of experimental results to the field are shown in Chapter 7 where the vertical distribution of tuna larvae has been explained by their thermal tolerance combining laboratory and field data. More applications are envisioned for the future such as including the transition from plantivory to piscivory as a bottleneck in early life growth and survival and therefore recruitment. For the two species this thesis provides knowledge on basic ecology that can be used to disentangle the effects that environmental variables have on specific vital rates of Atlantic bluefin tuna and Atlantic bonito larvae.

This thesis can have an impact in the aquaculture sector. Atlantic bluefin tuna and Atlantic bonito are the only two scombrids that are cultured in captivity in the Mediterranean Sea. The development of successful mass culture for the two species has been very challenging due to high mortality during the larval stage. Through several chapters of this thesis we have shown that cultured larvae that are fed *ad libitum* are in good nutritional condition. Therefore, this thesis supports the lack of malnutritional larvae feeding on the proposed diets few days after first feeding. This thesis also shows light hours and timing of feeding can be modified without influencing larval growth (Chapter 5) resulting in reduced rearing costs. Finally, this thesis describes the best timing to change from a planktivorous to a piscivirous diet which can be used to avoid cannibalism (Chapter 3). Besides, new methodological approaches have been used in this thesis to study larval growth and survival in cultured condition. Otolith microstructure analyses and biochemical analyses of condition are widely used in field studies but this is the first attempt to use them in these two species in captivity. Still, there is a long way forward since survival rates in captive conditions are still very low, ranging from 0.01% to 4.5% (Masuma *et al.*, 2008). In order to improve the aquaculture industry, further emphasis should be directed towards investigating larviculture of both species.

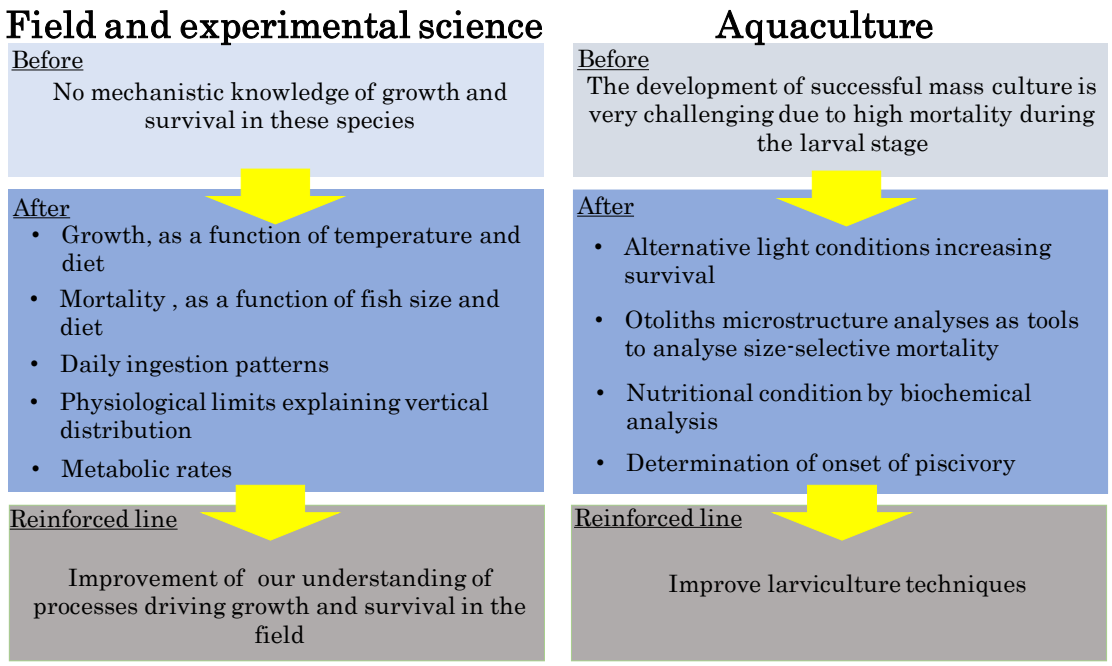


Figure 8.1. Schematic showing how this thesis has the potential to affect field and experimental science and aquaculture of Atlantic bonito and Atlantic bluefin tuna.

8.3 The difficulty of working with these species

The culture of these species is a very difficult task and so it is the experimental procedures. Atlantic bluefin tuna and Atlantic bonito have a very narrow environmental window for spawning (Reglero *et al.*, 2018b) which reduces the days available to work with these species. Spawning a large amount of eggs does not guarantee the ability to carry out experiments every year.

To date, the only option of obtaining Atlantic bluefin tuna eggs is from naturally spawning captive adults in sea cages. During the spawning season every day at least two workers moved to the cages by boat. Therefore, weather conditions sometimes prevent eggs collection from the cages (Gordoa and Carreras, 2014). Additionally, despite being buoyant, some eggs can be caught in transverse currents and be transported under the curtain and out of the cage (De la Gándara *et al.*, 2016; Reglero *et al.*, 2013). Only when enough eggs are collected then the experiments can be conducted. Ocasionally, eggs from other fish species may be collected together with Atlantic bluefin tuna eggs being difficult to distinguish them until the development of the embryo. After the eggs are obtained, critical issues that contributed to satisfactory egg viability after shipment included pre-packing temperature, insulation of transport boxes, and minimizing transit time (De la Gándara *et al.*, 2010). Even though the facility in Mazarrón is relatively close to the breeding cages (2 hours), critical issues may still happen. In 2016 juvenile tuna were introduced into recently built inland tanks that are expected to reach maturity after 4-5 years and could provide eggs.

Atlantic bonito eggs were collected from adults raised in the facility in 2013. Afterwards, after the dead of the adults, only in 2016 we were able to successfully fertilize AB eggs stripping adults captured from the almadraba trap in La Azohía (Cartagena). Stripping has several handicaps. The collaboration of fishermen is needed, enough mature adults of both sexes must be collected to ensure good egg quality and fertilization needs to be successful. As well as for ABFT, shipment to the facility might be a source of mortality. The obtention of enough viable eggs to carry out experiments might be an important limiting factor.

The culture of these species still fluctuated due to several unresolved confined problems that caused high mortalities, such as surface tension-related death and death at the bottom of the rearing tank by larval sinking, up to 10 days after hatching (De la Gándara *et al.*, 2012; Masuma *et al.*, 2011). Once they reach the post-flexion stage, there are two major causes of mortality. One is cannibalism and the other is collision with the rearing tank wall and net cages (Masuma *et al.*, 2011; Sawada *et al.*, 2005). Feeding transition phases generate two bottlenecks in the survival of both species (Reglero *et al.*, 2014a). These larvae are very sensitive and complicated for experimental work and the facilities of Mazarrón are unique worldwide in the culture of these species.

8.4 Future perspectives

Chapter 3. If the nutritional condition of the larvae does not show any specific pattern in growth differences during the larval stage other information is need and other aspects as energy consumption (*e.g.* swimming, specific dynamic action) should be investigated in order to better understand the larval species-specific strategies.

Chapter 4. Mortality during the larval stage is assumed to be size-dependent. In our study we found size selective mortality of the largest larvae during the piscivorous stage. However, size-selective mortality can vary depending on the larval stage and conditions. Further research will allow to determine the type of mortality and possible reasons affecting the earlier and alter stages in order to integrate all survival data to obtain more precise recruitment estimation.

Chapter 5. The specific optimum light/feeding regimes may be species-stage dependent. Manipulation of the photoperiod can affect feed-conversion ratios or efficiencies, demonstrating the importance of investigating the effect of photoperiod on feeding efficiency. The effect of endocrine factors on the appetite and growth of fish larvae should also be studied in the future. These differences in energy expenditure should be considered in further research before applying the results to the field.

Chapter 6. There is a need of empirically study how the oxygen measurements are being affected by the respirometry chambers, and how a conversion to the field is possible to do. Measures of swimming activity are therefore the next step.

Chapter 7. The results show that the vertical habitat of tuna and bonito larvae that spawn in temperate regions with strong thermal gradients is reduced to the first meters of the water column. Therefore, interactions between tuna species and their prey may be stronger in the first meters of the water column in the Mediterranean than in tropical areas with wider mixed layers due to physiological limitations forced by temperature. Besides, the dispersal of larvae towards nursery areas is expected to be strongly affected by processes at the surface of the water column in temperate areas. Further research is need in order to study fish larval prey-predator interactions in the first meters of the water column which could give us an answer of the survival strategy of bluefin tuna and bonito with predator interaction involved. Also, the data obtained should be used in dispersal predicted studied.

CONCLUDING REMARKS

CONCLUDING REMARKS

1. Size and not age is a good proxy for morphological development of Atlantic bluefin tuna and Atlantic bonito larvae, since it was found an overlap in the age at which different developmental stages were observed while there was no significant overlap in the larval length and weight among developmental stages. Growth differences are therefore apparent during the planktivorous phase of Atlantic bluefin tuna and Atlantic bonito larvae cultured under the same conditions, of the same age and with *ad libitum* conditions.

2. Under culture conditions, where fish larvae are fed *ad libitum*, the nutritional condition determined by the analysis of the nucleic acid, does not show differences between faster and slower growing individuals. The explanation of growth differences and developmental rates in those fast-growing fish larval species might be a difficult to identify since short time interval separate different stages and differences in growth strategies between individuals.

3. The nucleic acid ratios analysis show two different growth mechanisms of the cells between Atlantic bluefin tuna and Atlantic bonito larvae. From sizes between 5.1 ± 0.5 to 7.5 ± 0.6 mm in standard length, Atlantic bluefin tuna larvae switch from a higher proportion of hyperplasia (cell proliferation) to a higher proportion of hypertrophy (cell enlargement). However Atlantic bonito larvae from 5.7 ± 0.3 to 8.2 ± 0.3 mm standard length cell growth mechanism might be predominantly by continued hypertrophy. Based on those results, further research should be done in order to understand the rapid growth rate of bonito larvae compared to bluefin tuna whose larval stages share big morphological and feeding regimes similarities.

5. Daily increment formation of Atlantic bluefin tuna larvae from 15 to 27 days post hatch (dph) regardless of the diet compositions and growth rate has been validated for the first time at 25 ºC.

6. By the analysis of the otolith back-calculation, size selective mortality of the largest larvae was discovered independently of the timing to onset of piscivory. Being bigger might not be always the best strategy to survive and mortality do not always decline in culture conditions with increasing larval size in Atlantic bluefin tuna larvae. Smaller size at a given age could under certain conditions and stages of development confer a survival advantage of individuals members of a larval cohort when suitable small-sized prey is available.

7. Stomach content analysis of Atlantic bluefin tuna piscivorous larvae showed a continuous feeding in light and a clear interruption of feeding in darkness, which confirms their visual feeding mechanism. Evacuation time of a minimum of 1.5 hours was found, short evacuation time that might be caused by the high digestion capacity of the complete stomach when initiating piscivory and by the digestibility of preys. Besides a trend of increasing food consumption at the end of the day was observed.

8. Intermittent feeding can enhance larval growth and maintain or improve survival rates compared to continuous feeding. It may have a beneficial effect on ingestion rates, possibly because feeding is less satiation-limited, metabolic costs are lower or because food digestion is more efficient under these conditions.

9. The oxygen consumption of piscivorous Atlantic bluefin tuna larvae from 0.6 to 23 mg (17 to 30 dph at 26 ºC) was similar in light and darkness, which suggests similar swimming behavior and oxygen expenses. Inside a close chamber, with light conditions but without preys and foraging, the larvae might have regulated (decreased) their swimming activity with the consequent decrease in the oxygen consumption to reduce the cost of activity to find a balance between the lack of energy consumption and activity expenses which result in similar metabolic rates during light and darkness.

