# Trophic ecology of small pelagic fish in the northwestern Mediterranean

*Ecología trófica de peces pelágicos pequeños en el Mediterráneo noroccidental*

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# Trophic ecology of small pelagic fish in the northwestern Mediterranean.

Ecología trófica de peces pelágicos pequeños en el Mediterráneo noroccidental.

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**“Nature is a much more powerful**
**means of ascertaining *truth* than**
**authority or faith”**
Johannes Scotus Erigena (c. 810 - c. 877).

**“Rather than love, than money,**
**than fame, give me *truth*”**
Henry David Thoreau (1817 - 1862).

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*‘So long, and thanks for all the fish’.*

El Port de la Selva, septiembre de 2012.

## Estructura de la disertación.

Este manuscrito está estructurado en 2 Partes.

La primera parte (**Parte I**) está compuesta de una introducción general (**Introducción**), que expone la relevancia que los peces pelágicos pequeños tienen tanto para los ecosistemas marinos como a nivel socio-económico dada su importancia para la explotación pesquera. A continuación se presentan las 2 especies objetivo de esta tesis, anchoa europea (*Engraulis encrasicolus* Linnaeus, 1758) y sardina europea (*Sardina pilchardus* Walbaum, 1792), en el marco del mar Mediterráneo y, brevemente, se explican el contexto y los objetivos del proyecto europeo SARDONE, dentro del cual se inició el trabajo de la tesis. Seguidamente, se desarrolla una descripción general de las características hidrográficas y oceanográficas del golfo de León que permite enmarcar y entender el contexto físico y geográfico del área de estudio que pudiera ser relevante para la realización de esta tesis, junto con una exposición de las campañas oceanográficas realizadas en la zona con el objeto de obtener muestras para la realización del trabajo.

También dentro de la Introducción se desarrollan las razones por las cuales era importante realizar este trabajo, y, a continuación se exponen los objetivos generales que se pretenden alcanzar con esta tesis.

Posteriormente se desarrolla una **Discusión** de los resultados obtenidos durante la realización de la tesis, enumerando las principales **conclusiones** obtenidas, y a continuación se muestran posibles futuros trabajos y aplicaciones derivados de los resultados aquí obtenidos.

Tras esta discusión se detalla la **bibliografía** utilizada en la elaboración de esta primera parte. Finalmente, se incluye el **Informe de la directora**.

La segunda parte (**Parte II**) consta de 4 capítulos, cada uno de ellos correspondiente a un artículo científico publicado, o listo para publicar:

- **Capítulo 1**. Feeding behaviour of European pilchard: from late-larva to adult.
- **Capítulo 2**. Comparison of the diets of European anchovy *Engraulis encrasicolus* and sardine *Sardina pilchardus* juveniles in the Gulf of Lions during different seasons.
- **Capítulo 3**. Ontogenetic and seasonal changes in the feeding habits and trophic levels of two small pelagic fish species.
- **Capítulo 4**. Ecological understanding for fishery management: condition and growth of anchovy late larvae during different seasons in the Northwestern Mediterranean.

Como **anexos**, además, al final de la memoria, se adjuntan copias de los 2 artículos publicados en el formato original de la revista correspondiente.

# CONTENIDO

# PARTE I

## 1. Introducción.

### 1.1 Relevancia ecológica y socio-económica de los peces pelágicos pequeños.

Los peces pelágicos pequeños, principalmente pertenecientes al orden de los clupeiformes, juegan un papel crucial en los ecosistemas marinos ya que transfieren la energía de los niveles tróficos inferiores (plancton) a los grandes depredadores (peces, aves y mamíferos marinos) (Bakun, 1996, Cury *et al*., 2000).

Engráulidos como las anchoas del género *Engraulis* spp. y clupeidos como las sardinas *Sardina* spp. y *Sardinops* spp. cohabitan las mismas regiones, y sus poblaciones más importantes se distribuyen en las zonas costeras más productivas del planeta (zonas de afloramiento del Este del océano Pacífico y del Atlántico y el sistema de la corriente de Kuroshio, en las costas japonesas; Figura 1).

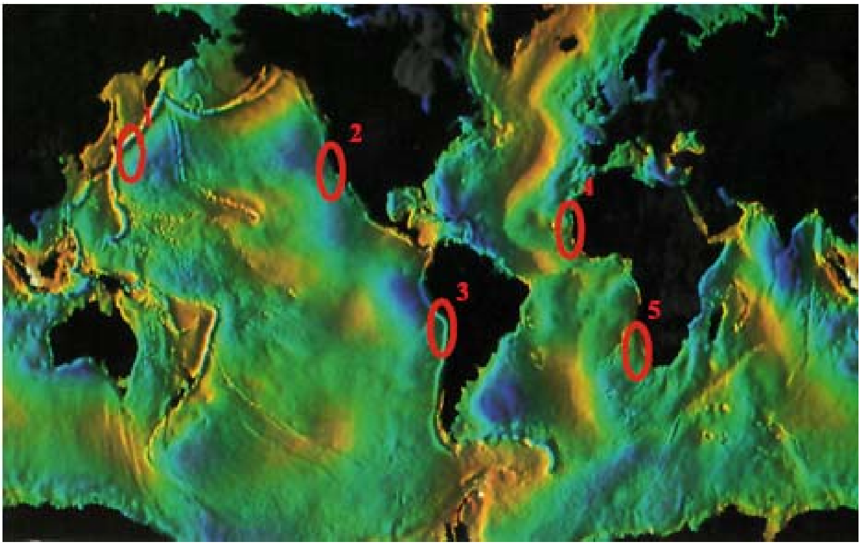

**Figura 1. Mapa en el que se señalan las 5 regiones costeras más productivas, en las que coinciden poblaciones de anchoa y de sardina. 1- Sistema de la corriente de Kuroshio; 2- Sistema de la corriente de California; 3- Sistema de la corriente de Humboldt; 4-Sistema de la corriente Canaria; 5- Sistema de la corriente de Benguela.**

Algunos trabajos ya han mostrado las fluctuaciones y alternancias de regímenes de abundancia que existen entre las poblaciones de sardina y anchoa en las áreas mencionadas (ver Lluch-Belda *et al*., 1992). Estos cambios de régimen probablemente sean debidos a las diferentes dinámicas tróficas que pueden presentar las dos especies determinadas que cohabiten cada región, así pues, dependiendo del régimen ambiental dominante en un momento dado, una de las especies se verá más favorecida que la otra (van der Lingen *et al*., 2006). Además, las alternancias entre las poblaciones pueden conducir a que la presión pesquera se centre sobre todo en la especie más abundante en ese momento, permitiendo una mejor recuperación de la otra (Schwartzlose *et al*., 1999).

Las importantes fluctuaciones temporales en las biomasas de estas especies de peces a nivel regional, principalmente debidas a su rápido crecimiento, su corta esperanza de vida, su tendencia a agruparse en grandes bancos y las fuertes y rápidas respuestas de sus poblaciones a la variabilidad ambiental, llevaron a muchos científicos y científicas, especialmente antes del desarrollo y expansión de la pesca industrial, a pensar que las actividades pesqueras no tenían un impacto determinante en las poblaciones de dichas especies pues su abundancia parecía estar influenciada exclusivamente por factores ambientales (Huxley, 1883). Sin embargo, esa creencia se ha ido refutando por completo desde finales del siglo pasado, y actualmente se acepta sin lugar a dudas que la intensa presión pesquera sobre los peces pelágicos pequeños puede resultar en una drástica reducción de sus poblaciones (Beverton, 1990, Freon *et al*., 2005). Además, una reducción notable en la disponibilidad trófica de este tipo de peces debida bien a la pesca, bien a condiciones ambientales determinadas, o bien a una combinación de ambas, tienen impactos directos y duraderos en el medio que podrían cambiar la estructura y función del ecosistema marino (Cury & Shannon, 2004, Pikitch *et* *al*., 2012).

Históricamente, los peces pelágicos pequeños han sido consumidos por la especie humana en todas o la mayoría de las áreas costeras en que podían ser encontrados. Actualmente, los relativamente bajos costes del combustible que se consume durante la pesca, gracias en parte al comportamiento de agrupación de estas especies y su tendencia a habitar zonas cercanas a la costa, han hecho que sus precios en el mercado sean especialmente reducidos. Además, su alto valor nutricional los ha convertido también en el componente fundamental en la elaboración de harinas y aceites de pescado, hasta el punto de que el 90 % de las capturas mundiales de estas especies son procesadas y utilizadas para fines agrícolas, acuícolas, ganaderos y otros productos industriales derivados (Alder *et al*., 2008). La economía de la pesca de peces pelágicos pequeños está, por tanto, fuertemente afectada por factores externos a la misma pesca, como pueden ser los precios de otros productos intensamente utilizados para la producción de piensos para alimentar ganado y peces carnívoros en producciones acuícolas, los cuales están a menudo fuera de la capacidad de control de los pescadores, e incluso de los propios productores de harinas y aceites de pescado.

Mientras el consumo del pescado procedente de la acuicultura y alimentado a base de estos piensos y aceites tiene lugar principalmente en los países desarrollados, la mayor parte del pescado usado para ser procesado y convertido en harinas de pescado para usos en esa acuicultura se pesca en las costas de países en vías de desarrollo, sobre todo de Sudamérica y África, y, puesto que los desembarques a escala mundial de este tipo de peces superan en la actualidad los 31.5 mill. Tm. año$^{-1}$ (aproximadamente el 37 %  de todas las capturas mundiales de peces marinos; Alder *et al*., 2008), se trata de especies determinantes para la seguridad alimentaria en muchos países.

## 1.2 Sardina y anchoa en el Mediterráneo.

Esta tesis se inició dentro del marco del proyecto europeo SARDONE[1] (SARDONE project, 2010), dedicado a desarrollar una serie de herramientas que permitieran un mayor conocimiento ecológico de las especies económica y ecológicamente más importantes del Mediterráneo (anchoa y sardina europeas, *Engraulis encrasicolus* y *Sardina pilchardus*, respectivamente, en el golfo de León, el mar Adriático y el mar Egeo), así como una mejor evaluación de los *stocks* y una gestión más sostenible de su pesca.

Los objetivos generales del proyecto se dirigían a detectar cuáles son los hábitats esenciales para los juveniles y las zonas de reproducción y crecimiento de ambas especies en el Mediterráneo y relacionarlas con las características oceanográficas y topográficas de cada una de las tres regiones mediante sondeos acústicos, lo que, a su vez, permitiría generar estimaciones del éxito en el reclutamiento en años futuros. También se pretendía cubrir el importante vacío de conocimiento relativo a la ecología trófica de las larvas grandes (>15 mm de longitud total (LT), que por consenso de las y los participantes en el proyecto se denominaron *late-larvae*, pero que aparecerán de aquí en adelante en esta sección simplemente como *larvas*) y los juveniles, perfeccionar la selectividad de las actuales artes de pesca y evaluar el impacto que tiene dicha pesca sobre las poblaciones de individuos inmaduros (larvas y juveniles) en zonas como el Adriático.

El periodo inicial del proyecto se dedicó en gran medida a la obtención de datos sobre los parámetros medioambientales y a la recogida de muestras. Tanto la recogida de las muestras de peces y plancton como de los datos oceanográficos se llevaron a cabo durante prospecciones acústicas en las tres áreas de estudio

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[1] Los resultados del proyecto son públicos y están accesibles en *http://cordis.europa.eu*.

(Egeo, Adriático y golfo de León), siguiendo un protocolo común de muestreo previamente establecido.

En el Mediterráneo noroccidental, *E. encrasicolus* y *S. pilchardus* son las especies más importantes en términos de biomasa y también de toneladas capturadas (Palomera *et al*., 2007), y, junto con la alacha, *Sardinella aurita*, y el espadín, *Sprattus sprattus*, representan casi el 50 % del total de capturas marinas en todo el Mediterráneo (Lleonart & Maynou, 2003).

La anchoa y la sardina son especies planctívoras que consumen, durante todas sus fases de desarrollo, un amplio rango de especies, lo que podría esperarse que se tradujera en un posible solapamiento de sus nichos tróficos. Sin embargo, las interacciones tróficas entre las dos especies habían sido, hasta el momento del inicio de esta tesis, escasamente estudiadas.

A pesar de que los periodos de reproducción de la anchoa y la sardina en el Mediterráneo noroccidental son diferentes, ocurriendo en primavera-verano y en otoño-invierno, respectivamente, (Palomera *et al*., 2007) y de que ambas especies tardan en torno a un año en alcanzar la madurez sexual (Blaxter & Hunter, 1982) es posible que los juveniles de ambas especies coincidan en ciertas épocas del año. Cuando esto llega a ocurrir, se podría esperar una competencia por el alimento entre los juveniles de ambas especies. De hecho, en el futuro, si continúan las tendencias regionales actuales de aumento de la temperatura (Salat & Pascual, 2011), incluso las larvas de ambas especies podrían llegar a estar presentes en el golfo de León al mismo tiempo y, por tanto, presumiblemente llegarían a competir también por el alimento.