10. Oxygen consumption increased isometrically with dry weight, being the mass scaling exponent being 1.0 in larvae weighted between 0.6 to 23 mg. The relationship is higher than the average of other species which is probably caused by the high oxygen consumption demand caused by the fast growth of the species adapted at high temperature (26 ºC) and under a piscivorous diet.

11. As confirmed from field and laboratory data, Atlantic bluefin tuna and Atlantic bonito larvae showed a temperature-dependent behavior with a significant preference for the higher water temperatures found above the thermocline. This result suggest that temperature is a significant cue for the vertical distribution of these species during their early life and that the vertical distribution of both species is spatially constrained by larval temperature tolerance.

12. No differences in the day and night distribution were observed for tuna species. This outcome evidence the lack of daily migration of tuna species in the Mediterranean Sea. Experimental studies help us to understand the mechanistic processes that might explain the relationship observed between temperature and larval vertical distribution obtained in the field.

REFERENCES

REFERENCES

Adachi, K., Kato, K., Yamamoto, M., Ishimaru, K., Kobayashi, T., Murata, O., Kumai, H., 2008. Pulsed expression of growth hormone mRNA in the pituitary of juvenile Pacific bluefin tuna under aquacultured conditions. Aquaculture 281(1), 158–161.

Alemany, F., 1997. Ictioplancton del Mar Balear. Ph.D. thesis, University of Illes Balears, Palma de Mallorca.

Alemany, F., Quintanilla, L., Velez-Belchí, P., García, A., Cortés, D., Rodríguez, J.M., Fernández de Puelles, M.L., González-Pola, C., López-Jurado, J.L., 2010. Characterization of the spawning habitat of Atlantic bluefin tuna and related species in the Balearic Sea (western Mediterranean). Prog. Oceanogr. 86(1–2), 21–38.

Anderson, J.T., 1988. A review of size dependent survival during pre-recruit stages of fishes in relation to recruitment. J. Northwest Atl. Fish. Sci. 8, 55–66.

Anonymous, 2007. Proposal for a Council Regulation amending Council Regulation (EC) No. 41/2007 as concerns the recovery plan for bluefin tuna recommended by the International Commission for the Conservation of Atlantic Tunas.

Anonymous, 2017. Report of the 2017 ICCAT bluefin tuna data preparatory meeting. Collective Volume of Scientific Papers ICCAT 74, 2268-2371.

Aranda, G., Abascal, F.J., Varela, J.L., Medina, A., 2013 Spawning behaviour and post-spawning migration patterns of Atlantic bluefin tuna (*Thunnus thynnus*) ascertained from satellite archival tags. PLoS ONE 8, e76445.

Auer, S.K., Salin, K., Anderson, G.J., Metcalfe, N.B., 2015. Aerobic scope explains individual variation in feeding capacity. Biology letters 11(11), 20150793.

Bailey, K.M., Houde, E.D., 1989. Predation on eggs and larvae of marine fishes and the recruitment problem. Adv. Mar. Biol. 25, 1–83.

Balbín, R., López-Jurado, J.L., Flexas, M.M., Reglero, P., Vélez-Velchí, P., González-Pola, C., Rodríguez, J.M., García, A., Alemany, F., 2014. Interannual variability of the early summer circulation around the Balearic Islands: driving factors and potential effects on the marine ecosystem. J. Marine Syst. 138, 70–81.

Benetti, D.D., Partridge, G.J., Stieglitz, J., 2016. Overview on status and technological advances in tuna aquaculture around the world. In: Benetti, D.D., Partridge, G.J., Buentello, A. (Eds.), Advances in Tuna Aquaculture. From Hatchery to Market. Academic press, London, pp. 1–19.

Bergeron, J.P., 1997. Nucleic acids in ichthyoplankton ecology: a review, with emphasis on recent advances for new perspectives. J. Fish. Biol. 51(sA), 284-302.

Billerbeck, J.M., Lankford Jr, T.E., Conover, D.O., 2001. Evolution of intrinsic growth and energy acquisition rates. I. Trade-offs with swimming performance in *Menidia menidia*. Evolution 55(9), 1863–1872.

Björnsson, B.T., Johansson, V., Benedet, S., Einarsdottir, I.E., Hildahl, J., Agustsson, T., Jönsson, E., 2002. Growth hormone endocrinology of salmonids: regulatory mechanisms and mode of action. Fish Physiol. Biochem. 27(3–4), 227–242.

Blanco, E., Reglero, P, Ortega, A., de la Gándara, F., Fiksen, Ø., Folkvord, A., 2017. The effects of light, darkness and intermittent feeding on the growth and survival of reared Atlantic bonito and Atlantic bluefin tuna larvae. Aquaculture 479, 233–239.

Blanco, E., Reglero, P., Ortega, A., De la Gándara, F., Folkvord, A., 2018. Size-selective mortality of laboratory-reared Atlantic bluefin tuna larvae: evidence

from microstructure analysis of otoliths during the piscivorous phase. Journal of Experimental Marine Biology and Ecology 509, 36-43.

Blank, J.M., Morrissette, J.M., Farwell, C.J., Price, M., Schallert, R.J., Block, B.A., 2007. Temperature effects on metabolic rate of juvenile Pacific bluefin tuna *Thunnus orientalis*. J. Exp. Biol. 210(23), 4254–4261.

Blank, J.M., Morrissette, J.M., Landeira-Fernandez, A.M., Blackwell, S.B., Williams, T.D., Block, B.A., 2004. In situ cardiac performance of Pacific bluefin tuna hearts in response to acute temperature change. J. Exp. Biol. 207(5), 881-890

Block, B.A., Dewar, H., Blackwell, S.B., Williams, T.D., Prince, E.D., Farwell, C.J., Boustany, A., Teo, S.L., Seitz, A., Walli, A., Fudge, D., 2001. Migratory movements, depth preferences, and thermal biology of Atlantic bluefin tuna. Science 293(5533), 1310–1314.

Block, B.A., Teo, S.L., Walli, A., Boustany, A., Stokesbury, M.J., Farwell, C.J., Weng, K.C., Dewar, H., Williams, T.D., 2005. Electronic tagging and population structure of Atlantic bluefin tuna. Nature 434(7037), 1121.

Boehlert, G.W., Mundy, B.C., 1994. Vertical and onshore–offshore distributional patterns of tuna larvae in relation to physical habitat features. Mar. Ecol. Prog. Ser. 107, 1–13.

Boustany, A.M., Reeb, C.A., Block, B.A., 2008. Mitochondrial DNA and electronic tracking reveal population structure of Atlantic bluefin tuna (*Thunnus thynnus*). Mar. Biol. 156(1), 13–24.

Boyce, D.G., Tittensor, D.P., Worm, B., 2008. Effects of temperature on global patterns of tuna and billfish richness. Mar. Ecol. Prog. Ser. 355, 267–276.

Brett, J.R., Groves, T.D.D., 1979. Physiological energetics. In: Hoar, W.S., Randall, D.J., Brett, J.R. (Eds.), Fish Physiology, Vol.8. New York, NY: Academic Press, pp. 279–352.

Brill, R.W., 1979. Effect of Body Size on the Standard Metabolic-Rate of Skipjack Tuna, *Katsuwonus pelamis*. Fish. Bull. 77(2), 494–498.

Brill, R.W., 1987. On the standard metabolic rates of tropical tunas, including the effect of body size and acute temperature change. Fish. Bull. 85(1), 25–35.

Brothers, E.B., Prince, E.D., Lee, D.W., 1983. Age and growth of young-of-the-year bluefin tuna, *Thunnus thynnus*, from otolith microstructure. NOAA Tech. Rep. NMFS 8, 49-59.

Brown, J.A., Wiseman, D., Kean, P., 1997. The use of behavioural observations in the larviculture of cold-water marine fish. Aquaculture 155(1), 297-306.

Buckley, L.J., 1984. RNA-DNA ratio: an index of larval fish growth in the sea. Mar. Biol. 80, 291–298.

Buckley, L., Caldarone, E., Ong, T.L., 1999. RNA–DNA ratio and other nucleic acid-based indicators for growth and condition of marine fishes. Hydrobiologia 401, 265–277.

Buckley, L.J., Turner, S.I., Halavik, T.A., Smigielski, A.S., Drew, S.M., Laurence, G.C., 1984. Effects of temperature and food availability on growth, survival, and RNA-DNA ratio of larval sand lance (*Ammodytes americanus*). Mar. Ecol. Prog. Ser. 15(1), 91–97.

Buentello, J.A., Pohlenz, C., Margulies, D., Scholey, V.P., Wexler, J.B., Tovar-Ramírez, D., Neill, W.H., Hinojosa-Baltazar, P., Gatlin, III, D.M., 2011. A preliminary study of digestive enzyme activities and amino acid composition of early juvenile yellowfin tuna (*Thunnus albacares*). Aquaculture 312(1–4), 205–211.

Caldarone, E.M., Clemmesen, C.M., Berdalet, E., Miller, T.J., Folkvord, A., Holt, G. J., Olivar, M.P., Suthers, I.M., 2006. Intercalibration of four

spectrofluorometric protocols for measuring RNA/DNA ratios in larval and juvenile fish. Limnol. Oceanogr.: Methods 4(5), 153–163.

Campana, S.E., 1990. How reliable are growth back-calculations based on otoliths?. Can. J. Fish. Aquat. Sci. 47(11), 2219–2227.

Campana, S.E., 1999. Chemistry and composition of fish otoliths: pathways, mechanisms and applications. Mar. Ecol. Prog. Ser. 188, 263–297.

Campana, S.E., Jones, C.M., 1992. Analysis of otolith microstructure data. In: Stevenson, D.K., Campana, S.E. (Eds.), Otolith microstructure examination and analysis. Can. Spec. Publ. Fish. Aquat. Sci. Department of Fisheries and Oceans, Ottawa, 117, pp. 73–100.

Cañavate, J.P., Zerolo, R., Fernández-Díaz, C., 2006. Feeding and development of Senegal sole (*Solea senegalensis*) larvae reared in different photoperiods. Aquaculture 258(1), 368–377.

Catalán, I.A., Tejedor, A., Alemany, F., Reglero, P., 2011. Trophic ecology of Atlantic bluefin tuna *Thunnus thynnus* larvae. J. Fish Biol. 78(5), 1545–1560.

Catalán, I.A., Vollset, K.W., Morales-Nin, B., Folkvord, A., 2011. The effect of temperature gradients and stomach fullness on the vertical distribution of larval herring in experimental columns. J. Exp. Mar. Biol. Ecol. 404, 26–32.

Carlsson, J., McDowell, J.R., Carlsson, J.E.L., Graves. J.E., 2007. Genetic identity of YOY bluefin tuna from the eastern and western Atlantic spawning areas. J. Heredity 98(1), 23-28.

Cermeño, P., Quílez-Badia, G., Ospina-Alvarez, A., Sainz-Trápaga, S., Boustany, A. M., Seitz, A.C., Tudela, S., Block, B.A., 2015. Electronic tagging of Atlantic bluefin tuna (*Thunnus thynnus*, L.) reveals habitat use and behaviors in the Mediterranean Sea. PLoS ONE 10(2), e0116638.

Chabot, D., Steffensen, J.F., Farrell, A.P., 2016. The determination of standard metabolic rate in fishes. J. Fish Biol. 88(1), 81–121.

Chambers, R.C., Miller, T.J., 1995. Evaluating fish growth by means of otolith increment analysis: Special properties of individual-level longitudinal data. In: Secor, D.H., Dean, J.M., Campana, S.E. (Eds.), Recent Developments in Fish Otolith Research. University of South Carolina Press: Colombia, SC, pp. 155–175.

Chícaro, M.A., 1997. Starvation percentages in field caught Sardina pilchardus larvae off southern Portugal. Sci. Mar. 61, 507–516.

Chícharo, L., Chícharo, M.A., 1995. The RNA:DNA ratio as a useful indicator of the nutritional condition in juveniles of *Ruditapes decussatus*. Sci. Mar. 59 Suppl. 1, 95–101.

Chícharo, M.A., Chícharo, L., 2008. RNA:DNA ratio and other nucleic acid derived indices in marine ecology. Int. J. Mol. Sci. 9(8), 1453–1471.

Chícharo, L., Chícharo, M.A., Alves, F., Amaral, A., Pereira, A., Regala, J., 2001. Diel variation of the RNA/DNA ratios in *Crassostrea angulata* (Lamarck) and *Ruditapes decussatus* (Linnaeus 1758) (Mollusca: Bivalvia). J. Exp. Mar. Biol. Ecol. 259(1), 121–129.

Chícharo, M.A., Chícharo, L., Valdés, L., López-Jamar, E., Ré, P., 1998. Estimation of starvation and diel variation of the RNA/DNA ratios in field-caught *Sardina pilchardus* larvae off the north of Spain. Mar. Ecol. Prog. Ser. 273–283.

Clarke, A., Johnston, N.M., 1999. Scaling of metabolic rate with body mass and temperature in teleost fish. J. Anim. Ecol. 68(5), 893–905.

Clemmesen, C.M., 1987. Laboratory studies on RNA/DNA ratios of starved and fed herring (*Clupea harengus*) and turbot (*Scophthalmus maximus*) larvae. ICES J. Mar. Sci. 43(2), 122–128.

Clemmesen, C., 1994. The effect of food availability, age or size on the RNA/DNA ratio of individually measured herring larvae: laboratory calibration. Mar. Biol. 118(3), 377–382.

Collette, B.B., Reeb, C., Block, B.A., 2001. Systematics of the tunas and mackerels (Scombridae). Fish Physiol. 19.

Coma, R., Ribes, M., Serrano, E., Jiménez E., Salat J., Pascual, J., 2009. Global warming-enhanced stratification and mass mortality events in the Mediterranean. Proc. Natl. Acad. Sci. U.S.A. 106, 6176–6181.

Cort, J.L., 2007. El enigma del atún rojo reproductor del Atlántico Nororiental. Bedia Artes Gráficas, S.C., Santander. 64 pp.

Cort, J.L., Martínez, D., 2010. Possible effects of the bluefin tuna (*Thunnus thynnus*) recovery plan in some Spanish fisheries. Collect. Vol. Sci. Pap. ICCAT 65(3), 868–874.

Cushing, D.H., 1990. Plankton production and year-class strength in fish populations: an update of the match/mismatch hypothesis. Adv. Mar. Biol. 26, 249–293.

Davenport, J., Lönning, S., 1980. Oxygen uptake in developing eggs and larvae of the cod, *Gadus morhua* L. J. Fish Biol. 16(3), 249–256.

Davis, T.L.O., Jenkins, G.P., Young, J.W., 1990. Diel patterns of vertical distribution in larvae of southern bluefin *Thunnus maccoyii*, and other tuna in the East Indian Ocean. Mar. Ecol. Prog. Ser. 59, 63–74.

De la Gándara, F., Mylonas, C.C., Covès, D., Bridges, C.R., (Eds.), 2010. SELFDOTT Report 2009. 279 pp.

De la Gándara, F., Mylonas., C.C., Covés, D., Bridges, C.R. (Eds.), 2012. SELFDOTT Report 2010-2011.488 pp.

De la Gándara, F., Ortega, A., Belmonte, A., Mylonas, C.C., 2011. Spontaneous spawning of Atlantic bluefin tuna *Thunnus thynnus* kept in captivity. In: Proceedings of Aquaculture Europe, Rhodes (Greece), October 18–21, 249–250.

De la Gándara, F., Ortega, A., Blanco, E., Reglero, P., 2014. Buoyancy of Atlantic bluefin tuna (*Thunnus thynnus*) eggs obtained from captive broodstock spontaneous spawning events. In: Proceedings of Aquaculture Europe, San Sebastián (Spain), October 14–17, pp. 313–314.

De la Gándara, F., Ortega, A., Blanco, E., Viguri, J. Reglero, P., 2013. La flexión de la notocorda en larvas de Atún Rojo, *Thunnus thynnus* (L, 1758) cultivadas a diferentes temperaturas. In: Actas del XIV Congreso Nacional de Acuicultura, Gijón (Spain), September 23–25, pp. 180–181.

De la Gándara, F., Ortega, A., Buentello, A., 2016. Tuna Aquaculture in Europe. In: Benetti, D.D., Partridge, G.J., Buentello, A. (Eds.), Advances in Tuna Aquaculture. From hatchery to market. Academic press, London, pp.115–157.

Demir, M., 1963. Synopsis of biological data on bonito, *Sarda sarda* (Bloch). FAO Fish Rep. 6, 101–129.

De Silva, C.D., Premawansa, S., Keembiyahetty, C.N., 1986. Oxygen consumption in *Oreochromis niloticus* (L.) in relation to development, salinity, temperature and time of day. J. Fish Biol. 29(2), 267–277.

De Silva, C.D., Tytler, P., 1973. The influence of reduced environmental oxygen on the metabolism and survival of herring and plaice larvae. Neth. J. Sea Res. 7, 345–362.

Dewar, H., Graham, J., 1994. Studies of tropical tuna swimming performance in a large water tunnel-Energetics. J. Exp. Biol. 192(1), 13–31.

Dickhut, R.M., Deshpande, A.D., Cincinelli, A., Cochran, M.A., Corsolini, S., Brill, R. W., Secor, D.H., Graves, J.E., 2009. Atlantic bluefin tuna (*Thunnus thynnus*)

population dynamics delineated by organochlorine tracers. Environ. *Sci.* Technol. 43(22), 8522–8527.

Dickson, K.A., Graham, J.B., 2004. Evolution and consequences of endothermy in fishes. Physiol. Biochem. Zool. 77(6), 998-1018.

Duray, M., Kohno, H., 1988. Effects of continuous lighting on growth and survival of first-feeding larval rabbitfish, *Siganus guttatus*. Aquaculture 72(1–2), 73–79.

FAO, 2015. FishstatJ. Available at: http://www.fao.org/fishery/statistics/software/fishstat/en.

Faria, A.M., Chícharo, M.A., Gonçalves, E.J., 2011. Effects of starvation on swimming performance and body condition of pre-settlement *Sparus aurata* larvae. Aquatic Biol. 12(3), 281–289.

Fernández-Polaco, J., Llorente, I., 2016. Tuna economics and markets. In: Benetti, D.D., Partridge, G.J., Buentello, A. (Eds.), Advances in Tuna Aquaculture. From hatchery to market. Academic press, London, pp. 333–350.

Fielder, D.S., Bardsley, W.J., Allan, G.L., Pankhurst, P.M., 2002. Effect of photoperiod on growth and survival of snapper *Pagrus auratus* larvae. Aquaculture 211(1), 135–150.

Fiksen, Ø., Jørgensen, C., 2011. Model of optimal behaviour in fish larvae predicts that food availability determines survival, but not growth. Mar. Ecol. Prog. Ser. 432, 207–219.

Fiksen, Ø., Jørgensen, C., Kristiansen, T., Vikebø, F., Huse, G., 2007. Linking behavioural ecology and oceanography: larval behaviour determines growth, mortality and dispersal. Mar. Ecol. Prog. Ser. 347, 195–205.

Finn, R.N., Fyhn, H.J., Evjen, M.S., 1995. Physiological energetics of developing embryos and yolk-sac larvae of Atlantic cod (*Gadus morhua*). I. Respiration and nitrogen metabolism. Mar. Biol. 124(3), 355–369.

Finn, R., Rønnestad, I., Van der Meeren, T., Fyhn, H., 2002. Fuel and metabolic scaling during the early life stages of Atlantic cod *Gadus morhua*. Mar. Ecol. Prog. Ser. 243, 217–234.

Foley, C.J., Bradley, D.L., Höök, T.O., 2016. A review and assessment of the potential use of RNA: DNA ratios to assess the condition of entrained fish larvae. Ecol. Indic. 60, 346–357.

Folkvord, A., Fiksen, Ø., Høie, H., Johannessen, A., Otterlei, E., Vollset, K.W., 2009. What can size distributions within cohorts tell us about ecological processes in fish larvae?. Sci. Mar. 73(S1), 119–130.

Folkvord, A., Johannessen, A., Moksness, E., 2004. Temperature-dependent otolith growth in Norwegian spring-spawning herring (*Clupea haren*gus L.) larvae. Sarsia: North Atlantic Marine Science, 89(5), 297–310.

Folkvord, A., Koedijk, R., Grahl-Nielsen, O., Meier, S., Rydland Olsen, B., Blom, G., Otterlei, E., Imsland, A.K., 2018. You are what you eat? Differences in lipid composition of cod larvae reared on natural zooplankton and enriched rotifers. Aquac. Nutr. 24(1), 224–235.

Folkvord, A., Koedijk, R.M., Lokøy, V., Imsland, A.K., 2010. Timing and selectivity of mortality in reared Atlantic cod revealed by otolith analysis. Environ. Bio. Fish. 89(3–4), 513–519.

Folkvord, A., Øiestad, V., Kvenseth, P.G., 1994. Growth patterns of three cohorts of Atlantic cod larvae (*Gadus morhua* L.) studied in a macrocosm. ICES J. Mar. Sci.: Journal du Conseil. 51(3), 325–336.

Folkvord, A., Vollset, K. W., Catalán, I.A., 2015. Differences in growth and survival between cod *Gadus morhua* and herring *Clupea harengus* early stages co-reared at variable prey concentrations. J. Fish Biol. 87(5), 1176–1190.

REFERENCES

Folkvord, A., Ystanes, L., Johannessen, A., Moksness, E., 1996. RNA: DNA ratios and growth of herring (*Clupea harengus*) larvae reared in mesocosms. Mar. Biol. 126(2), 591–602.

Foreman, T.J., Ishizuka. Y., 1990. Giant bluefin tuna off southern California, with a new California size record. Calif. Fish Game 76, 181–186.

Fromentin, J.M., Powers, J.E., 2005. Atlantic bluefin tuna: population dynamics, ecology, fisheries and management. Fish Fish. 6(4), 281–306.

Fuiman, L.A., 1983. Growth gradients in fish larvae. J. Fish Biol. 23(1), 117–123.

Fujimoto, K., Yamamoto, T., Sudo, M., Haga, Y., Kurata, M., Okada, T., Shigeru, M., Sawada, Y., Kumai, H., 2008. Neutral lipid deposition in larval and juvenile Pacific bluefin tuna, *Thunnus orientalis*, under different rearing temperatures. Aquac. Sci. 56(1), 19–30.

Galuardi, B., Royer, F., Golet, W., Logan, J., Neilson, J., Lutcavage, M., 2010. Complex migration routes of Atlantic bluefin tuna (*Thunnus thynnus*) question current population structure paradigm. Can. J. Fish. Aquat. Sci. 67(6), 966–976.