En el golfo de León, la pesquería de peces pelágicos pequeños, que se realiza tanto con redes de cerco como de arrastre pelágico (Lleonart & Maynou, 2003) sobre un *stock* que comparten Francia y España, se centra casi exclusivamente en *E. encrasicolus* y *S. pilchardus*, y, a pesar de que siguen siendo las especies más pescadas, las toneladas capturadas han descendido en los últimos años (Figura 2). Y la misma tendencia han seguido las biomasas disponibles estimadas de sus poblaciones, desde unas 472000 Tm en 2005 hasta unas 50000 Tm en 2010 (siempre incluyendo todas las demás especies de pelágicos pequeños; GFCM, 2011). Además, el número de embarcaciones dedicadas a su explotación en el golfo de León ha descendido desde 56 en 2002 a menos de 20 en la actualidad, mientras que, por ejemplo, el número total de embarcaciones de arrastre se ha mantenido estable en torno a 100.

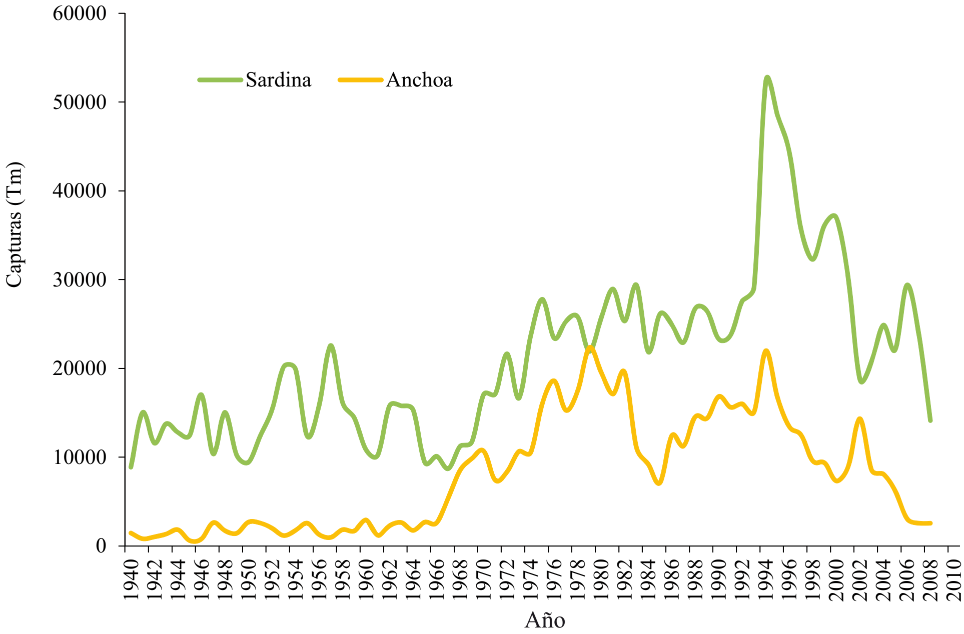

**Figura 2. Capturas anuales desde 1940 a 2010, en toneladas (Tm), de anchoa (naranja) y sardina (verde) en el Mediterráneo occidental. Origen de los datos: Gobierno de España y Govern de Catalunya.**

Sumado a esto, se ha detectado (ver en GFCM, 2011) que la talla modal de las anchoas del golfo de León a una determinada edad ha descendido progresivamente desde 13-14.5 cm en 2005 a 10-13 cm en 2009-2010, y las anchoas más grandes (>15 cm TL) casi han desaparecido. En los años 2009 y 2010, las anchoas de 2 o más años de edad estaban escasamente representadas o ausentes, y más del 80 % de la biomasa de la población estaba compuesta por individuos de 1 año de edad.

En el mismo informe se detallan tendencias similares para el caso de la sardina, con los individuos adultos más pequeños (<12.5 cm LT) llegando a representar en 2009 y 2010 más del 80 % de la biomasa de la población.

Junto con anchoa y sardina, en el golfo de León también aparecen otras especies de peces pelágicos pequeños y medianos y que son a menudo capturados por las mismas embarcaciones, como la caballa (*Scomber sconbrus*), el estornino (*S. japonicus*), el chicharro (*Trachurus trachurus*), el jurel (*T. mediterraneus*), la merluza (*Merlucius merlucius*), la alacha (*Sardinella aurita*), el pagel (*Pagellus* spp.), la bacaladilla (*Micromesistius poutassou*) y el espadín (*Sprattus sprattus*).

La pesquería de pelágicos pequeños en el golfo de León es, por tanto, multiespecífica y el esfuerzo pesquero sobre la anchoa no puede ser separado del esfuerzo sobre la sardina, o viceversa, por lo que la gestión de esta pesquería debe considerar ambas especies en conjunto, o incluso desarrollarse a través de una aproximación ecosistémica.

Puesto que numerosas especies animales, incluida la nuestra, dependen de estos peces, es esencial gestionar su explotación de forma sostenible y aplicando el principio de precaución cuando no existan datos suficientes para garantizar la gestión adecuada.

## 1.3 Área de estudio. El golfo de León.

La subcuenca noroccidental del mar Mediterráneo se extiende desde el mar Tirreno, al este, hasta el canal de Eivissa en el suroeste, con el mar de Liguria y el golfo de León en el norte, y cubre un área de unos 200000 km$^2$. En particular, el golfo de León presenta una amplia plataforma continental, con una profundidad generalmente menor de 200 m, lo que, entre otros motivos, hace a esta región relativamente rica en vida marina (Estrada, 1996).

### 1.3.1 Caraterísticas ambientales.

La complejidad del régimen hidrodinámico del golfo de León ha sido ampliamente abordada por diversos autores (p. ej., Lacombe & Tchernia, 1960, Millot, 1991, Bethoux *et al*., 1999, Millot, 1999, Tsimplis *et al*., 2006), aunque algunas características concretas están aún sometidas a debate (Millot, 2005, 2009, Bergamasco & Malazotte-Rizzoli, 2010). La circulación en la cuenca occidental está fuertemente influenciada por el flujo de aguas atlánticas, las cuales forman, al pasar el estrecho de Gibraltar, las llamadas Aguas Atlánticas Modificadas (MAW, siglas del inglés Modified Atlantic Waters), una capa de 100-200 m de profundidad caracterizada por una creciente salinidad, debida a la evaporación y la mezcla con aguas mediterráneas, desde unos 36.5 en torno a Gibraltar hasta 38-38.3 en el Mediterráneo noroccidental, y por una temperatura media bajo la capa de mezcla de 14-15º C (Millot, 1999).

En el Mediterráneo noroccidental, las MAW fluyen en sentido suroeste desde el mar de Liguria, siguiendo el talud continental a lo largo del golfo de León hasta el mar Catalán. Estas corrientes son conocidas, en conjunto, como la Corriente del Norte (Millot, 1992) (Figura 3). Esta corriente, de unos 30-50 km de anchura, se caracteriza por velocidades que oscilan entre los ~50 cm $s^{-1}$ en la superficie y su zona central y unos pocos cm $s^{-1}$ a más profundidad. La Corriente del Norte, además, sufre una marcada variabilidad estacional en lo que respecta a su flujo (Bethoux *et al*., 1980), y tiene importantes caracteres locales y de mesoescala, principalmente debidos a la abundancia de cañones a lo largo de todo el talud y de los patrones locales de viento, que influyen en la producción primaria de la región (Estrada, 1996).

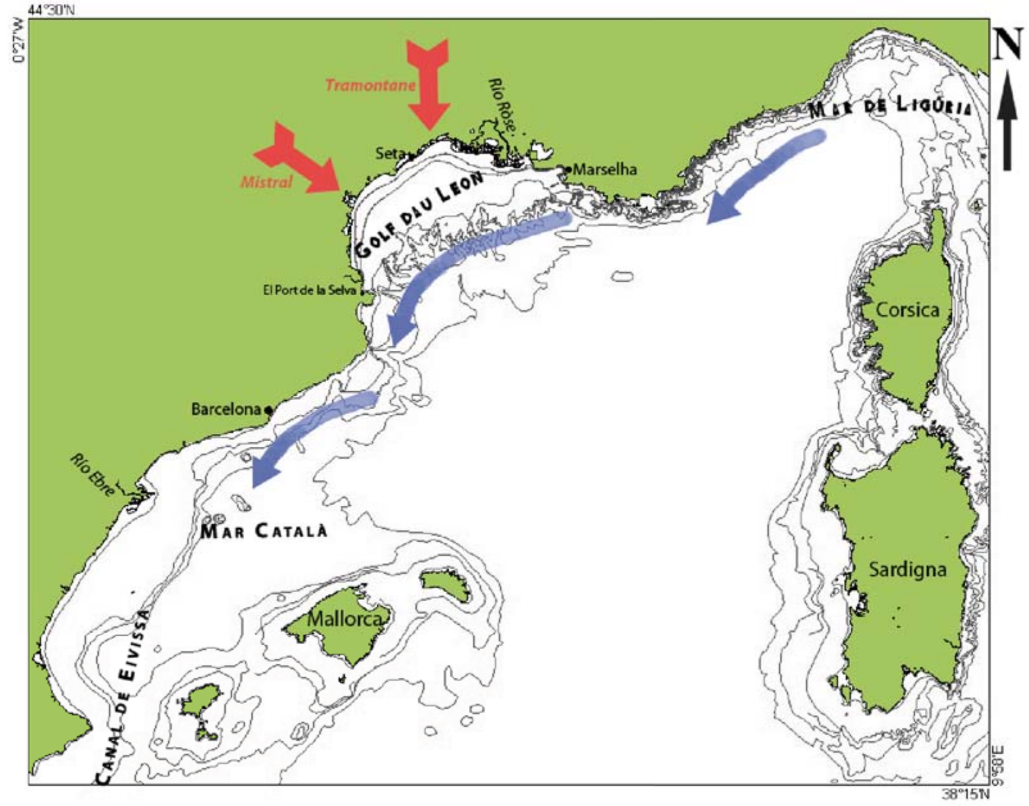

**Figura 3. Mapa del Mediterráneo noroccidental donde se señalan los vientos dominantes del norte y noroeste (flechas rojas) y la Corriente del Norte (flechas azules)**

Desde otoño hasta primavera, los vientos del suroeste pueden soplar con cierta intensidad, pero los vientos más fuertes en la región son los del norte y del noroeste, Mistral y Tramontana, respectivamente (Figura 3), que pueden soplar durante todo el año (Millot, 1990), a veces con especial violencia. Estos vientos inducen un desplazamiento de las aguas superficiales, más cálidas, desde la costa hacia mar abierto, originándose así eventos localizados de afloramiento (Johns *et al*., 1992).

Otro importante rasgo hidrográfico del golfo de León en invierno, especialmente cuando soplan los vientos fríos y secos del noroeste, es la formación de agua densa sobre la plataforma continental debido a la evaporación y el enfriamiento de las capas de aguas superiores. Las aguas densas pueden extenderse sobre el fondo marino y deslizarse por la plataforma y el talud continental, formando el denominado efecto de ‘cascading’ (Ulses *et al*., 2008).

La estratificación estacional de las aguas superficiales, una característica común en todo el Mediterráneo, es especialmente pronunciada en el golfo de León. A mediados de primavera se forma una termoclina, separando una capa de agua inferior (con una temperatura mínima de 13.5º C) de otra superficial (10-20 m) con una temperatura media de 20º C, pero con valores que alcanzan hasta 25º C en agosto. Al final de otoño, debido a la pérdida de calor, la temperatura superficial ha descendido lo suficiente como para atenuarse el gradiente térmico y la estratificación desaparece casi por completo, especialmente en la zona de mar abierto del golfo (Millot, 1990).

Las descargas de los ríos también marcan los patrones hidrológicos del golfo de León, en especial las provenientes del río Ródano, con un flujo medio de 1700 $m^3 s^{-1}$ (Thill *et al*., 2001). Estas aguas del Ródano se pueden llegar a detectar incluso en la plataforma continental de mar Catalán (Castellón *et al*., 1985) gracias a que son desplazadas por la Corriente del Norte (Bethoux, 1980).

El régimen climático en el Mediterráneo está altamente influenciado por la Oscilación del Atlántico Norte (NAO, siglas del inglés North Atlantic Oscillation), siendo particularmente afectadas por ésta la cantidad de precipitaciones, las descargas de los ríos y la variabilidad del nivel del mar (Tsimplis *et al*., 2006). También la Oscilación Atlántica Multidecadal (AMO, siglas del inglés Atlantic Multidecadal Oscillation), de mayor amplitud temporal, tiene efectos en las condiciones climáticas de la región (Lejeusne *et al*., 2010). El grado en el que tanto la NAO como la AMO, o incluso otras oscilaciones más regionales, pueden llegar a impactar sobre la vida marina en el Mediterráneo occidental está aún siendo discutido (Lejeusne *et al*., 2010, Martín *et al*., 2011).

De cualquier modo, lo que sí está demostrado es que el Mediterráneo noroccidental viene experimentando un aumento en la temperatura superficial en las últimas décadas (Salat & Pascual, 2011), cuyas consecuencias están aún por definir, y que ha habido un descenso en el volumen de descargas del río Ródano (Mikhailova, 2006) que podría conducir a una reducción en la productividad de la zona (Darnaude *et al*., 2004).