García, A., Cortés, D., Quintanilla, J., Rámirez, T., Quintanilla, L., Rodríguez, J.M., Alemany, F., 2013. Climate-induced environmental conditions influencing interannual variability of Mediterranean bluefin (*Thunnus thynnus*) larval growth. Fish. Oceanogr. 22, 273–287.

García, A., Cortés, D., Ramírez, T., Fehri-Bedoui, R., Alemany, F., Rodríguez, J.M., Carpena, A., Álvarez, J.P., 2006. First data on growth and nucleic acid and protein content of field-captured mediterranean bluefin (*Thunnus thynnus*) and albacore (*Thunnus. alalunga*) larvae: a comparative study. Sci. Mar. 70(S2), 67–78.

Giguère, L.A., Cote, B., St-Pierre, J.F., 1988. Metabolic rates scale isometrically in larval fishes. Mar. Ecol. Prog. Ser. 50, 13–19.

Gillooly, J.F., Brown, J.H., West, G.B., Savage, V.M., Charnov, E.L., 2001. Effects of size and temperature on metabolic rate. Science, 293(5538), 2248-2251.

Glazier, D.S., 2015. Is metabolic rate a universal "pacemaker" for biological processes?. Biol. Rev. 90, 377–407.

Gooding, R.M., Neill, W.H., Dizon, A.E., 1981. Respiration rates and low-oxygen tolerance in skipjack tuna, *Katsuwonus pelamis*. Fish. Bull. 79, 31–48.

Gordoa, A., Carreras, G., 2014. Determination of temporal spawning patterns and hatching time in response to temperature of Atlantic bluefin tuna (*Thunnus thynnus)* in the Western Mediterranean. PLoS ONE 9(3), e90691.

Gordoa, A., Sanz, N., Viñas, J., 2015. Individual spawning duration of captive Atlantic bluefin tuna (*Thunnus thynnus*) revealed by mitochondrial DNA analysis of eggs. PLoS ONE 10(8), e0136733.

Graham, J.B., Dickson, K.A., 2000. The evolution of thunniform locomotion and heat conservation in scombrid fishes: new insights based on the morphology of *Allothunnus fallai*. Zool. J. Linnean. Soc. 129(4), 419–466.

Graham, J.B., Dickson, K.A., 2004. Tuna comparative physiology. J. Exp. Biol. 207(23), 4015–4024.

Graham, J.B., Laurs, R.M., 1982. Metabolic rate of the albacore tuna *Thunnus alalunga*. Mar. Biol. 72(1), 1–6.

Habtes, S., Muller-Karger, F.E., Roffer, M.A., Lamkin, J.T., Muhling, B.A., 2014. A comparison of sampling methods for larvae of medium and large epipelagic fish species during spring SEAMAP ichthyoplankton surveys in the Gulf of Mexico. Limnol. Oceanogr.: Methods 12, 86–101.

Hart, P.R., Hutchinson, W.G., Purser, G.J., 1996. Effects of photoperiod, temperature and salinity on hatchery-reared larvae of the greenback flounder (*Rhombosolea tapirine* Günther, 1862). Aquaculture 144(4), 303–311.

Hecht, T., Pienaar, A.G., 1993. A review of cannibalism and its implications in fish larviculture. J. World Aquac. Soc. 24(2), 246–261.

Hjort, J., 1914. Fluctuations in the great fisheries of northern Europe viewed in the light of biological research. Rapp.P.-V. Reun. Cons. Int. Explor. Mer. 20, 1–228.

Honryo, T., Tanaka, T., Guillen, A., Wexler, J.B., Cano, A., Margulies, D., Scholey, V.P., Stein, M.S., Sawada, Y., 2016. Effect of water surface condition on survival, growth and swim bladder inflation of yellowfin tuna, *Thunnus albacares* (Temminck and Schlegel), larvae. Aquac. Res. 47(6), 1832–1840.

Horodysky, A.Z., Cooke, S.J., Brill, R.W., 2015. Physiology in the service of fisheries science: why thinking mechanistically matters. Rev. Fish Biol. Fisher. 25(3), 425–447.

Horodysky, A.Z., Cooke, S.J., Graves, J.E., Brill, R.W., 2016. Fisheries conservation on the high seas: linking conservation physiology and fisheries ecology for the management of large pelagic fishes. Conserv. Physiol. 4, cov059.

Houde, E.D., 1989. Comparative growth, mortality, and energetics of marine fish larvae: temperature and implied latitudinal effects. Fish. Bull. 87, 471–495.

Houde, E.D., 2008. Emerging from Hjort's shadow. J. Northwest Atl. Fish. Sci. 41, 53–70.

Houde, E.D., Schekter, R.C., 1983. Oxygen uptake and comparative energetics among eggs and larvae of three subtropical marine fishes. Mar. Biol. 72, 283–293.

Houlihan, D.F., Pedersen, B.H., Steffensen, J.F., Brechin, J., 1995. Protein synthesis, growth and energetics in larval herring (*Clupea harengus*) at different feeding regimes. Fish Physiol. Biochem. 14(3), 195–208.

Hunter, J.R., 1980. The feeding behavior and ecology of marine fish larvae. In: Bardach, J.E., Magnuson, J.J., May, R., Reinart, J. (Eds.), Fish Behavior and Its Use in the Capture and Culture Fishes. ICLARM Conference Proceedings 5, pp. 512. International Center for Living Aquatic Resources Management, Manila, Philippines. pp. 287–330.

Hunter, J.R., Kimbrell, C.A., 1980. Early life history of Pacific mackerel, *Scomber japonicus*. Fish. Bull. 78, 89–101.

Hunter, J.R., Sanchez, C., 1976. Diel changes in swimbladder inflation of the larvae of the northern anchovy, *Engraulis mordax.* Fish. Bull. 74, 845–855.

Hunt von Herbing, I., Turnbough, M., 2011. Bioenergetics of growth in commercially important developing fishes. In: Holt, J. (Eds.), Larval Fish Nutrition. Oxford: Blackwell Publishing, pp. 249–263.

Hydes, D.J., Aoyama, M., Aminot, A., Bakker, K., Becker, S., Coverly, S., Daniel, A., Dickson, A.G., Grosso, O., Kerouel, R., Van Ooijen, J., Sato, K., Tanhua, T., Woodward, E.M.S., Zhang, J.Z., 2010. Determination of dissolved nutrients (N, P, SI) in seawater with high precision and inter-comparability using gas-segmented continuous flow analyses. IOCCP Report 14, ICPO Publication Series 134, version 1.

ICCAT, 2011. Report of the 2010 ICCAT bluefin data preparatory meeting. Collect. Vol. Sci. Pap. ICCAT, 66, 65 pp.

ICCAT, 2014. Report of the 2014 Atlantic Bluefin Tuna Stock Assessment session, Madrid, Spain, September, 2014.

ICES, 2004. Recruitment studies: Manual on precision and accuracy of tools. By Belchier, M., Clemmesen, C., Cortes, D., Doan, T., Folkvord, A., García, A.,

Geffen , A., Høie, H., Johannessen, A., Moksness, E., De Pontual., H., Ramirez, T., Schnack, D., Sveinsbo, B. D. Schnack, and B. Sveinsbo. ICES Tech. Mar. Env. Sci., No. 33. 35 pp.

Illing, B., Moyano, M., Berg, J., Hufnagl, M., Peck, M.A., 2018. Behavioral and physiological responses to prey match-mismatch in larval herring. Estuar. Coast. Shelf Sci. 201, 82–94.

Ingram, G.W., Alvarez-Berastegui, D., Reglero, P., Balbín, R., García, A., Alemany, F., 2015. Indices of larval bluefin tuna (*Thunnus thynnus*) in the western Mediterranean Sea (2001–2013). ICCAT SCRS/2015/035.

Ingram, G.W., Alvarez-Berastegui, D., Reglero, P., Balbín, R., García, A., Alemany, F., 2017. Incorporation of habitat information in the development of indices of larval bluefin tuna (*Thunnus thynnus*) in the western Mediterranean Sea (2001-2005 and 2012-2013). Deep Sea Research II 140, 203–211.

Ingram, G.W., Richards, W.J., Lamkin, J.T. and Muhling, B., 2010. Annual indices of Atlantic bluefin tuna (*Thunnus thynnus*) larvae in the Gulf of Mexico developed using delta-lognormal and multivariate models. Aquat. Living Resour. 23, 35–47.

Itoh, T., Shiina, Y., Tsuji, S., Endo, F., Tezuka, N., 2000. Otolith daily increment formation in laboratory reared larval and juvenile bluefin tuna *Thunnus thynnus*. Fish. Sci. 66(5), 834–839.

IUCN, 2018 The IUCN Red List of Threatened Species. Version 2018. See https://newredlist.iucnredlist.org/

Javahery, S., Nekoubin, H., Moradlu, A.H., 2012. Effect of anaesthesia with clove oil in fish (review). Fish Physiol. Biochem. 38, 1545–1552.

Jenkins, G.P., Young, J.W., Davis, T.L., 1991. Density dependence of larval growth of a marine fish, the southern bluefin tuna, *Thunnus maccoyii*. Can. J. Fish. Aquat. Sci. 48(8), 1358–1363.

Johnsson, J.I., Björnsson, B.T., 1994. Growth hormone increases growth rate, appetite and dominance in juvenile rainbow trout, *Oncorhynchus mykiss*. Anim. Behav. 48(1), 177-186.

Juan-Jordá, M.J., Mosqueira, I., Freire, J., Dulvy, N.K., 2013. Life in 3-D: life history strategies in tunas, mackerels and bonitos. Rev. Fish Biol. Fisher. 23(2), 135–155.

Kaji, T., 2003. Bluefin tuna larval rearing and development: State of the art. In: Bridges C.R., García, A., Gordin, H. (Eds.), Domestication of the bluefin tuna *Thunnus thynnus thynnus*. Zaragoza: CIHEAM, Cahiers Options Méditerranéennes, pp. 85–89.

Kaji, T., Shoji, J., Aoyama, M., Tanaka, M., 2002. Highly specialized development of the digestive system in piscivorous scombrid larvae. Fish. Sci. 68(sup1), 884-887.

Kaji, T., Tanaka, M., Oka, M., Takeuchi, H., Ohsumi, S., Teruya, K., Hirokawa, J., 1999. Growth and morphological development of laboratory-reared yellowfin tuna *Thunnus albacares* larvae and early juveniles, with special emphasis on the digestive system. Fish. Sci. 65(5), 700–707.

Kaji, T., Tanaka, M., Takahashi, Y., Oka, M., Ishibashi, N., 1996. Preliminary observations on development of Pacific bluefin tuna *Thunnus thynnus* (Scombridae) larvae reared in the laboratory, with special reference to the digestive system. Mari. Freshw. Res. 47(2), 261–269.

Karakulak, F.S., Ozturk, B., Yildiz, T., 2016. From ocean to farm: capture-based aquaculture of bluefin tuna in the eastern Mediterranean Sea. In: Benetti, D.D., Partridge, G.J., Buentello, A. (Eds.), Advances in Tuna Aquaculture. From hatchery to market. Academic press, London, pp. 59-76.

Kendall, A.W.Jr., Ahlstrom, E.H., Moser, H.G., 1984. Early life history stages of fishes and their characters. In: Moser, H.G., Richards, W.J., Cohen, D.M., Fahay, M.P., Kendall, A.W., Richardson, S.L. (Eds.), Ontogeny and systematics of fishes. Special publication 1. American Society of Ichthyologists and Herpetologists, Lawrence, KS, pp. 11–22.