En relación directa con la variabilidad oceanográfica estacional aquí descrita, la comunidad planctónica en el Mediterráneo noroccidental también experimenta cambios en sus dinámicas y estructura a lo largo del año (Calbet *et al*., 2001). Esta comunidad planctónica y la inestabilidad de los parámetros oceanográficos juegan un papel fundamental en la estructura y la viabilidad de la red trófica marina del golfo de León, como se pretende demostrar en esta tesis.

Todas estas características hacen del golfo de León una región relativamente productiva en comparación con el resto del Mediterráneo (Estrada, 1996, Salat, 1996), que se puede considerar un mar oligotrófico (Huertas *et al*., 2012). El golfo de León es, por estas razones, una zona de cría (reproducción y crecimiento) muy importante tanto para *E. encrasicolus* como para *S. pilchardus* (Giannoulaki *et al*., 2011, 2012) y sostiene una destacable pesquería de peces pelágicos pequeños (Palomera *et al*., 2007) cuya productividad está íntimamente asociada a las caracterísiticas ambientales previamente descritas (Lloret *et al*., 2001, 2004).

### 1.3.2 Campañas oceanográficas.

En el golfo de León, dentro del marco del proyecto SARDONE, se llevaron a cabo, entre 2007 y 2009, 4 campañas oceanográficas en distintas épocas del año con el fin de recolectar las muestras y los datos necesarios para el desarrollo de la presente tesis.

Dichas campañas se realizaron a bordo del *N/O L'Europe,* perteneciente al Institut Français de Recherche pour l'Exploitation de la Mer (Ifremer), en las fechas comprendidas entre el 28 de julio y el 9 de agosto de 2007 (PELMED07), el 8 y el 21 de diciembre de 2007 (JUVALION07), el 21 y 29 de julio de 2008 (PELMED08) y del 11 al 27 de enero de 2009 (JUVALION09).

Durante todas las campañas mencionadas se recogieron muestras de plancton en diferentes estaciones de muestreo usando una red de plancton WP2 estándar, con una luz de malla de 200 µm, y otra red de plancton Calvet, con luz de malla de 53 µm. Una vez a bordo, las muestras recogidas con la red WP2 se tamizaban a través de una malla de 3000 µm para obtener una muestra de individuos de entre 200 y 3000 µm (mesozooplancton), y las muestras recogidas con la red Calvet se tamizaban a través de una malla de 200 µm de luz para obtener la fracción del plancton de entre 53 y 200 µm (microplancton). A continuación, todas las muestras de plancton de dividían en 2 mitades iguales gracias a un separador de plancton Motoda (Motoda, 1959). Una mitad se preservaba en una solución de agua marina con formol tamponado al 4 %, que se utilizaría para análisis cualitativos de la comunidad planctónica, y la otra mitad se congelaba a bordo (-20º C) para posteriores estimaciones de biomasa y/o análisis alternativos (p. ej., lípidos/ácidos grasos o isótopos estables).

El análisis cualitativo del plancton se realizaba en el laboratorio, y los individuos eran identificados hasta el nivel taxonómico más bajo posible con un estéreo-microscopio (Leica MZ12) con un aumento de hasta 100×. Las muestras de mesozooplancton se analizaban en alícuotas que representaban el 10 % de la muestra, hasta contabilizar, como mínimo, 400 copépodos. Se calculaban también el número de individuos de cada taxón identificado y sus abundancias (ind $\mathrm{m}^{-3}$).

Con un CTD Seabird 19 se tomaron medidas de temperatura, salinidad y fluorescencia en la columna vertical de cada una de las estaciones de muestreo del plancton, desde la superficie hasta 100 m de profundidad, o hasta el fondo si la profundidad era menor.

Las muestras de anchoas y sardinas de todas las tallas estudiadas se recogieron mediante redes de arrastre pelágico. A bordo del *N/O L'Europe* la red usada era una red de arrastre pelágico estándar, equipada con un copo de malla pequeña (longitud de malla: 5 mm; ISO 1107), que se remolcaba a una velocidad media de 3.6 nudos durante unos 30-40 min. Para localizar y estimar la abundancia de anchoas y sardinas en el mar durante las campañas, se usaron sondas multihaz Simrad EK500, operando a 38, 120 y 200 kHz (Diachok *et al.*, 2001), a lo largo de transectos perpendiculares a la línea de costa a una velocidad de 8 nudos, desde un fondo de 15-20 m hasta el talud.

## 1.4 Justificación del trabajo.

Como ya se ha mencionado en esta introducción, el papel ecológico de los peces pelágicos pequeños es crucial, como también lo es su relevancia económica, tanto en el Mediterráneo como en el resto del mundo.

Sin embargo, existen aún importantes huecos que rellenar en el conocimiento acerca de las larvas y los juveniles de anchoa, y de las sardinas en general, en el Mediterráneo noroccidental. En todo el conjunto del mar Mediterráneo es necesario un urgente y sustancial esfuerzo para mejorar y ampliar la información científica disponible para los gestores, y, particularmente, la Comisión General de Pesca del Mediterráneo (GFCM, del inglés General Fisheries Commission for the Mediterranean) recomienda llevar a cabo estudios concernientes a las edades no maduras de peces pelágicos pequeños, con vistas a mejorar las estimas del reclutamiento de estas especies.

Con el fin de evaluar y estudiar dichos parámetros, se puso en marcha el ya definido proyecto SARDONE, desde el cual se diseñó una tarea con las siguientes metas particulares: 1. establecer una imagen completa y exhaustiva de la ecología trófica de estas especies, 2. estudiar sus tasas de crecimiento durante las fases previas al reclutamiento, y 3. comparar su alimentación y su crecimiento durante dichas fases entre diferentes puntos del Mediterráneo y bajo condiciones ecológicas y oceanográficas dispares.

En particular, esta tesis se ha desarrollado dentro de, aunque sin limitarse a, las tareas relativas a ampliar los conocimientos sobre la ecología alimenticia de juveniles y larvas de anchoa y sardina en el golfo de León. Además, dada la importancia de la sardina en la región y la escasez de estudios concernientes a su biología, se ha hecho un esfuerzo aún mayor, fuera del marco del proyecto SARDONE, en el estudio de la ecología trófica de esta especie.

Un aspecto de especial interés ecológico es el de las interacciones tróficas entre anchoas y sardinas durante todas sus fases de desarrollo. Estas dos especies comparten el mismo hábitat pelágico durante toda su vida, y, por tanto, potencialmente podrían o bien llegar a ser competidores por las mismas presas o bien mostrar una repartición de los  recursos alimenticios. Desentrañar esta cuestión proporcionaría información esencial sobre la biología de estas dos especies y sobre cómo podrían evolucionar sus poblaciones en el cambiante medio marino.

Hasta la fecha, los estudios relacionados con la ecología alimenticia de la anchoa en el Mediterráneo noroccidental se han centrado, sobre todo, en la dieta de los adultos (Tudela & Palomera, 1997, Plounevez & Champalbert, 2000), y se ha puesto escasa atención en la dependencia que su comportamiento trófico puede tener en su morfología y en su ontogenia durante las fases larvarias (Tudela *et al*., 2002) y juveniles. En el caso de la sardina, la falta de conocimientos se extiende a todas sus tallas, incluyendo adultos, con la única excepción del trabajo de Lee (1961) y de otros dos estudios realizados en la región (Rasoanarivo *et al*., 1991 y Morote *et al*., 2010), aunque estos dos últimos centrados exclusivamente en larvas <15 mm LT.

Los estudios de los contenidos estomacales de estos peces proporcionan información directa sobre sus dietas (Tudela & Palomera, 1997, Conway *et al*., 1998, Plounevez & Champalbert, 2000, Borme *et al*., 2009, Morote *et al*., 2010), pero en ocasiones su análisis no es suficiente para evaluar cuáles han sido sus presas durante un periodo más largo anterior a la captura del pez, por lo que otros métodos, como por ejemplo el análisis de isótopos estables, podrían ser necesarios (Post, 2002, Polunin & Pinnegar, 2008). Además, la observación de las estructuras morfológicas relacionadas con la alimentación, que pueden verse modificadas durante el desarrollo ontogénico de los peces o ser utilizadas de un modo u otro en función de las características ambientales, también puede ser útil para estimar las dietas y compararlas entre especies (Castillo-Rivera *et al*., 1996).

Aunque los impactos del cambio climático sobre la productividad marina aún se están dilucidando, parece obvio, especialmente en latitudes templadas, que las consecuencias del calentamiento global en las pesquerías serán, en general, de una enorme trascendencia (Lehodey *et al*., 2006, Barange *et al*., 2009). El calentamiento general de los océanos ya está afectando a la distribución de ciertas especies pelágicas en el Mediterráneo (Sabatés *et al*., 2009), que podrían competir con anchoas y sardinas, y estos cambios en la composición y la estacionalidad de las especies podrían causar, a su vez, un desacoplamiento trófico entre las etapas de desarrollo más tempranas de los peces y sus presas, comprometiendo de este modo el éxito de su reclutamiento. Además de todo esto, la presión pesquera puede aumentar la sensibilidad de las poblaciones de peces a los cambios climatológicos (Hsieh *et al*., 2006).

## 2. Objetivos.

La comparación de los hábitos alimenticios de la anchoa y la sardina en diferentes estaciones del año, y bajo una situación de cambio climático, podría ayudar a desentrañar qué agentes medioambientales favorecen o dificultan la viabilidad de las poblaciones de estos peces en el Mediterráneo (Brown *et al*., 2004, Jennings *et al*., 2008).

Esta tesis tiene la finalidad de examinar y comparar, de manera exhaustiva, los comportamientos alimenticios de sardinas y anchoas en diferentes fases de su desarrollo, y durante distintas épocas del año, en el golfo de León, a la vez que pretende mostrar el análisis de algunos de los caracteres morfológicos relacionados con la alimentación de estas especies, como son las branquispinas y los ciegos pilóricos.

Como uno de los objetivos principales de esta tesis, se plantea un análisis en profundidad del comportamiento trófico de la sardina en todas sus fases de desarrollo (desde 15 mm LT) y en épocas distintas del año, con el objeto de describir de forma detallada su dieta y de identificar posibles cambios alimenticios que pudieran ocurrir a lo largo del desarrollo de la especie o en función de las condiciones ambientales, como ya se ha demostrado que ocurre en ésta y en otras especies similares en otros lugares (Bulgakova, 1996, Bode *et al*., 2004, Garrido *et al*., 2007, Tanaka *et al*., 2008).

Bajo la hipótesis de un posible solapamiento trófico entre sardinas y anchoas se propuso la comparación de los hábitos alimenticios de los juveniles en torno al punto de desarrollo en el que se espera que estas especies sean capaces de cambiar de tipo de dieta gracias a la presencia de, por ejemplo, branquispinas o ciego pilóricos. Por tanto, además de comprobar la hipótesis expuesta, este trabajo pretende establecer a qué talla ambas especies han desarrollado de manera completamente funcional sus estructuras morfológicas relacionadas con la alimentación.

Otro de los objetivos fundamentales de la presente tesis es proporcionar una idea general de las relaciones tróficas entre ambas especies, desde larvas hasta adultos, en distintas épocas del año (verano, otoño e invierno), y, por tanto, bajo diferentes condiciones ambientales, mediante el análisis de isótopos estables ($\delta^{13}C$ y $\delta^{15}N$), con el fin de determinar posibles cambios estacionales y/o ontogénicos en la selección de las fuentes de alimento y en los niveles tróficos de las dos especies. Además, se pretende esclarecer qué tipo de limitaciones pueden tener los estudios de dieta basados en el análisis directo de contenidos estomacales.

Esta memoria también expone el análisis de la condición nutricional de larvas de anchoa de tamaños similares encontradas en diferenrres épocas del año. Este análisis se basa en la hipótesis de partida de que las larvas presentes en épocas con condiciones ambientales consideradas como menos favorables, tendrían una condición nutricional inferior a la de las larvas encontradas en épocas más favorables y, por tanto, una probabilidad también menor de sobrevivir y reclutar.

Esta tesis, en resumen, pretende desarrollar un análisis en profundidad de la ecología trófica de las dos especies más importantes de peces pelágicos pequeños en el Mediterráneo, sardina y anchoa, y proporcionar resultados relevantes que permitan determinar, de la manera más precisa posible, los efectos que los futuros cambios ambientales pueden tener sobre sus poblaciones.

## 3. Discusión general y conclusiones.

### 3.1 Discusión general.

Desde los primeros estudios acerca de la ecología trófica de los peces pelágicos pequeños, hasta bien pasada la mitad del siglo XX, estas especies se consideraban estrictamente fitófagas (ver Checkley *et al*., 2009, y las citas que ahí se mencionan), pero desde finales del siglo pasado se ha venido comprobando que tanto anchoas como sardinas se alimentan también de zooplancton, aunque con una mayor proporción de fitoplancton en zonas con importantes afloramientos (van der Lingen *et al*., 2009). Como han demostrado ya otros autores (Bulgakova, 1996, Garrido *et al*., 2007, Tanaka *et al*., 2008), la capacidad de los clupeiformes para cambiar de un tipo de alimentación a otro les convierte en oportunistas, y les permite ser tróficamente flexibles, con una buena capacidad de adaptación a los cambios ambientales. Sin embargo, esta capacidad de adaptación no se presenta de igual modo en todas las especies de clupeiformes ni en todos los lugares que habitan.

En esta tesis, centrada en la anchoa y la sardina del golfo de León, hemos mostrado, a través de un detallado análisis de sus dietas y de sus dinámicas tróficas, de qué manera estas dos especies pueden interactuar entre ellas y con el medio, considerando especialmente la posibilidad de que se vean afectadas por las perturbaciones derivadas del cambio climático.

El primer capítulo expone que en la sardina, si bien tiene una dieta casi estrictamente carnívora al final de su etapa larvaria y hasta poco después de la metamorfosis, el desarrollo de sus branquispinas y ciegos pilóricos la habilitan para empezar a efectuar un posible cambio de dieta cuando alcanzan los 4 cm de longitud estándar (LE), y que al alcanzar los 7 cm LE ya ha desarrollado por completo dichas estructuras, por lo que es capaz de alimentarse de fitoplancton con total efectividad, como también se observa en sus contenidos estomacales. Aunque la importancia numérica de las diatomeas en los estómagos de juveniles y adultos de sardina es muy destacable, su relevancia se ve bastante reducida frente a presas más grandes y menos abundantes (p. ej., larvas de decápodos) cuando se analiza la importancia de las presas según su aporte de carbono. Se muestra además, aunque sin la posibilidad de demostrarlo con total certeza debido a la falta de replicaciones en las muestras, que se alimentan predominantemente de día.

Gracias a los resultados expuestos en el segundo capítulo se observa que los juveniles de anchoa y de sardina en el golfo de León no presentan competencia alimenticia interespecífica debido a sus ya marcadas diferencias en el número y disposición de sus branquispinas y en el número de ciegos pilóricos, que posibilitan que los juveniles de sardina puedan alimentarse de fitoplancton, mientras que los de anchoa siguen basando su dieta principalmente en copépodos. Por tanto, es de esperar que su adaptación a posibles cambios ambientales siga también patrones diferentes. Por ejemplo, en un supuesto escenario futuro en el que la comunidad planctónica tenga una especial abundancia de las especies de copépodos más pequeños (Conversi *et al*., 2009), con un aumento en la proporción de fitoplancton (Edwards, 2009) o incluso con un descenso en la proporción general de copépodos (Molinero *et al*., 2005), la población de sardina sería la más favorecida frente a la de anchoas.

Por otro lado, si, tal y como algunos autores apuntan (Gladan *et al*., 2009, Lejeusne *et al*., 2010), la comunidad planctónica se ve modificada hacia un dominio de las especies fitoplanctónicas no silíceas en lugar de diatomeas, que son la principal presa, en número, de las sardinas, serían éstas las que encontrarían mayores dificultades para alimentarse.

El tercer capítulo resume las interacciones tróficas de las dos especies, sardina y anchoa, desde larvas grandes (>15 mm LT) hasta adultos, demostrando que los estudios de isótopos estables (en particular $\delta^{13}C$ y $\delta^{15}N$) pueden ser necesarios para complementar las observaciones de los contenidos estomacales en especies como anchoa y sardina, y más aún si se pretende averiguar la dieta que han seguido durante un periodo más largo de lo que pueden mostrar los contenidos estomacales (que suelen ser las horas anteriores a la captura). El estudio muestra que el microplancton es la fuente principal de alimento de las larvas de ambas especies, pero que después se alimentan también en gran medida de cladóceros, en verano, y de apendicularias, probando, por un lado, que existe un cambio de dieta a lo largo del desarrollo y, por otro, que el análisis de isótopos estables es esencial para identificar presas potenciales, como por ejemplo las apendicularias, cuya contribución a la dieta cuando se observan directamente los contenidos estomacales suele subestimarse (Capitanio *et al*., 1997), al ser estas presas fácilmente digeribles (Capitanio *et al*., 2005). Hemos observado también que los niveles tróficos de ambas especies son similares, y desarrollado un innovador método de evaluación de sus preferencias alimenticias modificando el cálculo del índice de selectividad de Ivlev (Ivlev, 1961) basándolo en los datos de estimación de dieta del modelo de mezcla isotópica SIAR.

A pesar de que los estudios de dieta de las larvas han presentado dificultades durante el desarrollo del proyecto, al ser muy difícil encontrar contenidos estomacales en su tubo digestivo, hemos tenido la oportunidad de realizar un análisis sobre la condición nutricional y el crecimiento de las larvas de anchoa en ambientes muy contrastados. La aparición en diciembre de larvas de anchoa, de forma inesperada teniendo en cuenta el periodo de reproducción conocido (de abril a octubre; Palomera *et al*., 2007), nos sugirió la idea de realizar un estudio que nos permitiera determinar la influencia del ambiente en el desarrollo y posterior reclutamiento de esta especie. Las diferencias en la condición nutricional de ambas cohortes de larvas se evaluó comparando su composición lipídica y haciendo una estima de sus tasas de crecimiento a partir del análisis de los otolitos. La condición nutricional de las larvas de diciembre no difiere de la condición de las larvas de igual tamaño capturadas en verano, que es la época en que ocurre el pico de reproducción de esta especie; por lo tanto, no se puede confirmar si una de las dos cohortes de larvas tendría más probabilidades de supervivencia y de reclutar que la otra, y serían necesarias nuevas investigaciones para poder llegar a una conclusión más clara al respecto. Sin embargo, el hecho de que la tasa de crecimiento diario de las larvas de verano sí sea mayor que en diciembre induce a pensar que, ante una probable extensión del periodo de puesta debido a un calentamiento de las aguas superficiales, la temperatura sí influiría de manera significativa en el desarrollo de las larvas de anchoa, como otros autores han demostrado (Aldanondo *et al*., 2008). Estos resultados son especialmente relevantes en un contexto ecológico donde existe una tendencia positiva en el aumento de la temperatura, lo que podría implicar que la anchoa en el golfo de León extendiera su periodo de reproducción. Ello supondría la posibilidad de que larvas de anchoa y sardina coincidieran en el mismo momento, ya que la sardina inicia la puesta en otoño, y, por lo tanto, se generara una competencia trófica que podría comprometer a una de las dos especies.

Esta tesis demuestra que anchoa y sardina, a pesar de ser dos especies muy cercanas, han adaptado sus facultades y sus hábitos ecológicos en el Mediterráneo noroccidental con el fin de evitar la competencia interespecífica en todo lo posible, no sólo alternando sus periodos reproductivos sino también adaptando sus comportamientos tróficos. Aunque no hay indicios de limitación en la disponibilidad de alimento en la región en ninguna época, las larvas de ambas especies parecen alimentarse de presas muy similares (capítulo 3). Por lo tanto, en el caso de que llegaran a coincidir las larvas de ambas especies en la misma estación (capítulo 4), sí que existiría la posibilidad de solapamiento trófico entre ambas poblaciones, añadiendo un factor más de estrés a éstas. Cuando los individuos de las dos especies alcanzan los 4 cm LE, la posibilidad de solapamiento trófico se reduce gracias a que se empiezan a diferenciar las estructuras de filtración y digestión de alimento (capítulo 2) y, particularmente, la sardina (capítulo 1) puede empezar a alimentarse de presas que, por su reducido tamaño, no podría consumir antes de haber desarrollado dichas estructuras (ciegos pilóricos y branquispinas). Por tanto, cabría esperar que la adaptación de las dos especies ante cambios ambientales fuese diferente. Sin embargo, la escasa certeza aún existente respecto a los efectos que el posible aumento de temperatura puede tener sobre determinados factores oceanográficos y, especialmente, sobre la comunidad planctónica, no permiten establecer con seguridad de qué forma se verán afectados los peces pelágicos pequeños en el Mediterráneo. A este desconocimiento sobre la posible evolución en las dinámicas tróficas de la anchoa y la sardina se le ha de añadir el hecho de que tanto el espadín como la alacha, otras dos especies de peces pelágicos pequeños, son ahora relativamente más abundantes en el golfo de León que en años anteriores, añadiendo consecuentemente un factor más de presión sobre las poblaciones de anchoa y sardina.

Por lo tanto, la gestión de la pesca de estas especies, posiblemente el único factor casi por completo predecible de los que afectan a estas poblaciones, debería considerarse desde el principio de precaución e incluir medidas basadas en una visión más ecosistémica que únicamente en la dinámica poblacional de cada especie de forma independiente.

## 3.2 Conclusiones.

- La observación de los contenidos estomacales de sardina en el golfo de León indicó que esta especie tiene una dieta casi estrictamente carnívora al final de su etapa larvaria y hasta poco después de la metamorfosis. El desarrollo de branquispinas y ciegos pilóricos, que se inicia entre los 2-4 cm LE, habilitan a esta especie para empezar a efectuar un cambio de dieta cuando alcanzan los 4 cm LE, por lo que es capaz de alimentarse de fitoplancton a esta talla, y a partir de 7 cm LE ya posee una funcionalidad completa de dichas estructuras. Tanto juveniles como adultos de sardina se alimentaron en gran medida de cladóceros durante el verano, época en que estos están presentes, mientras que en épocas con mayor abundancia de plancton, como ocurría en invierno, ambos grupos de edad adquirieron un comportamiento alimenticio más filtrador, siendo entonces las diatomeas su principal presa. Las especies pequeñas de copépodos, como Corycaeidae o *Microsetella* spp., aparecían en casi todos los estómagos de juveniles y adultos, tanto en verano como en invierno, en unas proporciones también importantes y siendo seleccionadas con mayor preferencia que las demás presas. Al analizar la dieta en contenido en carbono de las presas encontradas en los estómagos, las larvas de decápodos, debido a su gran tamaño, adquirían una gran importancia en juveniles y adultos de sardina de ambas épocas.

- Los juveniles de anchoa del golfo de León basaron su alimentación principalmente en copépodos pequeños como Corycaeidae, *Microsetella* spp., *Oncaea* spp. y Clauso-Paracalanidae durante todo el año, aunque los cladóceros fueron también muy importantes en su dieta en verano. Al estimar la composición de la dieta en base al peso seco de las presas en lugar de en base a su abundancia numérica, las larvas de decápodos aparecieron como la principal fuente de alimento de los juveniles de anchoa en invierno.

- Los juveniles de anchoa y de sardina en el golfo de León no presentaron competencia alimenticia interespecífica en ninguna de las épocas estudiadas debido a las ya marcadas diferencias en el número de branquispinas y de ciegos pilóricos que presentan. Por tanto, es de esperar que su adaptación a posibles cambios ambientales siga también patrones diferentes, en función de la evolución de la comunidad planctónica. Una tendencia en la comunidad planctónica hacia un mayor dominio de especies más pequeñas y/o fitoplanctónicas sería más favorable para la sardina dado su comportamiento trófico, mientras que si, por el contrario, los cambios ambientales tienden a generar una mayor abundancia de especies planctónicas relativamente más grandes, la más favorecida podría ser la anchoa.

- Los análisis de isótopos estables han resultado ser una herramienta necesaria para complementar las observaciones de los contenidos estomacales en los estudios de dieta, por ejemplo, para detectar presas que al ser fácilmente digeribles podrían ser subestimadas con los métodos de observación directa, como ocurrió con las apendicularias, las cuales aparecieron como presas muy importantes tanto para anchoa como para sardina, especialmente en otoño. El análisis de isótopos estables demostró la existencia de un cambio de dieta a lo largo del desarrollo en sardina y anchoa, así como también que los niveles tróficos de ambas especies son similares.

- La similitud en la condición nutricional encontrada en las larvas de anchoa capturadas en agosto y en diciembre impide afirmar si una de las dos cohortes de larvas podría tener más probabilidades de supervivencia que la otra. Sin embargo, la tasa de crecimiento diario de las larvas de verano fue sustancialmente mayor que en diciembre, indicando que, en el golfo de León, la temperatura puede ser un factor más limitante que la disponibilidad de alimento para el desarrollo de estas larvas. Por otra parte, la presencia de larvas de anchoa en diciembre parece indicar la extensión del periodo de puesta de esta especie, probablemente debido a la tendencia actual en el aumento de la temperatura del agua. Sin embargo, la viabilidad de estas larvas en el golfo de León sí podría estar comprometida por una posible competencia trófica con las larvas de sardina.