Killen, S.S., 2014. Growth trajectory influences temperature preference in fish through an effect on metabolic rate. J. Anim. Ecol. 83(6), 1513-1522.

Killen, S.S., Atkinson, D., Glazier, D.S., 2010. The intraspecific scaling of metabolic rate with body mass in fishes depends on lifestyle and temperature. Ecology letters, 13(2), 184-193.

Killen, S.S., Costa, I., Brown, J. A., Gamperl, A.K., 2007. Little left in the tank: metabolic scaling in marine teleosts and its implications for aerobic scope. Proc. R. Soc. *B* 274(1608), 431−438.

Kimura, S., Kato, Y., Kitagawa, T., Yamaoka, N., 2010. Impacts of environmental variability and global warming scenario on Pacific bluefin tuna (*Thunnus orientalis*) spawning grounds and recruitment habitat. Prog. Oceanogr. 86, 39−44.

Kiørboe, T., Munk, P., Richardson, K., 1987. Respiration and growth of larval herring *Clupea harengus*: relation between specific dynamic action and growth efficiency. Mar. Ecol. Prog. Ser. 122, 135−145.

Koched, W., Hattour, A., Alemany, F., García, A. Said, K., 2013. Spatial distribution of tuna larvae in the Gulf of Gabes (eastern Mediterranean) in relation with environmental parameters. Mediterr. Mar. Sci. 14, 5–14.

Korsmeyer, K.E., Dewar, H., 2001. Tuna metabolism and energetics. Fish Physiol. 19, 35-78.

Kubo, T., Sakamoto, W., Murata, O., Kumai, H., 2008. Whole-body heat transfer coefficient and body temperature change of juvenile Pacific bluefin tuna *Thunnus orientalis* according to growth. Fish. Sci. 74(5), 995−1004.

Lankford Jr, T.E., Billerbeck, J.M., Conover, D.O., 2001. Evolution of intrinsic growth and energy acquisition rates. II. Trade-offs with vulnerability to predation in *Menidia menidia*. Evolution, 55(9), 1873−1881.

Leggett, W.C., Deblois, E., 1994. Recruitment in marine fishes: is it regulated by starvation and predation in the egg and larval stages? Neth. J. Sea Res. 32, 119–134.

Leis, J.M., 2006. Are larvae of demersal fishes plankton or nekton?. Adv. Mar. Biol. 51, 57−141.

Lejeusne, C., Chevaldonné, P., Pergent-Martini, C., Boudouresque, C.F., Perez, T., 2010. Climate change effects on a miniature ocean: the highly diverse, highly impacted Mediterranean Sea. *Trends* Ecol. Evol. 25, 250−260.

Lionello, P., Gacic, M., Gomis, D., Garcia-Herrera, R., Giorgi, F., Planton, S., Trigo, R., Theocharis, A., Tsimplis, M.N., Ulbrich, U., Xoplaki, U.U., 2012. Program focuses on climate of the Mediterranean region. EOS Transactions, American Geophysical Union 93, 105−106.

Litvak, M.K., Leggett, W.C., 1992. Age and size-selective predation on larval fishes: the bigger-is-better hypothesis revisited. Mar. Ecol. Prog. Ser. 81, 13−24.

Llopiz, J.K., 2013. Latitudinal and taxonomic patterns in the feeding ecologies of fish larvae: a literature synthesis. J. Marine Syst. 109, 69−77.

Llopiz, J.K, Hobday, A.J., 2015. A global comparative analysis of the feeding dynamics and environmental conditions of larval tunas, mackerels and billfishes. Deep Sea Res. II 113, 113−124.

Llopiz, J.K., Muhling, B.A., Lamkin, J.T., 2015. Feeding dynamics of Atlantic bluefin tuna (*Thunnus thynnus*) larvae in the Gulf of Mexico. Collect. Vol. Sci. Pap. ICCAT 71, 1710-1715.

Llopiz, J.K., Richardson, D.E., Shiroza, A., Smith, S.L., Cowen, R.K., 2010. Distinctions in the diets and distributions of larval tunas and the important role of appendicularians. Limnol.Oceanogr. 55(3), 983–996.

Lutcavage, M.E., Brill, R.W., Skomal, G.B., Chase, B.C., Goldstein, J.L., Tutein, J., 2000. Tracking adult North Atlantic bluefin tuna (*Thunnus thynnus*) in the northwestern Atlantic using ultrasonic telemetry. Mar. Biol. 137(2), 347–358.

Lutcavage, M.E., Brill, R.W., Skomal, G.B., Chase, B.C., Howey, P.W., 1999. Results of pop-up satellite tagging of spawning size class fish in the Gulf of Maine: do North Atlantic bluefin tuna spawn in the mid-Atlantic?. Can. J. Fish. Aquat. Sci. 56(2), 173–177.

Macías, D., Gómez-Vives, M.J., García, S., Ortiz de Urbina, J.M., 2005. Reproductive characteristics of Atlantic bonito (*Sarda sarda*) from the south western Spanish Mediterranean. Col. Vol. Sci. Pap. ICCAT 58 (2), 470–483.

Majkowski, J., Arrizabalaga, H., Carocci, F., 2011. Tuna and tuna-like species. In: FAO Fisheries and Aquaculture Department (Eds.), Review of the state of world marine fishery resources. FAO Fisheries and Aquaculture Technical Papers 569, 227–243.

Margulies, D., 1997. Development of the visual system and inferred performance capabilities of larval and early juvenile scombrids. *Mar. Freshw. Behav. Phy.* 30(2), 75–98.

Margulies, D., Scholey, V.P., Wexler, J.B., Olson, R.A., Suter, J.M., Hunt, S.L., 2007. A review of IATTC research on the early life history and reproductive biology of scombrids conducted at the Achotines Laboratory from 1985 to 2005. La Jolla, CA, IATTC. (Special Report, 16), 67 pp.

Margulies, D., Scholey, V.P., Wexler, J.B., Stein, M.S., 2016. Research on the reproductive biology and early life history of yellowfin tuna *Thunnus albacares* in Panama. In: Benetti, D.D., Partridge, G.J., Buentello, A. (Eds.), Advances in Tuna Aquaculture. From hatchery to market. Academic press, London, pp. 77-114.

Malzahn, A. M., Clemmesen, C., Rosenthal, H., 2003. Temperature effects on growth and nucleic acids in laboratory-reared larval coregonid fish. Mar. Ecol. Prog. Ser. 259, 285-293.

Masuma, S., Takebe, T., Sakakura, Y., 2011. A review of the broodstock management and larviculture of the Pacific northern bluefin tuna in Japan. Aquaculture 315(1), 2–8.

Mather, F.J., Mason, J.M., Jones, A.C., 1995. Historical document: life history and fisheries of Atlantic bluefin tuna. NOAA Technical Memorandum, NMFS-SEFSC-370.

Matsumoto, W.M., 1958. Description and distribution of larvae of four species of tuna in central Pacific waters. Fish. Bull. 58, 31–72.

McFarlane, M.B., Cripe, D.J., Thompson, S.H., 2000. Larval growth and development of cultured Pacific bonito. J. Fish Biol. 57, 134–144.

McGurk, M.D., 1986. Natural mortality of marine pelagic fish eggs and larvae: role of spatial patchiness. Mar. Ecol. Prog. Ser. 34 (3), 227–242.

McKenzie, D. J., Lund, I., Pedersen, P.B., 2008. Essential fatty acids influence metabolic rate and tolerance of hypoxia in Dover sole (*Solea solea*) larvae and juveniles. Mar. Biol. 154(6), 1041.

McLeod, I.M., Rummer, J.L., Clark, T.D., Jones, J.P., McCormick, M.I., Wenger, A. S., Munday, P.L., 2013. Climate change and the performance of larval coral

reef fishes: the interaction between temperature and food availability. Conserv. Physiol.1, cot024. doi: 10.1093/conphys/cot024

Meekan, M.G., Fortier, L., 1996. Selection for fast growth during the larval life of Atlantic cod *Gadus morhua* on the Scotian Shelf. Mar. Ecol. Prog. Ser. 137, 25–37.

Mena, C., Reglero, P., Ferriol, P., Torres, A.P., Aparicio-González, A., Balbín, R., Santiago, R., Moyá, G., Alemany, F., Agawin, N.S.R., 2016. Prokaryotic picoplankton spatial distribution during summer in a haline front in the Balearic Sea, western Mediterranean. Hydrobiologia, 779(1), 243–257.

Meyer, S., Caldarone, E.M., Chícharo, M.A., Clemmesen, C., Faria, A. M., Faulk, C., Folkvord, A., Holt, G.J., Hoie, H., Kanstinger, P., Malzahn, A., Moran, D., Petereit, C., Stottrup, J.G., Peck, M.A., 2012. On the edge of death: Rates of decline and lower thresholds of biochemical condition in food-deprived fish larvae and juveniles. J. Marine Syst. 93, 11–24.

Mielgo, R., 2007. It's the tuna stupid…!. January 2007, issue: Croatia. pp.54.

Miyashita, S., Hattori, N., Sawada, Y., Ishibashi, Y., Nakatsukasa, H., Okada, T., Murata, O., Kumai, H., 1999. Ontogenetic change in oxygen consumption of bluefin tuna, *Thunnus thynnus*. Aquacult. Sci. 47(2), 269–275.

Miyashita, S., Kato, K., Sawada, Y., Murata, O., Ishitani, Y., Shimizu, K., Yamamoto, S., Kumai, H., 1998. Development of digestive system and digestive enzyme activities of larval and juvenile bluefin tuna, *Thunnus thynnus*, reared in the laboratory. Aquac. Sci. 46(1), 111–120.

Miyashita, S., Sawada, Y., Okada, T., Murata, O., Kumai, H., 2001. Morphological development and growth of laboratory-reared larval and juvenile *Thunnus thynnus* (Pisces: Scombridae). Fish. Bull. 99(4), 601-617.

Miyashita, S., Tanaka, Y., Sawada, Y., Murata, O., Hattory, N., Takii, K., Mukai, Y., Kumai, H., 2000. Embryonic development and effects of water temperature on hatching of the bluefin tuna, *Thunnus thynnus*. Suisan Zoshoku 48, 199–207.

Morote, E., Olivar, M.P., Pankhurst, P.M., Villate, F., Uriarte, I., 2008. Trophic ecology of bullet tuna *Auxis rochei* larvae and ontogeny of feeding-related organs. Mar. Ecol. Prog. Ser. 353, 243–254.

Mosegaard, H., Folkvord, A., Wright, P.J., 2002. V. Some uses of individual age data. Ecological applications. In: Panfili, J., Troadec, H., Pontual, H. De., Wright, P.J. (Eds.), Manual of fish sclerochronology. Brest: Ifremer–IRD co-edition, pp. 167–178.

Moyano, M., Candebat, C., Ruhbaum, Y., Álvarez-Fernández, S., Claireaux, G., Zambonino-Infante, J.L., Peck, M.A., 2017. Effects of warming rate, acclimation temperature and ontogeny on the critical thermal maximum of temperate marine fish larvae. PLoS ONE 12(7), e0179928.

Moyano, M., Garrido, S., Teodósio, M.A., Peck, M. A., 2014. Standard metabolism and growth dynamics of laboratory-reared larvae of *Sardina pilchardus*. J. Fish Biol. 84(4), 1247–1255.