## 3.3 Posibles aplicaciones y futuras perspectivas.

La descripción de las dietas de anchoas y sardinas durante sus fases larvaria, juvenil y adulta en el golfo de León (alguna de las cuales era hasta ahora completamente desconocida) tienen la utilidad potencial de formar parte de los datos tróficos necesarios para mejorar los actuales modelos ecológicos (Ecopath / Ecosim) que se han desarrollado recientemente para el Mediterráneo (Coll & Libralato, 2012): en el golfo de León (Banaru *et al.*, 2012), en el mar Catalán (Coll *et al.*, 2006), en el mar Adriático (Coll *et al.*, 2007, Libralato *et al.*, 2010) y en el mar Egeo (Tsagarakis *et al*., 2010). Los peces pelágicos pequeños son componentes fundamentales en cualquier modelo ecológico marino, incluso cuando no se consideran los importantes efectos de elementos antropogénicos (p. ej., pesca) dentro de la modelización (Libralato *et al*., 2005). Estos modelos se basan en interacciones tróficas y posibilitan definir cuantitativamente la estructura y el funcionamiento de los ecosistemas marinos explotados (Christensen & Pauly, 1992). Detallar las distintas fracciones del desarrollo ontogénico de las especies clave incluidas en estos modelos es uno de los aspectos más importantes que aún se están desarrollando. De igual modo, este trabajo permitirá además ampliar la comprensión de las dinámicas del reclutamiento de estas especies en un contexto ecosistémico.

Esta aproximación no proporcionará una imagen directa de una solución inmediata de los problemas de gobernación de las actividades pesqueras, pero la utilización en conjunto tanto de estas evidencias científicas y ecológicas basadas en la observación del medio, como de los resultados obtenidos de las modelizaciones será básica en el desarrollo y la aplicación de una gestión pesquera ecosistémica y sostenible.

Paralelamente a los modelos ecológicos que los resultados de esta tesis puedan ayudar a desarrollar, sería deseable también realizar estimaciones, mediante experimentos en laboratorio o en el mar, de los requerimientos energéticos de anchoa y sardina para estimar, a su vez, el flujo de materia y energía que se canaliza desde los niveles tróficos más bajos hasta los más altos. De esta forma, se obtendría un conocimiento muy preciso de la estructura del ecosistema pelágico. Asimismo, estudios de la ecología trófica de los potenciales depredadores de anchoa y sardina serían un paso necesario para tener una idea más completa del funcionamiento de dicho ecosistema, al tiempo que podría ayudar a explicar mejor ciertos comportamientos de las especies de pelágicos pequeños que se ven influidos por la presencia de depredadores.

Entre estos comportamientos que áun requieren un estudio más detallado, pero cuya idea general se ha podido vislumbrar en esta tesis, destacan los relacionados con los ciclos circadianos de sardina y de anchoa y los momentos del día que estas especies elijen para alimentarse. Por tanto, un análisis más exhaustivo de los índices de repleción estomacal, con muestras que comprendan varios ciclos consecutivos de 24 horas, es casi de obligado desarrollado. Este índice de repleción permite evaluar la tasa de consumo diario de las especies (Tudela & Palomera, 1995), que es un elemento básico para el desarrollo de cualquier modelo trófico.

Dada la importancia que ciertas especies de pequeños pelágicos están adquiriendo en el golfo de León (como la alacha o el espadín) y de la importante presencia de otras más habituales y con hábitos alimenticios posiblemente similares a los de anchoas y sardinas, como el jurel o la caballa en sus fases juveniles, es indudable la necesidad de estudiar también sus dinámicas tróficas y las interacciones entre todas estas especies.

Por último, uno de los aspectos más relevantes de estas especies, más allá de las cuestiones meramente ecológicas, es su papel tan crucial en la seguridad alimentaria de muchas comunidades humanas costeras, especialmente en países en desarrollo, siendo éste un tema que, desde un punto de vista más personal, requiere una aproximación también social, política y económica para permitir, basándose en asunciones biológicas, una gestión de estos recursos que pueda garantizar la soberanía alimentaria de los pueblos que dependen de dichos recursos.

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## 5. Informe de la directora.

El doctorant David Costalago Meruelo presenta la seva tesi doctoral titulada ‘**Trophic ecology of small pelagic fish in the northwestern Mediterranean**’ / ‘**Ecología trófica de peces pelágicos pequeños en el Mediterráneo noroccidental**’.

La directora de tesi, Dra. Isabel Palomera Laforga, informa que la tesi està composta de quatre treballs de gran qualitat científica en format article. Tres dels treballs han estat sotmesos a revistes científiques internacionals incloses al Science Citation Index (SCI), dos dels quals han estat publicats, els altres dos seran sotmesos en breu. A continuació es detalla la contribució científica que ha realitzat el doctorant en cadascun dels articles, així com el factor d’impacte (segons Thomson Institute for Scientific Information) de les revistes.

1. **Feeding behaviour of European pilchard: from late-larva to adult.**

Autors: David Costalago, Isabel Palomera.
Revista (probable): Journal of Sea Research.
Factor Impacte 2007-11: 2.68.

El doctorant ha contribuït en tot el treball de mostreig de plancton i peixos a les campanyes oceanogràfiques com a responsable, en l’anàlisi de continguts estomacals i en el tractament de les dades i redacció de l’article.

2. **Comparison of the diets of European anchovy *Engraulis encrasicolus* and sardine *Sardina pilchardus* juveniles in the Gulf of Lions during different seasons.**

Autors: David Costalago, Isabel Palomera, Valentina Tirelli.
Revista (sotmès): Marine Biology.
Factor Impacte 2007-11: 2.47.

El doctorant ha contribuït en tot el treball de mostreig de plancton i peixos a les campanyes oceanogràfiques com a responsable, en l'anàlisi de continguts estomacals i el tractament de les dades i redacció de l'article.

3. **Ontogenetic and seasonal changes in the feeding preferences and trophic levels of two small pelagic fish species.**

Autors: David Costalago, Joan Navarro, Ignacio Álvarez-Calleja, Isabel Palomera.
Revista: Marine Ecology Progress Series 460 (2012).
Factor Impacte 2007-11: 3.09.

El doctorant ha contribuït en tot el treball de mostreig de plancton i peixos a les campanyes oceanogràfiques com a responsable, en la preparació de mostres per l'anàlisi d'isòtops estables i el tractament de les dades i redacció de l'article.

4. **Ecological understanding for fishery management: Condition and growth of anchovy late larvae during different seasons in the Northwestern Mediterranean.**

Autors: David Costalago, Samuele Tecchio, Isabel Palomera, Ignacio Álvarez-Calleja, Andrés Ospina-Alvarez, Sacha Raicevich.
Revista: Estuarine, Coastal and Shelf Science 93 (2011).
Factor Impacte 2007-11: 2.62.

El doctorant ha contribuït en el mostreig de les larves, en la preparació de mostres per l'anàlisi de àcids grassos, en l'anàlisi de lípids i el tractament de les dades i la redacció de l'article.

Els coautors participants en els articles que componen aquesta tesi no han utilitzat, implícitament o explícitament, cap d'aquests treballs per a l'elaboració d'altres tesis doctorals.

Signat:

Dra. Isabel Palomera Laforga
Directora de la Tesi
Barcelona, 17 de setembre de 2012

# PARTE II

## CAPÍTULOS - PUBLICACIONES

# Capítulo 1. COMPORTAMIENTO ALIMENTICIO DE LA SARDINA EUROPEA: DE LARVA A ADULTO.

**Resumen.** En este trabajo analizamos la importancia relativa de distintos tipos de presas de sardina en todas sus fases de desarrollo, desde larvas grandes hasta adultos. Hemos utilizado dos metodologías diferentes en el análisis de los contenidos estomacales para analizar la dinámica trófica de la sardina, y descrito cómo se relaciona el comportamiento trófico de esta especie con el desarrollo ontogénico de sus estructuras corporales relacionadas con la alimentación, como las branquispinas y los ciegos pilóricos. Esto es esencial si pretendemos describir cómo utiliza la sardina los recursos planctónicos en la región y discutir hasta qué punto sus poblaciones en el Mediterráneo podrían verse afectadas por cambios ambientales. Mostramos que los cladóceros, en verano, y las diatomeas, en invierno, fueron las presas más importantes, en número, para juveniles y adultos. Sin embargo, en relación al contenido en carbono, las larvas de decápodos fueron las presas más importantes en todas las estaciones, lo que indica la importancia de tener en cuenta la metodología utilizada para inferir la dieta de la sardina. Considerando la composición del plancton en el medio, las especies de copépodos pequeños eran seleccionadas preferentemente por las sardinas de todas las tallas y en todas las estaciones. También mostramos que los ciegos pilóricos empiezan a desarrollarse cuando la sardina alcanza los 4-5 cm de longitud estándar (LE), y terminan su desarrollo a una talla próxima a 8 cm LE, mientras que las branquispinas parecen ser completamente funcionales al alcanzar las sardinas una talla de 7 cm LE. Ello implica que pueden filtrar pequeñas partículas con total eficacia a partir de los 7-8 cm LE en adelante. En vista de la ventaja energética que representa la alimentación por filtración para las especies bien adaptadas a este método de captura de presas, como es la sardina, una eventual limitación en la disponibilidad de presas más pequeñas, tal y como prevén algunos autores que pudiera ocurrir en el Mediterráneo, podría tener consecuencias negativas para esta especie. Este trabajo demuestra que las poblaciones de sardina, dada su alta dependencia directa en los niveles tróficos inferiores, pueden verse fuertemente afectadas por alteraciones ambientales y de la comunidad planctónica.

# Feeding behavior of European pilchard: from late-larva to adult.

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**ABSTRACT.** We assessed the relative importance of different prey types of sardine, from late-larvae to adults. Two different methodologies of analysis of stomach contents were used in order to describe the trophic dynamics of sardine and how its feeding behavior is related to the ontogenetical development of its feeding body structures, such as gill rakers and pyloric caeca. This is essential if we want to accurately depict how sardine utilize the planktonic resources in the area and to discuss to which extent sardine population could be affected by environmental changes in the Mediterranean sea. We showed that cladocerans in summer and diatoms in winter were numerically the most important prey types for both juveniles and adults. However, regarding the carbon contents, decapod larvae were the most important prey in all seasons, so differences in the methodology should be taken into account when analyzing sardine diets. Considering the plankton composition, small copepods were highly selected by sardines at all ages and in both seasons. We also saw that pyloric caeca started growing when sardine were about 4-5 cm SL, and stopped developing when sardine reached around 8 cm SL, while gill rakers appeared to be completely functional when sardines reached 7 cm SL. Therefore, filter-feeding of small particles could be done with total efficacy from 7-8 cm SL on. In light of the energetic advantage of filter-feeding for a well-adapted species on filtering food as sardine, prospective limited availability of small particles, as some authors hypothesize it could happen in the Mediterranean, could have negative consequences for sardine. This work demonstrates that sardine populations, given their extremely high dependence on the lower marine trophic levels, can be strongly affected by alterations in the environment and the planktonic community.

KEY WORDS: *Sardina pilchardus* · northwestern Mediterranean · ontogeny · trophic ecology · small pelagic fish

## 1. Introduction

Nowadays, most marine fish ecologists accept that the dietary habits of a fish species may depend upon both the availability of the preys (Frederiksen *et al.*, 2006) and the anatomy of the fish (Gerking, 1994). However, the mechanisms that fishes employ for feeding are diverse. In clupeids, generally 2 different feeding methods are assumed, namely particulate (selective) and filter (non-selective) feeding (James, 1986). The switch from one feeding mode to the other depends mainly on the concentration of food (Bulgakova, 1996), and can be shifted also following the presence and abundance of particular preys (van der Lingen, 1994) if the ontogenetical development of the individuals allows it (Turingan *et al.*, 2005). Therefore, changes in the lowest trophic level of the ecosystem can have drastic consequences in the fish recruitment (Cushing, 1990, Beaugrand *et al.*, 2003), particularly in the small pelagic fish species, which preys are exclusively planktonic (Durbin, 1979, Blaxter & Hunter, 1982, Checkley *et al.*, 2009).

The European sardine or pilchard *Sardina pilchardus* is a fast growing and short-lived small pelagic fish species. It is one of the most important fish resources all along its distribution area in the northeast Atlantic, from the North Sea to the Senegalese coast, including the Mediterranean and the Black seas. Studies about its feeding dynamics have been carried out in the Spanish Atlantic coast, where the diets of juveniles and adults of sardine have been found to be entirely composed by plankton (Bode *et al.* 2004, Garrido *et al.*, 2007, 2008) and where adults are able to perform both filter and particulate feeding, therefore adults presenting a higher fraction of phytoplankton in their stomachs than juveniles. On the other hand, in the same region, it is been showed that sardine larvae feed basically on copepods (Conway *et al.*, 1994, Munuera & González-Quirós, 2006).

In the western Mediterranean sea there are very few works regarding this issue, with 2 classical studies (Massuti & Oliver, 1948, Lee, 1961) that considered sardine to be mainly zooplanktivorous, especially after reaching the juvenile stage, and only 2 other more recent studies by Rasoanarivo *et al.* (1991) and Morote *et al.* (2010), in the Gulf of Lions and in the Catalan sea, respectively, both focused strictly on larvae smaller than 15 mm SL. Also, concerning all the ontogenetical stages, from larvae to adults, Costalago *et al.* (2012) studied sardine diet in the Gulf of Lions using stable isotopes.

Therefore, given the scarcity of information on the trophic ecology of sardine in the western Mediterranean, with this work we intend to fill that gap of knowledge and to contribute to better understand the functioning of the pelagic system in the northwestern Mediterranean. In addition to this gap, the ecological models based on trophic web interactions and capable to quantitatively describe the structure and functioning of exploited marine ecosystems (e.g. Ecopath/Ecosim, see Banaru *et al.*, 2012, Coll *et al.*, 2006) have described sardine in the northwestern Mediterranean as a key species in the trophic web; nevertheless, they used data on the diet of sardine from other regions but the northwestern Mediterranean, like the East Mediterranean (Demirhindi, 1961) or the Atlantic coast of Spain (Bode *et al.*, 2004, Garrido *et al.*, 2008). Moreover, to detail ontogenetic fractions of key species included in the models (like sardine), from an ecological point of view, is one of the most important elements to be developed.

The area of study, the Gulf of Lions, in the northwestern Mediterranean, is highly productive compared to the generally oligotrophic Mediterranean sea (Huertas *et al.*, 2012), mainly due to the water discharges from the Rhône river and the dominant northern winds that spread the nutrients and particulate organic matter all along the continental shelf (Estrada, 1996, Salat, 1996) and can even cause occasional small upwelling events (Forget & André, 2007). This area constitutes, in addition, a very important nursery habitat for both European anchovy (*Engraulis encrasicolus*) and sardine (Giannoulaki *et al.*, 2011, 2012). Thus, the Gulf of Lions holds an important fishery of small pelagic fish, particularly of anchovy and sardine (Lleonart & Maynou, 2003, Palomera *et al.*, 2007), that is directly linked to the above described environmental features (Lloret *et al.*, 2004).

However, according to the General Fisheries Commission for the Mediterranean (GFCM; see GFCM, 2011), during the last decade sardine in the Gulf of Lions has experienced a decrease both in tonnes of catches and in population biomass; in addition, their mean size-at-age has also decreased in the last years (Voulgaridou & Stergiou, 2003, D. Roos, personal communication for data from 2005 on), with potential consequences for the viability of the population. To maintain this commercial fishing activity on this population without jeopardizing the future of the stock, management must be based on comprehensive researches about the ecology of the exploited species and the environment. So, environmental and trophodymanic drivers must be soundly studied because fish biomass trends, and therefore all subsequent anthropogenic actions such as fisheries, depend enormously on these drivers (Fu *et al.*, 2012).

In this work we assessed the relative importance of different prey types of sardine using 2 methodologies of analysis of stomach contents. The first method takes into account the number of every prey item in the stomachs, so an overestimation of smaller preys that are easily filtered during the feeding process can be expected; and the second method estimates the carbon content of prey items, given an idea of the actual importance that each prey type can have in the carbon flux from one trophic level to the next.

Also, a first attempt to find a pattern in the daily ration and consumption rates was carried out and described in the present study. Allegedly because of the practical difficulties that this type of observations have at sea, to our knowledge, there are no previous field studies concerning the daily feeding activity of sardine in the region. The information obtained through a complete analysis of the daily ration could be useful, not only to elucidate the extent of the relevance of the vision of sardine for its feeding behavior (Hubbs & Blaxter, 1986), but also to validate the ongoing development of bioenergetic models for small pelagic fish species (Urtizberea *et al.*, 2008).

The final aim of this study was to describe the trophic dynamics of sardine from late-larvae to adults through the analysis of their stomach contents and how the feeding behavior is related to the ontogenetical development of its feeding body structures such as gill rakers and pyloric caeca. This is essential if we want to accurately depict how sardine utilize the available planktonic resources in the area and to discuss the way that prospective changes in the plankton community could affect the sardine populations in the Mediterranean sea.

## 2. Materials and methods

### *2.1. Study area and sample collection*

The study was conducted in the Gulf of Lions (Figure 1), one of the most productive areas of the northwestern Mediterranean (Salat, 1996). In terms of fish biomass, it is also the most important area of the Mediterranean for small pelagic fish species (Barangé *et al.*, 2009).

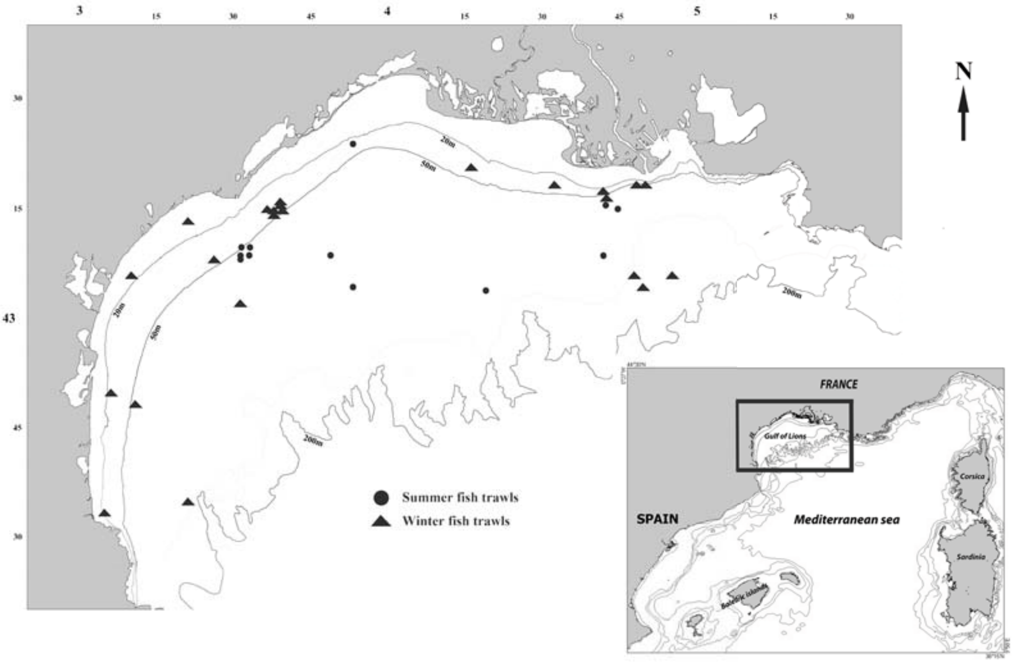

**Fig. 1. Study area (gulf of Lions, NW Mediterranean), indicating fish sampling locations in summer and winter.**

Two cruises were performed in 2 different seasons (summer and winter) on board the *N/O L'Europe* (Ifremer, France). The summer cruise (PELMED07) was conducted in July-August 2007, and the winter cruise (JUVALION09) was conducted during January 2009.

Plankton samples were collected during each season (16 plankton sampling stations in summer and 13 in winter) using a standard WP2 net with a mesh size of 200 µm and sieved through a 3,000 µm plankton mesh to obtain the 200-3000 µm mesozooplankton fraction. Also, to obtain the 53 to 200 µm fraction of microplankton, a scaled-down WP2 net with a mesh size of 53 µm was used, and the samples were sieved through a 200 µm plankton mesh. All plankton samples were split with a Motoda plankton splitter (Motoda, 1959). One-half of each sample was preserved in buffered 4% formaldehyde-seawater solution for subsequent qualitative analyses of plankton community composition, whereas the other half was frozen (-21º C) on board for biomass measurements.

We collected sardine juveniles (Standard Length (SL) range: 4.0-11.0 cm) and adults (SL range: 11-19.5 cm) in both cruises and also late-larvae (SL range: 2.2-3.7 cm) in winter, as resumed in Table 1. The size from which individuals were considered adults (11 cm) was based on the observations by Tsagarakis *et al.* (2012) of the shift in schooling behavior (10.7 cm), almost coinciding with the minimum landing size for sardine in the Mediterranean (11 cm, EC 1967/2006). In order to perform the daily ration analysis, consecutive hauls were performed during a 24-hour cycle in each cruise, every 3 hours in PELMED07 and every 4 hours in JUVALION09. All specimens were caught with a pelagic trawling net equipped with a small-mesh codend (mesh length 5 mm, ISO 1107) and towed at an average speed of 3.6 knots over a 30-40 min period. The samples were immediately frozen (-21º C) after sorting on board by groups of age.

**Table 1. Mean standard lengths (SL) and SL ranges, in cm, of sardines caught during the summer 2007 and winter 2009 cruises.**

| | Late-larva | Juveniles | | Adults | |
|---|---|---|---|---|---|
| | Winter | Summer | Winter | Summer | Winter |
| N. of individuals caught | 334 | 223 | 272 | 367 | 340 |
| Mean SL | 3.09 | 7.73 | 9.52 | 13.05 | 12.42 |
| SL range | 2.2-3.7 | 4.0-10.9 | 7.8-10.9 | 11-17.5 | 11-19.5 |

## *2.2. Plankton analysis*

Qualitative analysis of plankton was performed in the laboratory, and individuals were identified to the lowest taxonomical level possible under the stereomicroscope (Wild M12, at 100× magnification). The mesozooplankton samples were analyzed in aliquots representing about 10 % of the sample and repeated until counting at least 400 copepods in each; additional subsamples were also taken for any other abundant organism (i.e. cladocerans during summer). Microplankton samples were subsampled differently: 1 to 2 % of the original volume was analyzed to estimate the presence of nauplii, dinoflagellates, ciliates and diatoms; small copepods (mainly copepodites) were analyzed in volumes sufficient to count at least 400 individuals. Individuals of each identified taxon were counted and abundances (ind $m^{-3}$) calculated. The genera *Paracalanus*, *Ctenocalanus*, *Clausocalanus* and *Parvocalanus* were classified as the 'Clauso-Paracalanidae' group.

Differences between seasons regarding plankton biomass and abundance were calculated with non parametric Kruskal-Wallis tests. Significance levels were adopted at $p < 0.05$.

## *2.3. Diet composition analysis*

In total, the stomachs of 334 late-larvae, 145 juveniles and 268 adults of sardine were dissected and open under a stereomicroscope. The contents in the intestine were discarded to reduce bias caused by different rates of digestion and gut passage times or cod-end feeding (Hyslop, 1980). No items were found in the esophagus, so regurgitation due to sampling stress was discarded. Only food items that could be identified were recorded (e.g., van der Lingen, 2002). For juveniles and adults, pools of the contents of up to 20 stomachs for each tow, when available, were diluted to a known volume of filtered seawater as in van der Lingen (2002), and stomachs of late-larvae were analyzed individually. All the preys were counted and identified up to the lowest possible taxonomical level and their widths and lengths were measured, if distinguishable, using the software ImageJ 1.4 (Abramoff *et al.*, 2004).

A SIMPER analysis was applied in order to elucidate the most important prey species. Only prey species with a percentage of contribution >10 % are showed in the results. The SIMPER analysis also provided the average dissimilarity between seasons regarding the prey compositions of both juveniles and adults. One-way analysis of similarity (ANOSIM) was used to test significance of the differences in the diet composition between seasons and size classes. PRIMER software package (Version 6.1.9) (Clarke & Warwick, 2001) was used to perform both SIMPER and ANOSIM analysis, grouping prey species categories based on Euclidean distances and on Bray-Curtis mean similarities, respectively.

The contribution of every prey item to the diet was calculated with the index of relative importance (IRI, Pinkas *et al.*, 1971) based on the equation:

$$IRI = (\%W_i + \%N_i) * \%FO_i$$

where $W$ = dry weight of prey type $i$ in µg, $N$ = number of individuals of prey $i$ in stomachs and $FO$ = frequency of occurrence of prey $i$ in stomachs. $W$ was obtained from estimations by Uye (1982), Saiz & Calbet (2007) and Borme *et al.* (2009).

Prey selectivity was estimated by means of Ivlev´s diet selectivity index, E

(Ivlev, 1961) for each case analyzed. The value of the index was calculated with the equation:

$$E = (r_i - p_i) / (r_i + p_i)$$

where $r_i$ is the proportion of prey item *i* in the fish stomach and $p_i$ is the proportion of prey item *i* available from the marine environment. Fraction samples of mesozooplankton and microplankton were considered together, as total plankton, for the calculation of the Ivlev's index.

Carbon contents of every prey type, used for comparison with numerical contents, were estimated using equations and tables from Espinoza & Bertrand (2008) and Borme *et al.* (2009).

### *2.4. Anatomycal analysis*

For the morphological study of fish, different measures were taken: body wet weight (BW) in grams, and standard and total body lengths (SL and TL, respectively) in millimeters. 13 specimens of late-larvae between 2.6-4.0 cm SL, 56 of juveniles between 4.0-11.0 cm SL and 67 of adults between 11.0-19.5 cm SL were randomly selected from the 2 cruises to count the number of pyloric caeca and to measure the characteristics of gill rakers, following the procedures of Tanaka *et al.* (2006), in the first branchial arch (lower or ceratohypobranchial branch) of the left side of the body (Figure 2): the number of gill rakers was counted, and the length of the gill rakers ($L_{GR}$) and the width of the gill raker spacings ($S_{GR}$) in mm were measured. The $L_{GR}$ was averaged from five long gill rakers in the middle of the gill arch and the $S_{GR}$ from five gill raker spacings at the basis of these rakers. Data on number of pyloric caeca after 12 cm SL were not presented. Measurements were also made with the software ImageJ 1.4.