Moyano, M., Illing, B., Christiansen, L., Peck, M.A., 2018. Linking rates of metabolism and growth in marine fish larvae. Mar.Biol. 165(1), 5.

Moyano, M., Illing, B., Peschutter, P., Huebert, K. B., Peck, M.A., 2016. Thermal impacts on the growth, development and ontogeny of critical swimming speed in Atlantic herring larvae. Comp. Biochem. Physiol. A: Mol. Integr. Physiol. 197, 23–34.

Muhling., B.A., Lamkin, J.T., Alemany, F., García, A., Farley, J., Ingram Jr, W.G., Álvarez-Berastegui, D., Reglero, P., Láiz-Carrión, R. 2017. Reproduction and

larval biology in tunas, and the importance of restricted area spawning grounds. Rev. Fish Biol. Fisher. 27, 697–732.

Munk, P., 1995. Foraging behaviour of larval cod (*Gadus morhua*) influenced by prey density and hunger. Mar. Biol. 122, 205–212.

Mylonas, C.C., De La Gándara, F., Corriero, A., Ríos, A.B., 2010. Atlantic bluefin tuna (*Thunnus thynnus*) farming and fattening in the Mediterranean Sea. Rev. Fish. Sci. 18(3), 266–280.

Nakagawa, Y., Kurata, M., Sawada, Y., Sakamoto, W., Miyashita, S., 2011. Enhancement of survival rate of Pacific bluefin tuna (*Thunnus orientalis*) larvae by aeration control in rearing tank. Aquat. Living Resour. 24(4), 403-410.

Nakamura, I., 1990. Scombridae. In: Gon, O., Heemstra, P.C. (Eds.). Fishes of the Southern Ocean. J.L.B. Smith Institute of Ichthyology, Grahamstown, pp. 404–405.

Nelson, J.A., 2016. Oxygen consumption rate v. rate of energy utilization of fishes: a comparison and brief history of the two measurements. J. Fish Biol. 88(1), 10–25.

Nilsson, G.E., Östlund-Nilsson, S., Penfold, R., Grutter, A.S., 2007. From record performance to hypoxia tolerance: respiratory transition in damselfish larvae settling on a coral reef. Proc. R. *Soc.* Lond. B 274, 79–85.

Norin, T., Clark, T.D., 2016. Measurement and relevance of maximum metabolic rate in fishes. J. Fish Biol. 88(1), 122–151.

Olivar, M.P., Diaz, M.V., Chícharo, M.A., 2009. Tissue effect on RNA:DNA ratios of marine fish larvae. Sci. Mar. 73(S1), 171–182.

Ortega, A., 2015. Full cycle culture of two scombrid species: Atlantic bluefin tuna (*Thunnus thynnus*, L. 1758) and Atlantic bonito (*Sarda sarda*, Bloch 1793). Ph.D. Thesis. University of Murcia (Spain), 224 pp.

Ortega, A., De la Gándara, F., 2009. Efecto de diferentes esquemas de alimentación sobre crecimiento y supervivencia de larvas de Bonito Atlántico, *Sarda sarda*. In: Actas del XII Congreso Nacional de Acuicultura, Madrid, November 24–26, pp. 198–199.

Ortega, A., De la Gándara, F., 2017. Closing the life cycle of the Atlantic bluefin tuna *Thunnus thynnus* in captivity. Aquaculture Europe, Dubrovnik, Croatia, October 17-20.

Ortega, A., De la Gándara, F., Blanco, E., Reglero, P., Viguri, F., 2014. Effect of photoperiod and light intensity on larval rearing of bluefin tuna *Thunnus thynnus*, In: Proceeding of Aquaculture Europe, San Sebastían, 14–17 October, pp .929–930.

Ortega, A., Mourente, G., 2010. Comparison of lipid profiles from wild caught eggs and unfed larvae of two scombroid fish: northern bluefin tuna (*Thunnus thynnus* L., 1758) and Atlantic bonito (*Sarda sarda* Bloch, 1793). Fish Physiol. Biochem. 36(3), 461–471.

Ortega, A., Viguri, F., De la Gándara, F., 2013. Cierre del ciclo biológico en cautividad del bonito atlántico, *Sarda sarda* (Bloch, 1793), In: Actas del XIV Congreso Nacional de Acuicultura, Gijón, September 23-25, pp. 286–287.

Osse, J.W.M., Van den Boogaart, J.G.M., 1995. Fish larvae, development, allometric growth, and the aquatic environment. In: ICES Marine Science Symposia, Copenhagen, Denmark, 201, pp. 21–34.

Ottolenghi, F., 2008. Capture-based aquaculture of bluefin tuna. Capture-based aquaculture. Global overview. FAO Fish. Tech. Papers 508, 169–182.

Owen, R.W., 1989. Microscale and fine scale variations of small plankton in coastal and pelagic environments. J. Mar. Res. 47(1), 197–240.

Panella, G., 1971. Fish otoliths: daily growth layers and periodical patterns. Science 173 (4002), 1124-1127.

Papandroulakis, N., Scholey, V.P., De la Gándara, F., Benetti, D.D., Margulies, D., 2010. Evidence of positive influence of prolonged photophase on growth and survival during the larval rearing of yellow fin tuna (*Thunnus albacares*). In: Proceedings of Aquaculture Europe 2010, Porto (Portugal), October 5–8, p. 970.

Partridge, G.J., Benetti, D.D., Stieglitz, J.D., Hutapea, J., McIntyre, A., Chen, B., Scholey, V.P., 2011. The effect of a 24-hour photoperiod on the survival, growth and swim bladder inflation of pre-flexion yellowfin tuna (*Thunnus albacares*) larvae. Aquaculture 318(3), 471–474.

Peck, M.A., Buckley, L.J., 2008. Measurements of larval Atlantic cod (*Gadus morhua*) routine metabolism: temperature effects, diel differences and individual-based modeling. J. Appl. Ichthyol. 24(2), 144–149.

Peck, M.A., Huebert, K.B., Llopiz, J.K., 2012. Intrinsic and extrinsic factors driving match–mismatch dynamics during the early life history of marine fishes. Adv. Ecol. Res. 47, 177–302.

Peck, M.A., Moyano, M., 2016. Measuring respiration rates in marine fish larvae: challenges and advances. J. Fish Biol. 88(1), 173–205.

Pepin, P., Dower, J.F., Davidson, F.J.M., 2003. A spatially explicit study of prey–predator interactions in larval fish: assessing the influence of food and predator abundance on larval growth and survival. Fish. Oceanogr. 12(1), 19–33.

Pepin, P., Robert, D., Bouchard, C., Dower, J.F., Falardeau, M., Fortier, L., Jenkins, G.P., Leclerc, V., Levesque, K., Llopiz, J.K., Meekan, M.G., Murphy, H.M., Ringuette, M., Sirois, P., Sponaugle, S., 2015. Once upon a larva: revisiting the relationship between feeding success and growth in fish larvae. ICES J. Mar. Sci. 72(2), 359–373.

Pepin, P., Shears, T.H., De Lafontaine, Y., 1992. Significance of body size to the interaction between a larval fish (*Mallotus villosus*) and a vertebrate predator (*Gasterosteus aculeatus*). Mar. Ecol. Prog. Ser. 81, 1–12.

Peterson, I., Wroblewski, J.S., 1984. Mortality rate of fishes in the pelagic ecosystem. Can. J. Fish. Aquat. Sci. 41(7), 1117–1120.

Pittman, K., Yúfera, M., Pavlidis, M., Geffen, A. J., Koven, W., Ribeiro, L., Zambonino-Infante, J.L., Tandler, A., 2013. Fantastically plastic: fish larvae equipped for a new world. Rev. Aquacult. 5(s1), 224–267.

Porter, S.M., 2001. Effects of size and light on respiration and activity of walleye pollock (*Theragra chalcogramma*) larvae. J. Exp. Mar. Biol. Ecol. 256, 253–265.

Pörtner, H.O., Peck, M., 2010. Climate change effects on fishes and fisheries: towards a cause-and-effect understanding. J. Fish Biol. 77, 1745–1779.

Post, J.R., Lee, J.A., 1996. Metabolic ontogeny of teleost fishes. Can. J. Fish. Aquat. Sci. 53(4), 910–923.

Priede, I.G., 1985. Metabolic scope in fishes. In: Tytler, P., Calow, P. (Eds.), Fish energetics. Springer, Dordrecht, pp. 33–64.

Puvanendran, V., Brown, J.A., 2002. Foraging, growth and survival of Atlantic cod larvae reared in different light intensities and photoperiods. Aquaculture 214(1), 131– 151.

Rabe, J., Brown, J.A., 2000. A pulse feeding strategy for rearing larval fish: an experiment with yellowtail flounder. Aquaculture 191(4), 289–302.

Ramírez, T., Cortés, D., García, A., 2001. Growth of North Alboran Sea sardine larvae estimated by otolith microstructure, nucleic acids and protein content. J. Fish Biol. 59, 403–415

Reglero, P., Balbín, R., Ortega, A., Álvarez-Berastegui, D., Gordoa, A., Torres, A.P., Moltó, V., Pascual, A., De la Gándara, F., Alemany, F., 2013. First attempt to assess the viability of blue fin tuna spawning events in offshore cages located in an a priori favourable larval habitat. Sci. Mar. 77, 585–594.

Reglero, P., Blanco, E., Alemany, F., Ferrá, C., Alvarez-Berastegui, D., Ortega, A., De la Gándara, F., Aparicio-González, A., Folkvord, A., 2018a. Vertical distribution of Atlantic bluefin tuna *Thunnus thynnus* and bonito *Sarda sarda* larvae is related to temperature preference. Mar. Ecol. Prog. Ser. 594, 231–243.

Reglero, P., Blanco, E., Ortega, A., Fiksen, Ø., De la Gándara, F., Seoka, M., Viguri, F.J., Folkvord, A., 2015a. Prey selectivity in piscivorous bluefin tuna larvae reared in the laboratory. J. Plankton Res. 37, 2–5.

Reglero, P., Ciannelli, L., Alvarez-Berastegui, D., Balbín, R., López-Jurado, J.L., Alemany, F., 2012. Geographically and environmentally driven spawning distributions of tuna species in the western Mediterranean Sea. Mar. Ecol. Prog. Ser. 463, 273–284.

Reglero, P., Ortega, A., Balbín, R., Abascal, F.J., Medina, A., Blanco, E., De la Gándara, F., Alvarez-Berastegui, D, Hidalgo, M., Rasmuson, L., Alemany, F., Fiksen, Ø., 2018b. Atlantic bluefin tuna spawn at suboptimal temperatures for their offspring. Proc. R. Soc. B 285, 20171405.

Reglero, P., Ortega, A., Blanco, E., Fiksen, Ø., Viguri, F.J., De la Gándara, F., Folkvord, A., 2014a. Size-related differences in growth and survival in piscivorous fish larvae fed different prey types. Aquaculture 433, 94–101.

Reglero, P., Santos, M., Balbín, R., Laíz-Carrión, R., Alvarez-Berastegui, D., Ciannelli, L., Jiménex, E., Alemany, F., 2017. Environmental and biological characteristics of Atlantic bluefin tuna and albacore spawning habitats based on their egg distributions. Deep Sea *Res.* Part II: Top Stud. Oceanogr. 140, 105–116.

Reglero, P., Tittensor, D.P., Álvarez-Berastegui, D., Aparicio-González, A., Worm, B., 2014b. Worldwide distributions of tuna larvae: revisiting hypotheses on environmental requirements for spawning habitats. Mar. Ecol. Prog. Ser. 501, 207–224.