ANOVAs were used to test the significance of the slopes of the regressions between anatomical variables.

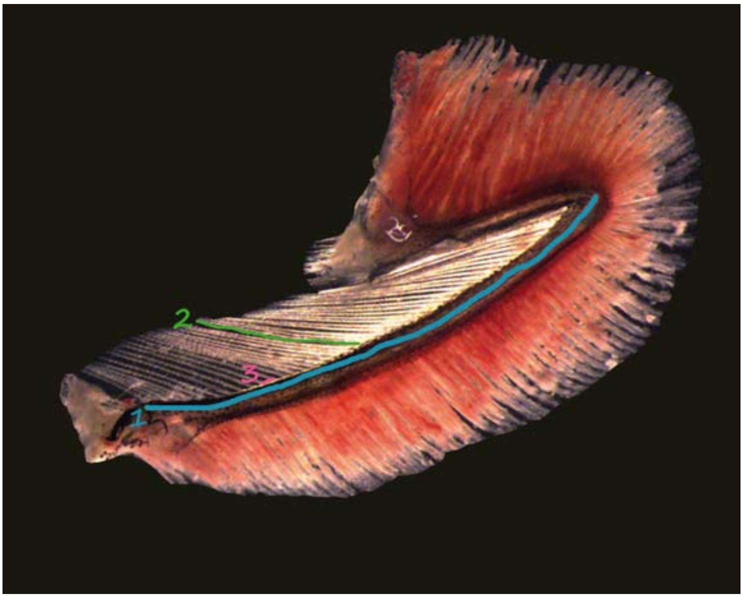

**Fig. 2. Photo of the first left branchial arch of a sardine adult, indicating how different structures were measured: 1) length of ceratohypobranchial arch (blue line); 2) length of a central gill raker (green line); 3) space between 2 consecutive gill rakers, at their bases (pink line).**

### *2.5. Daily ration analysis*

Up to 20 stomachs from each haul within the 24-h cycle in each season were dissected and the stomach contents removed carefully. Dissection took place under a stereo-microscope and the whole content of each stomach was extracted individually in a petri dish. As explained before for the diet analysis, the contents in the intestine were discarded in this analysis and no regurgitation was detected. Particular care was used in order to separate the stomach epithelium from preys. The contents of each stomach were filtered on pre-dried, pre-weighed Whatman GF/C filters (25 mm Ø) and subsequently dried at 60° C for 48 h. Dry weight (DW) of stomach content was measured to the nearest 0.1 mg.

The stomach fullness index for each individual was calculated by dividing the DW of the stomach contents by the total fish wet weight (wet BW), according to the equation:

$$SFI = (DW / wetBW) * 1000$$

Daily ration estimations were carried out by using 2 different models in which the mean SFI value from each tow was used:

a) Elliott & Persson (1978) model.

Consumption over a given time interval *t* is described by the equation:

$$C_t = \frac{Rt\left(S_t - S_0 e^{-Rt}\right)}{1 - e^{-Rt}}$$

where $C$, is consumption over time $t$, $S_t$, is the mean stomach fullness index over time $t$, $S_0$ is the mean stomach fullness index starting at time 0, and $R$ is the instantaneous gastric evacuation rate (Elliott & Persson, 1978). Total consumption over an entire cycle ($C_t$) is equal to the sum of partial consumptions calculated for the n time intervals between tows over a complete cycle:

$$C_t = \sum_{1}^{n} C_{tn}$$

b) Eggers (1979) model.

Consumption over a given time interval t is described by the equation:

$$C_t - (S_t - S_0) = \bar{S}Rt$$

where $C_t$, is consumption over the feeding interval considered, $S_t$, is the mean stomach fullness index at the end of the interval, $S_0$ is the mean stomach fullness index at the beginning of the interval, $S$ is the mean stomach fullness index over the entire interval, $R$ is the instantaneous evacuation rate, and $t$ is interval duration.

For both models gut evacuation rate ($R$) was calculated as described by the Elliot (1972) equation:

$$S_t = S_0\, e^{-Rt}$$

where $S_t$ is the mean stomach fullness index at time $t$, $S_0$ is the mean stomach fullness index at the beginning of the time interval and $R$ is the instantaneous evacuation rate.

A semi-logarithmic transformation of this equation gives a linear model:

$$ln\, S_t = ln\, S_0 - R_t$$

This equation was used to calculate $R$ for each consecutive pair of samples with declining mean stomach fullness index value. The maximum value calculated was selected to represent the instantaneous gastric evacuation rate.

## 3. Results

### *3.1. Plankton composition*

Information on total microplankton and mesozooplankton abundance (ind m$^{-3}$) and biomass (mg m$^{-3}$) during the surveys is summarized in Table 2. Abundance and biomass of both mesozooplankton and microplankton were higher in winter, except microplankton biomass, but no significant differences were found between the summer and winter abundances of both microplankton and mesozooplankton, neither mesozooplankton nor microplankton biomass were found significantly different between the 2 seasons.

**Table 2. Mesozooplankton and microplankton stock in terms of abundance and biomass during the 2 periods (Min: minimum, Max: maximum, SD: standard deviation).**

| | | | Summer | Winter |
|---|---|---|---|---|
| Mesozooplankton | Total abundance (ind m$^{-3}$) | Min | 3767.12 | 1211.68 |
| | | Max | 11881.19 | 34522.29 |
| | | Mean | 8024.19 | 14559.75 |
| | | SD | 2484.35 | 12273.61 |
| | Biomass (mg m$^{-3}$) | Min | 14.20 | 7.62 |
| | | Max | 54.57 | 484.14 |
| | | Mean | 33.65 | 53.25 |
| | | SD | 10.34 | 96.64 |
| Microplankton | Total abundance (ind m$^{-3}$) | Min | 11186.63 | 44731.10 |
| | | Max | 452339.33 | 487819.55 |
| | | Mean | 107734.67 | 299637.64 |
| | | SD | 138214.79 | 194726.17 |
| | Biomass (mg m$^{-3}$) | Min | 9.10 | 7.81 |
| | | Max | 907.21 | 335.12 |
| | | Mean | 190.01 | 52.91 |
| | | SD | 277.53 | 72.62 |

Plankton composition was mainly dominated by phytoplankton (mainly diatoms and dynoflagellates) and copepods during the 2 seasons (Figure 3a). The most abundante copepod species were, in summer, *Paracalanus parvus* (243602 ind m$^{-3}$), *Microsetella rosea* (243622 ind m$^{-3}$), *Candacia* spp. (55326 ind m$^{-3}$), *Calocalanus* spp. (55326 ind m$^{-3}$), and *Centropages typicus* (17061 ind m$^{-3}$), and, in winter, *Ctenocalanus* spp. (12353.57 ind m$^{-3}$), *Paracalanus* spp. (12206.59 ind m$^{-3}$), *Labidobcera* spp. (11139.83 ind m$^{-3}$),and *Oithona* spp. (7717.51 ind m$^{-3}$). Excluding copepods and phytoplankton, the most relatively abundant planktonic group in summer was mollusca and in winter similar percentages of mollusca, appendicularians and tintinnids were counted (Figure 3b). Cnidaria, Doliolida, Polychaeta, Echinodermata and Euphausiacea were also present in both seasons.

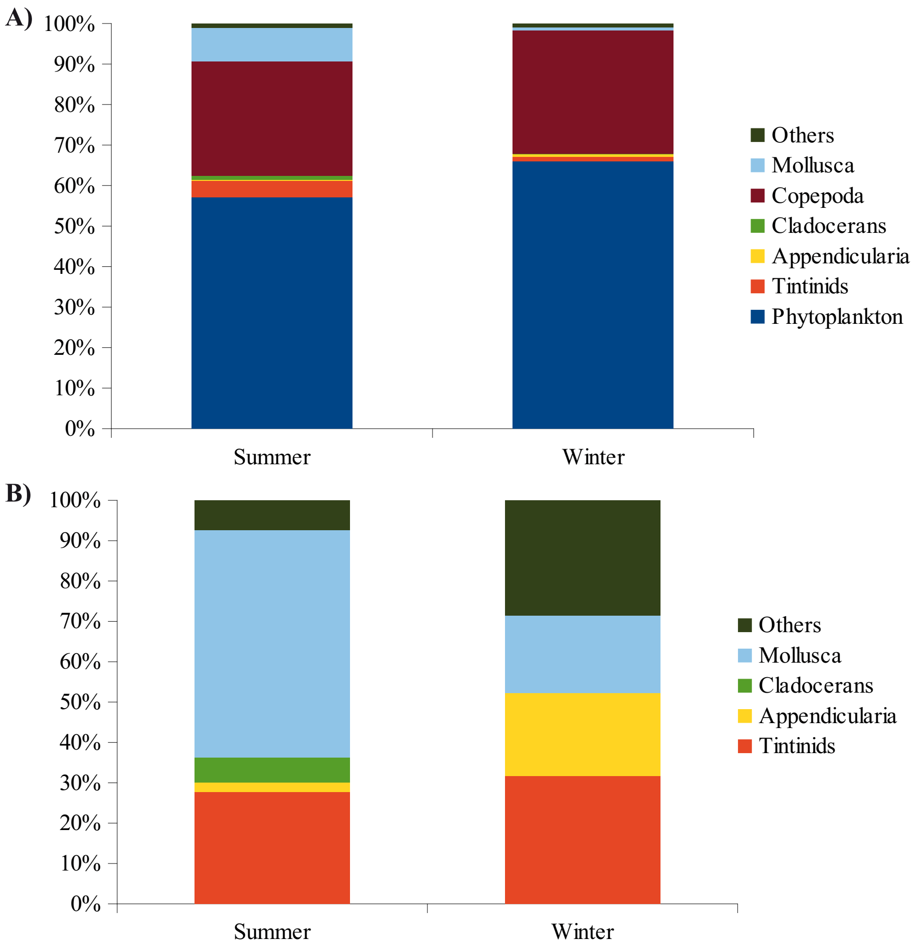

**Figure 3. Plankton relative abundances of A) the main groups in the environment, and B) excluding copepods and phytoplankton.**

### *3.2. Diet composition*

Only 6 out of the 334 stomachs of late-larvae analyzed contained preys, and only 2 of those preys could be identified (Corycaeidae), therefore the diet of sardine late-larvae is not presented in this work. Instead, we used the results from Costalago *et al.* (2012), based on isotopic signals, as indicative for sardine larvae diet.

As a percentage of the total number of preys, the stomachs of juveniles in summer contained mainly Cladocera (84.30 %), but only in 60 % of the stomachs analyzed (Table 3). In winter diatoms were the most abundant prey (72.26 %) and appeared in all the stomachs (Table 3). Sardine adults in summer had mainly Cladocera (42.95 %), *Oncaea* spp. (13.34 %) and other copepods (10.99 %), and all these prey types were found in all the stomachs (Table 3). In winter diatoms and appendicularians were the most abundant preys (66.61 % and 12.06 %, respectively) and the only prey type, together with other copepods, that were found in all the stomachs (Table 3).

The Index of Relative Importance (IRI, Table 3) confirmed the relevance of cladocerans in the diet of both size classes in summer, and the relevance of diatoms in the diet of sardine in winter. Also other crustaceans (mainly decapod larvae), besides their low numerical abundance in the stomachs, had a high IRI due to their relatively elevated weight.

For juveniles, regarding the SIMPER analysis of their dietary numerical composition, the average squared distance between seasons was 12.55 %, and the preys that better contributed to describe this dissimilarity were Cladocera (21.47 %), decapod larvae (16.28 %), *Temora* spp. (11.38 %), Clauso-Paracalanidae (10.19 %) and *M. rosea* (10.15 %). With a SIMPER analysis based on carbon composition of preys, the average squared distance between seasons was 30.45%, and the preys that better contributed to those results were decapod larvae (40.49 %), Cladocera (19.19 %) and unidentified copepods (14.36 %).