Reglero, P., Urtizberea, A., Torres, A.P., Alemany, F., Fiksen, Ø., 2011. Cannibalism among size classes of larvae may be a substantial mortality component in tuna. Mar. Ecol. Prog. Ser. 433, 205–219.

Reglero, P., Zaragoza, N., Blanco, E., De Gándara, F., Torres, A., Ortega, A., 2015b. Routine swimming speed of bluefin tuna larvae measured in the laboratory. 39th Annual Larval Fish Conference, Vienna, Austria, June 26-31.

Relini, L.O., Garibaldi, F., Cima, C., Palandri, G., Lanteri, L., Relini, M., 2005. Biology of Atlantic bonito, *Sarda sarda* (Bloch, 1793), in the western and central Mediterranean. A summary concerning a possible stock unit. Collect. Vol. Sci. Pap. ICCAT 58, 575–588.

Rey, J.C., Alot, E., Ramos, A., 1984. Sinopsis biológica del bonito, *Sarda sarda* (Bloch), del Mediterráneo y Atlántico Este. Collect. Vol. Sci. Pap. ICCAT 20(2), 469–502.

Rey, J.C., Cort, J.L., 1981. Migración de bonitos (*Sarda sarda*) y bacoreta (*Euthynnus alleteratus*) entre el Mediterráneo y el Atlántico. Col. Vol. Sci. Pap. ICCAT 15 (2), 346-347.

Richardson, D.E., Marancik, K.E., Guyon, J.R., Lutcavage, M.E., Galuardi, B., Lam, C.H., Walsh, H.J., Wildes, S., Yates, D.A., Hare, J.A., 2016. Discovery of a spawning ground reveals diverse migration strategies in Atlantic bluefin tuna (*Thunnus thynnus*). Proc. Natl. Acad. Sci. USA 113, 3299–3304.

Robert, D., Castonguay, M., Fortier, L., 2007. Early growth and recruitment in Atlantic mackerel *Scomber scombrus*: discriminating the effects of fast growth and selection for fast growth. Mar. Ecol. Prog. Ser. 337, 209–219.

Robert, D., Pepin, P., Dower, J.F., Fortier, L., 2014. Individual growth history of larval Atlantic mackerel is reflected in daily condition indices. ICES J. Mar. Sci. 71(4), 1001–1009.

Rodgers, G.G., Tenzing, P., Clark, T.D., 2016. Experimental methods in aquatic respirometry: the importance of mixing devices and accounting for background respiration. J. Fish Biol. 88(1), 65–80.

Rodríguez-Roda, J., 1966. Estudio de la bacoreta, Euthynnus alletteratus (Raf.), bonito, *Sarda sarda* (Bloch) y melva *Auxis thazard* (Lac.), capturados por las almadrabas españolas. Invest. Pesq. Barc. 30, 247-92.

Rodríguez, J.M., Alemany, F., Garcia A., 2017. A guide to the eggs and larvae of 100 common Western Mediterranean Sea bony fish species. FAO, Rome, Italy, 256 pp.

Rønnestad, I., Koven, W.M., Tandler, A., Harel, M., Fyhn, H.J., 1994. Energy metabolism during development of eggs and larvae of gilthead sea bream (*Sparus aurata*). Mar. Biol. 120, 187–196.

Rønnestad, I., Yúfera, M., Ueberschär, B., Ribeiro, L., Saele, Ø., Boglione, C., 2013. Feeding behaviour and digestive physiology in larval fish: current knowledge, and gaps and bottlenecks in research. Rev. Aquacult. 5(s1), S59–S98.

Rooker, J.R., Bremer, J.R.A., Block, B.A., Dewar, H., De Metrio, G., Corriero, A., Kraus, R.T., Prince, E.D., Rodríguez-Marin, E., Secor, D.H., 2007. Life history and stock structure of Atlantic bluefin tuna (*Thunnus thynnus*). Rev. Fish. Sci. 15, 265–310.

Rooker, J.R., Holt, G.J., 1996. Application of RNA:DNA ratios to evaluate the condition and growth of larval and juvenile red drum (*Sciaenops ocellatus*). Mar. Freshwater Res. 47(2), 283–290.

Rooker, J.R., Secor, D.H., De Metrio, G., Schloesser, R., Block, B.A., Neilson, J.D., 2008. Natal homing and connectivity in Atlantic bluefin tuna populations. Science 322(5902), 742–744.

Roy, B.C., Agawa, Y., Bruce, H.L., Ando, M., Okada, T., Sawada, Y., Tsukamasa, Y., 2014. Muscle growth in full-cycle cultured Pacific bluefin tuna *Thunnus orientalis* from early larval to juvenile stage: histochemical, immunohistochemical and ultrastructural observation. Fish. Sci. 80(5), 1009–1020.

Roy, B.C., Ando, M., Nakatani, M., Okada, T., Sawada, Y., Itoh, T., Tsukamasa, Y., 2012. Muscle fiber types, growth and development in the whole myotome of cultured Pacific bluefin tuna *Thunnus orientalis*. Fish. Sci. 78(2), 471–483.

Ruzicka, J.J., Gallager, S.M., 2006a. The importance of the cost of swimming to the foraging behavior and ecology of larval cod (*Gadus morhua*) on Georges Bank. Deep Sea Res. II 53, 2708–2734.

Ruzicka, J.J., Gallager, S.M., 2006b. The saltatory search behavior of larval cod (*Gadus morhua*). Deep Sea *Res.* Part II: Top Stud. Oceanogr. 53(23-24), 2735–2757.

Sabatés, A., Recasens, L., 2001. Seasonal distribution and spawning of small tunas (*Auxis rochei* and *Sarda sarda*) in the northwestern Mediterranean. Sci. Mar. 65, 95–100.

Saber, S., Muñoz, P., Ortiz de Urbina, J., Gómez-Vives, M.J., Rioja, P., Macías, D., 2015. Analysis of catch trends of skipjack tuna *Katsuwonus pelamis* (Linnaeus, 1758) from the recreational fishery in the western Mediterranean Sea (2006–2014). In: Volúmen de comunicaciones VIII Simposio Margen Continental Ibérico Atlántico, pp. 517-520.

Santamaria, N., Deflorio, M., De Metrio, G., 2005. Preliminary study on age and growth of juveniles of *Sarda sarda*, Bloch and *Euthynnus alletteratus*, Rafinesque, caught by clupeoids purse seine in the Southern Italian Seas. Collect. Vol. Sci. Pap. ICCAT 56(2), 630−643.

Sanzo, L., 1932. Uova e primi stadi larvali di *Pelamys sarda* Cuvier e Valenc. Memoria Comitato Talassogi Italia 188, 3–9.

Satoh, K., 2010. Horizontal and vertical distribution of larvae of Pacific bluefin tuna *Thunnus orientalis* in patches entrained in mesoscale eddies. Mar. Ecol. Prog. Ser. 404, 227−240.

Satoh, K., Tanaka, Y., Iwahashi, M., 2008. Variations in the instantaneous mortality rate between larval patches of Pacific bluefin tuna *Thunnus orientalis* in the northwestern Pacific Ocean. Fish. Res. 89(3), 248−256.

Satoh, K., Tanaka, Y., Masujima, M., Okazaki, M., Kato, Y., Shono, H., Suzuki, K., 2013. Relationship between the growth and survival of larval Pacific bluefin tuna, *Thunnus orientalis*. Mar. Biol. 160(3), 691−702.

Savage, V.M., Gillooly, J.F., Woodruff, W.H., West, G.B., Allen, A.P., Enquist, B.J., Brown, J.H., 2004. The predominance of quarter- power scaling in biology. Funct. Ecol. 18, 257–282.

Sawada, Y., Okada, T., Miyashita, S., Murata, O., Kumai, H., 2005. Completion of the Pacific bluefin tuna *Thunnus orientalis* (Temminck et Schlegel) life cycle. Aquac. Res. 36(5), 413−421.

Schultz, E.T., Conover, D.O., 1999. The allometry of energy reserve depletion: test of a mechanism for size-dependent winter mortality. Oecologia 119(4),474–483.

Secor, S.M., 2009. Specific dynamic action: a review of the postprandial metabolic response. J. Comp. Physiol. B 179(1), 1−56.

Seljeset, O., Vollset, K.W., Folkvord, A., Geffen, A.J., 2010. The role of prey concentration and size range in the growth and survival of larval cod. Mar. Biol. Res. 6(3), 251−262.

Sella, M., 1924. Caratteri differenziali di giovani stadi di *Orcynus thynnus* Ltkn. *O. alalonga* Risso, *Auxis bisus* Bp. Rendiconti atti della reale accademia nazionale del Lincei serie 5 (33), 300–305.

Seoka, M., Kurata, M., Kumai, H., 2007. Effect of docosahexaenoic acid enrichment in Artemia on growth of Pacific bluefin tuna *Thunnus orientalis* larvae. Aquaculture 270(1), 193−199.

Seoka, M., Kurata, M., Tamagawa, R., Biswas, A.K., Biswas, B.K., Yong, A.S.K., Kim, Y.S., Ji, S.C., Takii, K., Kumai, H., 2008. Dietary supplementation of salmon roe phospholipid enhances the growth and survival of Pacific bluefin tuna *Thunnus orientalis* larvae and juveniles. Aquaculture 275(1−4), 225−234.

Sepulveda, C., Dickson, K.A., 2000. Maximum sustainable speeds and cost of swimming in juvenile kawakawa tuna, *Euthynnus affinis*, and chub mackerel, *Scomber japonicus*. J. Exp. Biol. 203, 3089−3101.

Shi, Y., Zhang, G., Zhu, Y., Liu, J., 2010. Effects of photoperiod, temperature, and salinity on growth and survival of obscure puffer *Takifugu obscurus* larvae. Aquaculture 309(1), 103-108.

Shimose., T., Wells, R.J., 2015. Feeding ecology of bluefin tunas. In: Kitagawa, T., Kimura, S. (Eds.), Biology and ecology of bluefin tuna. CRC Press, pp.78-100.

Shoji, J., Maehara, T., Aoyama, M., Fujimoto, H., Iwamoto, A., Tanaka, M., 2001. Daily ration of Japanese Spanish mackerel *Scomberomorus niphonius* larvae. Fish. Sci. 67(2), 238–245.

Silva, L., Moyano, M., Illing, B., Faria, A.M., Garrido, S., Peck, M.A., 2015. Ontogeny of swimming capacity in plaice (*Pleuronectes platessa*) larvae. Mar. Biol. 162(4), 753–761.

Somarakis, S., Nikolioudakis, N., 2010. What makes a late anchovy larva? The development of the caudal fin seen as a milestone in fish ontogeny. J. Plankton Res. 32(3), 317–326.

Strasburg, D.W., 1960. Estimates of larval tuna abundance in the central Pacific. Fish. Bull. 60, 231–255.

Stuart, K.R., Drawbridge, M., 2012. The effect of photoperiod on larval culture performance of two marine finfish species. Aquaculture 360, 54–57.

Takashi, T., Kohno, H., Sakamoto, W., Miyashita, S., Murata, O., Sawada, Y., 2006. Diel and ontogenetic body density change in Pacific bluefin tuna, *Thunnus orientalis* (Temminck and Schlegel), larvae. Aquac. Res. 37(12), 1172–1179.

Takasuka, A., Aoki, I., Mitani, I., 2003. Evidence of growth-selective predation on larval Japanese anchovy *Engraulis japonicus* in Sagami Bay. Mar. Ecol. Prog. Ser. 252, 223–238.

Takasuka, A., Aoki, I., Mitani, I., 2004. Three synergistic growth-related mechanisms in the short-term survival of larval Japanese anchovy *Engraulis japonicus* in Sagami Bay. Mar. Ecol. Prog. Ser. 270, 217–228.

Takebe, T., Kurihara, T., Suzuki, N., Ide, K., Nikaido, H., Tanaka, Y., Shiozawa, S., Imaizumi, H., Masuma, S., Sakakura, Y., 2012. Onset of individual growth difference in larviculture of Pacific bluefin tuna *Thunnus orientalis* using fertilized eggs obtained from one female. Fish. Sci. 78(2), 343–350.

Tanaka, Y., Gwak, W.S., Tanaka, M., Sawada, Y., Okada, T., Miyashita, S., Kumai, H., 2007. Ontogenetic changes in RNA, DNA and protein contents of laboratory-reared Pacific bluefin tuna *Thunnus orientalis*. Fish. Sci. 73(2), 378–384.

Tanaka, Y., Kumon, K., Higuchi, K., Eba, T., Nishi, A., Nikaido, H., Shiozawa, S., 2015. Influence of the prey items switched from rotifers to yolk-sac larvae on growth of laboratory-reared Pacific bluefin tuna. Aquac. Sci. 63(4), 455–457.

Tanaka, Y., Kumon, K., Ishihi, Y., Eba, T., Nishi, A., Nikaido, H., Shiozawa, S., 2018. Mortality processes of hatchery-reared Pacific bluefin tuna *Thunnus orientalis* (Temminck et Schlegel) larvae in relation to their piscivory. Aquac. Res. 49(1), 11–18.

Tanaka, Y., Kumon, K., Nishi, A., Eba, T., Nikaido, H., Shiozawa, S., 2009. Status of the sinking of hatchery-reared larval Pacific bluefin tuna on the bottom of the mass culture tank with different aeration design. Aquac. Sci. 57(4), 587–593.

Tanaka, Y., Minami, H., Ishihi, Y., Kumon, K., Eba, T., Nishi, A., Nikaido, H., Shiozawa, S., 2010. Prey utilization by hatchery-reared Pacific bluefin tuna larvae in mass culture tank estimated using stable isotope analysis, with special reference to their growth variation. Aquac. Sci. 58(4), 501–508.

Tanaka, Y., Minami, H., Ishihi, Y., Kumon, K., Higuchi, K., Eba, T., Nishi, A., Nikaido, H., Shiozawa, S., 2014a. Differential growth rates related to initiation of piscivory by hatchery-reared larval Pacific bluefin tuna *Thunnus orientalis*. Fish. Sci. 80(6), 1205–1214.

Tanaka, Y., Minami, H., Ishihi, Y., Kumon, K., Higuchi, K., Eba, T., Nishi, A., Nikaido, H., Shiozawa, S., 2014b. Relationship between prey utilization and growth variation in hatchery-reared Pacific bluefin tuna, *Thunnus orientalis*

(Temminck et Schlegel), larvae estimated using nitrogen stable isotope analysis. Aquac. Res. 45(3), 537–545.

Tanaka, Y., Satoh, K., Iwahashi, M., Yamada, H., 2006. Growth-dependent recruitment of Pacific bluefin tuna *Thunnus orientalis* in the northwestern Pacific Ocean. Mar. Ecol. Prog. Ser. 319, 225–235.

Tanaka, Y., Satoh, K., Yamada, H., Takebe, T., Nikaido, H., Shiozawa, S., 2008. Assessment of the nutritional status of field-caught larval Pacific bluefin tuna by RNA/DNA ratio based on a starvation experiment of hatchery-reared fish. J. Exp. Mar. Biol. Ecol. 354(1), 56–64.

Teo, S.L.H., Boustany, A.M., 2016. Movements and habitat use of Atlantic bluefin tuna. In: Kitagawa, T., Kimura, S. (Eds.), Biology and ecology of bluefin tuna. CRC Press, pp. 137–188.

Theilacker, G., 1978. Changes in body measurements of larval northern anchovy, *Engraulis mordax*, and other fishes due to handling and preservation. Fish. Bull. 78, 685–692.

Tilley, J.D., Butler, C.M., Suárez-Morales, E., Franks, J.S., Hoffmayer, E.R., Gibson, D.P., Comyns, B.H., Ingram Jr, W.G., Blake, E.M., 2016. Feeding ecology of larval Atlantic bluefin tuna, *Thunnus thynnus*, from the central Gulf of Mexico. Bull. Mar. Sci. 92(3), 321–334.

Torres, J.J., Brightman, R. I., Donnelly, J., Harvey, J., 1996. Energetics of larval red drum, *Sciaenops ocellatus*. Part I: oxygen consumption, specific dynamic action, and nitrogen excretion. Fish. Bull. 94, 756–765.

Torres, A.P., Dos Santos, A., Balbín, R., Alemany, F., Massutí, E., Reglero, P., 2014. Decapod crustacean larval communities in the Balearic Sea (western Mediterranean): seasonal composition, horizontal and vertical distribution patterns. J. Marine Syst. 138, 112–126.

Torres, A.P., Reglero, P., Balbin, R., Urtizberea, A., Alemany, F., 2011. Coexistence of larvae of tuna species and other fish in the surface mixed layer in the NW Mediterranean. J. Plankton Res. 33, 1793–1812.

Tudela, S., Hidas, E., Graupera, E., Sainz Trápaga, S., Cermeño, P., Quílez-Badia G., 2011. Bluefin tuna migratory behavior in the western and central Mediterranean Sea revealed by electronic tags. Collect. Vol. Sci. Pap. ICCAT 66, 1157–69.

Ueyanagi, S., 1969. Observations on the distribution of tuna larvae in the Indo-Pacific Ocean with emphasis on the delineation of the spawning areas of the albacore, *Thunnus alalunga*. Bull. Far Seas Fish. Res. Lab., Shimizu 2, 177–254.

Uotani, I., Saito, T., Hiranuma, K., Nishikawa, Y., 1990. Feeding habit of bluefin tuna *Thunnus thynnus* larvae in the western North Pacific Ocean. Nippon Suisan Gakkaishi 56, 713–717.

Uriarte, A., Johnstone, C., Laiz-Carrión, R., García, A., Llopiz, J.K., Quintanilla, J.M., Lozano-Peral, D., Reglero, P., Alemany, F., 2017. First report on cannibalistic feeding behaviour in post-flexion bluefin larvae (*Thunnus thynnus*) of the Balearic Sea (NW Mediterranean). Collect. Vol. Sci. Pap. ICCAT 74(6), 2554–2562.

Urtizberea, A., 2009. From environment to survival. Foraging and bioenergetic models of anchovy and tuna larvae. Ph.D. dissertation, University of Bergen.

Vallés, R., Estévez, A., 2013. Light conditions for larval rearing of meagre (*Argyrosomus regius*). Aquaculture 376, 15–19.

Vollset, K.W., Catalán, I.A., Fiksen, Ø, Folkvord, A., 2013. Effect of food deprivation on distribution of larval and early juvenile cod in experimental vertical temperature and light gradients. Mar. Ecol. Prog. Ser. 475, 191–201.

Vollset, K.W., Fiksen, Ø., Folkvord, A., 2009. Vertical distribution of larval cod (*Gadus morhua*) in experimental temperature gradients. J. Exp. Mar. Biol. Ecol. 379, 16–22.

Watai, M., Ishihara, T., Abe, O., Ohshimo, S., Strussmann, C.A., 2017. Evaluation of growth-dependent survival during early stages of Pacific bluefin tuna using otolith microstructure analysis. Mar. Fresh. Res. 68(11), 2008–2017.

Weatherley, A.H., Gill, H.S., Lobo, A.F., 1988. Recruitment and maximal diameter of axial muscle fibres in teleosts and their relationship to somatic growth and ultimate size. J. Fish Biol. 33(6), 851–859.

Wegner, N.C., Sepulveda, C.A., Aalbers, S.A., Graham, J.B., 2013. Structural adaptations for ram ventilation: gill fusions in scombrids and billfishes. J. Morphol. 274(1), 108–120.

Wegner, N.C., Sepulveda, C.A., Bull, K.B., Graham, J.B., 2010. Gill morphometrics in relation to gas transfer and ram ventilation in high-energy demand teleosts: Scombrids and billfishes. J. Morphol. 271(1), 36–49.

Wexler, J.B., Margulies, D., Scholey, V.P., 2011. Temperature and dissolved oxygen requirements for survival of yellowfin tuna, *Thunnus albacares*, larvae. J. Exp. Mar. Biol. Ecol. 404, 63–72.

Wieser, W., 1995. Energetics of fish larvae, the smallest vertebrates. Acta Psychiatr. Scand. 154(3), 279–290.

Wieser, W., Forstner, H., Medgyesy, N., Hinterleitner, S., 1988. To switch or not to switch: partitioning of energy between growth and activity in larval cyprinids (Cyprinidae: Teleostei). Funct. Ecol. 499–507.

Wieser, W., Medgyesy, N., 1990a. Aerobic maximum for growth in the larvae and juveniles of a cyprinid fish, *Rutilus rutilus* (L.): implications for energy budgeting in small poikilotherms. Funct. Ecol. 4, 233–242.

Wieser, W., Medgyesy, N., 1990b. Cost and efficiency of growth in the larvae of two species of fish with widely differing metabolic rates. Proc. R. Soc. Lond. B: Biol. Sci. 242, 51–56.

Wood, S.N., 2006. Generalized additive models, an introduction with R. Chapman & Hall, London.

Woolley, L.D., Fielder, S.D., Qin, J.G., 2013. Swimbladder inflation associated with body density change and larval survival in southern bluefin tuna *Thunnus maccoyii*. Aquacult. Int. 21, 1233–1242.

Woolley, L.D., Qin, J.G., Thomson, M., Czypionka, A., 2009. Hatching success and early larval development of southern bluefin tuna (*Thunnus maccoyii)*. In: Proceedings of the 2nd Global COE Program Symposium of Kinki University, Adelaide, December 1–2, pp. 88–92.

Yamamoto, T., Teruya, K., Hara, T., Hokazono, H., Kai, I., Hashimoto, H., Furuita, H., Matsunari, H., Mushiake, K., 2009. Nutritional evaluation of rotifers in rearing tanks without water exchange during seed production of amberjack *Seriola dumerili*. Fish. Sci. 75(3), 697–705.

Yamashita, Y., Bailey, K.M., 1989. A laboratory study of the bioenergetics of larval walleye pollock, *Theragra chalcogramma*. Fish. Bull. 87, 525–536.

Young, J.W., Davis, T., 1990. Feeding ecology of larvae of southern bluefin, albacore and skipjack tunas (Pisces: Scombridae) in the eastern Indian Ocean. Mar. Ecol. Prog. Ser. 61, 17–29.

Yúfera, M., Ortiz-Delgado, J. B., Hoffman, T., Siguero, I., Urup, B., Sarasquete, C., 2014. Organogenesis of digestive system, visual system and other structures in Atlantic bluefin tuna (*Thunnus thynnus*) larvae reared with copepods in mesocosm system. Aquaculture 426, 126–137.

REFERENCES

Zarrad, R., Alemany, F., Rodríguez, J.M., Jarboui, O., López-Jurado, J.L., Balbin, R., 2013. Influence of summer conditions on the larval fish assemblage in the eastern coast of Tunisia (Ionian Sea, Southern Mediterranean). J. Sea Res. 76, 114–125.