For adults, SIMPER analysis based on numerical composition of preys showed that, with an average squared distance between seasons of 7.61 %, the major contribution of the preys to that dissimilarity was defined by phytoplankton (16.80 %). With a SIMPER analysis based on carbon content, the average squared distance between seasons was of 8.55 %, and the greatest contributing preys to that dissimilarity were decapod larvae (47.14 %) and unidentified cope-

**Table 3. Total stomach contents by weight (W), numerical abundance (N) of prey, frequency of occurrence (F) of prey in the stomachs and Index of Relative Importance (IRI)**

| | Juveniles | | | | | | | | Adults | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Summer | | | | Winter | | | | Summer | | | | Winter | | | |
| | W (%) | N (%) | F (%) | IRI (%) | W (%) | N (%) | F (%) | IRI (%) | W (%) | N (%) | F (%) | IRI (%) | W (%) | N (%) | F (%) | IRI (%) |
| *Acartia* spp. | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 |
| Appendicularia | 0.00 | 0.00 | 0 | 0.00 | 0.16 | 1.62 | 80 | 0.93 | 0.16 | 0.12 | 25 | 2.51 | 0.16 | 12.06 | 100 | 9.63 |
| *Calanus* spp. | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 |
| *Candacia* spp. | 0.00 | 0.00 | 0 | 0.00 | 6.06 | 0.68 | 40 | 1.75 | 6.04 | 0.25 | 12.7 | 1.27 | 6.01 | 1.05 | 33.3 | 1.85 |
| *Centropages* spp. | 0.00 | 0.00 | 0 | 0.00 | 0.21 | 0.29 | 20 | 0.06 | 0.00 | 0.00 | 0 | 0.00 | 0.21 | 0.17 | 16.7 | 0.05 |
| Cladocerans | 1.21 | 84.30 | 60 | 51.47 | 0.00 | 0.00 | 0 | 0.00 | 1.13 | 42.95 | 100 | 14.29 | 1.12 | 5.42 | 16.7 | 0.86 |
| Corycaeidae | 0.50 | 4.09 | 80 | 3.69 | 0.47 | 0.88 | 20 | 0.18 | 0.47 | 3.50 | 75 | 7.85 | 0.47 | 0.52 | 16.7 | 0.13 |
| Diatoms | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 73.46 | 100 | 47.75 | 0.00 | 8.24 | 75 | 8.32 | 0.22 | 66.61 | 100 | 52.65 |
| *Euterpina* spp. | 0.00 | 0.00 | 0 | 0.00 | 0.12 | 0.56 | 20 | 0.08 | 0.01 | 4.37 | 62.5 | 6.69 | 0.00 | 0.00 | 0 | 0.00 |
| Foraminiferans | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 1.75 | 100 | 1.14 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 |
| *Microsetella rosea* | 0.06 | 3.58 | 60 | 2.19 | 0.05 | 1.92 | 100 | 1.28 | 0.05 | 5.37 | 87.5 | 9.29 | 0.05 | 0.52 | 50 | 0.23 |
| Molluscs | 0.00 | 0.00 | 0 | 0.00 | 0.22 | 0.09 | 20 | 0.04 | 0.22 | 1.50 | 50 | 5.15 | 0.00 | 0.69 | 33.3 | 0.18 |
| *Oithona* spp. | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0 | 0 | 0.00 |
| *Oncaea* spp. | 0.00 | 0.00 | 0 | 0.00 | 0.10 | 0.95 | 40 | 0.37 | 0.10 | 13.34 | 100 | 11.34 | 0.10 | 1.57 | 83.3 | 1.10 |
| Other Copepods | 3.64 | 1.02 | 40 | 1.87 | 3.46 | 10.78 | 100 | 9.26 | 3.45 | 10.99 | 100 | 11.10 | 3.43 | 8.74 | 100 | 9.58 |
| Other crustaceans | 92.26 | 0.34 | 40 | 37.16 | 87.52 | 0.96 | 60 | 34.51 | 87.17 | 0.32 | 37.5 | 3.81 | 86.69 | 1.57 | 33.3 | 23.18 |
| Paracalanidae | 0.32 | 1.02 | 40 | 0.54 | 0.30 | 3.23 | 80 | 1.83 | 0.30 | 2.62 | 62.5 | 6.51 | 0.30 | 0.17 | 16.7 | 0.06 |
| Polychaeta larvae | 0.00 | 0.00 | 0 | 0.00 | 0.34 | 0.14 | 20 | 0.06 | 0.00 | 0.00 | 0 | 0.00 | 0.34 | 0.17 | 16.7 | 0.07 |
| *Temora* spp. | 0.93 | 1.36 | 40 | 0.92 | 0.88 | 0.07 | 20 | 0.12 | 0.88 | 2.37 | 50 | 5.24 | 0.87 | 0.69 | 33.3 | 0.41 |
| Tintinids | 0.00 | 0.00 | 0 | 0.00 | 0.07 | 2.24 | 40 | 0.60 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 |

pods (12.93 %).

According to the SIMPER analysis of the number of preys, in summer, the average squared distance between juveniles' and adults' diets was 12.99 %, mainly defined by Cladocera (13.71 %) and phytoplankton (11.10 %); in winter, the average squared distance between size classes was 3.38 %, and the main preys explaining that dissimilarity were tintinnids (18.88 %) and Clauso-Paracalanidae (11.13 %). With the SIMPER analysis of the carbon content of preys, in summer the average squared distance between diets of juveniles and adults was 22.59 %, and mainly defined by decapod larvae (40.34 %), Cladocera (15.30 %) and unidentified copepods (14.71 %); in winter, the average squared distance between size classes was 4.73 %, and the main preys explaining that dissimilarity were decapod larvae (62.25 %) and *Candacia* spp. (13.52 %).

An ANOSIM analysis confirmed the significant differences in the diet composition between seasons (significance level of sample statistic: 0.001 % and 0.01 %, for data based on number of prey and carbon fraction of prey, respectively) and also the differences between juveniles and adults (0.3 % and 0.008 %, for data based on number of prey and carbon fraction of prey, respectively).

Ivlev´s index of prey selectivity (Figure 4) showed that sardine juveniles in summer ate preferably copepods Harpacticoidae and cladocerans, while in winter *Candacia* spp., Corycaeidae and phytoplankton (radiolarians and diatoms) were the preferred preys. Sardine adults in summer selected mainly Corycaeidae, *Microsetella* spp. and cladocerans, and in winter Corycaeidae, *Temora* spp., *Centropages* spp., together with cladocerans and appendicularians were the most positively selected preys.

Proportion of carbon content of preys showed the importance of the group 'other crustaceans' (composed basically by decapod larvae) in both juveniles and adults of sardine during the 2 seasons (Figure 5), while the numerical percentage of prey types showed a greater relevance of cladocerans in summer and diatoms in winter (Figure 5).

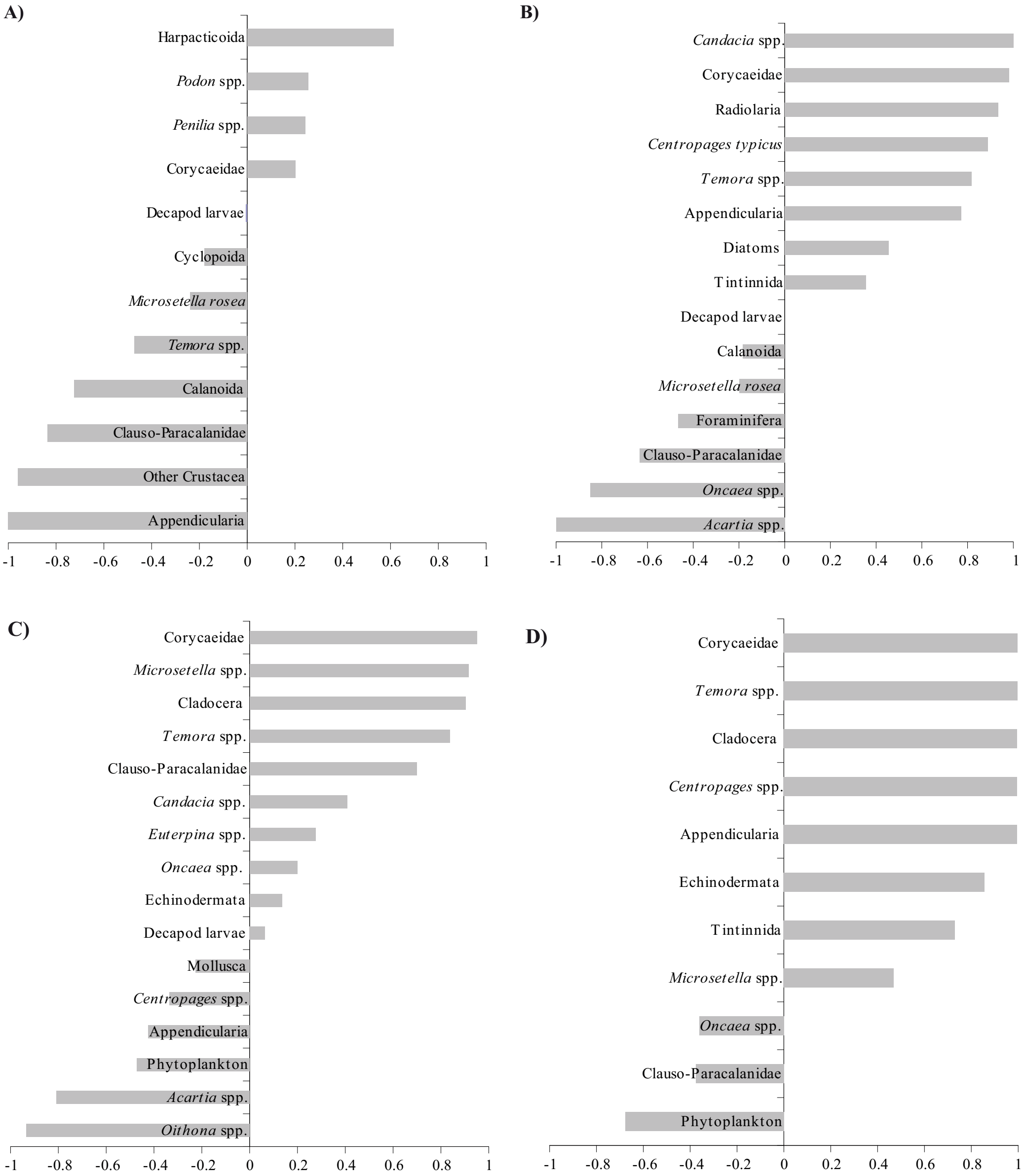

**Figure 4. Ivlev's dietary indexes for sardine: A) juveniles in summer; B) juveniles in winter; C) adults in summer; D) adults in winter.**

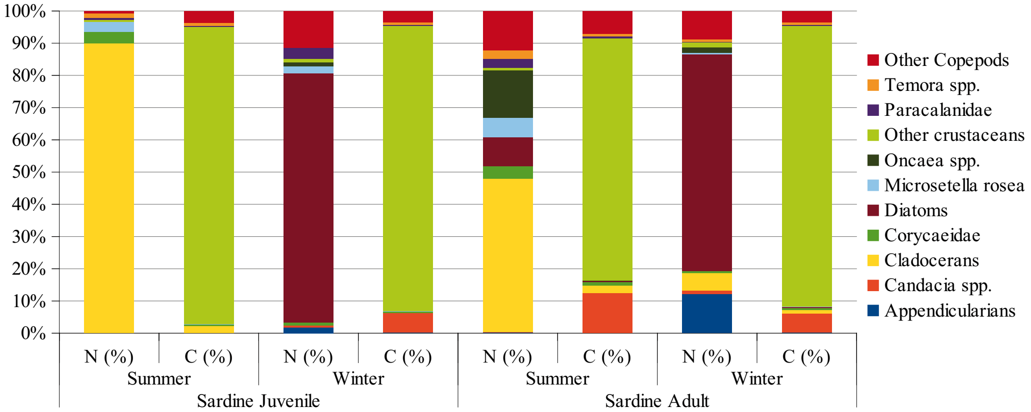

**Figure 5. Preys in the stomachs of sardine juveniles and adults, during summer and winter, in percentages of number (N) and of carbon content (C).**

### *3.3. Development of trophic-related structures*

All the measured morphological parameters' means are resumed in Table 4.

**Table 4. Means ± Standard deviations of anatomical parameters of sardine.**

| | Late-larvae | Juveniles | Adults |
|---|---|---|---|
| Standard length (cm) | 3.15 | 7.28 | 13.61 |
| Piloric caeca | 0 | 90.39 | 98.55 |
| Gill rakers | 20.17 | 43.15 | 55.67 |
| Arch length (mm) | 2.68 | 8.76 | 18.87 |
| Gill raker spacing (mm) | 0.12 | 0.20 | 0.15 |
| Gill raker length (mm) | 0.65 | 3.14 | 5.19 |

Sardines between 2 and 2.5 cm SL had already around 10 short (< 5 mm) gill rakers in the first branchial arch, but none pyloric caeca. The number of gill rakers (GR) maintained a significantly positive trend ($p < 0.05$) along all the sizes (Figure 6a), but the slope in the GR / SL relation was lower after 5 cm SL.

Pyloric caeca in sardines started developing when fishes reach 4 cm of SL. Once pyloric caeca appeared, their number was always significantly increasing positively ($p < 0.05$) until 12 cm SL, where their number leveled off between 120-140. Although the number of pyloric caeca after 12 cm SL remained within that range, we did not present these data because of the high uncertainty when trying to count the exact number of caeca, produced by the accumulation of fat around them (Figure 6b).

The mean gill rakers length ($L_{GR}$) was always positively correlated with the SL (Figure 6c), going from around 1 mm in 2.5 cm SL larvae up to almost 20 mm in 17 cm SL larvae. The density of gill rakers ($D_{GR}$; number of GR / length of branchial arch in mm) showed a negative correlation with SL (Figure 6d), started from around 7.5 GR $mm^{-1}$ branchial arch in the smallest larvae analyzed, and down to less than 4 GR $mm^{-1}$ branchial arch in sardines of 17 cm SL.

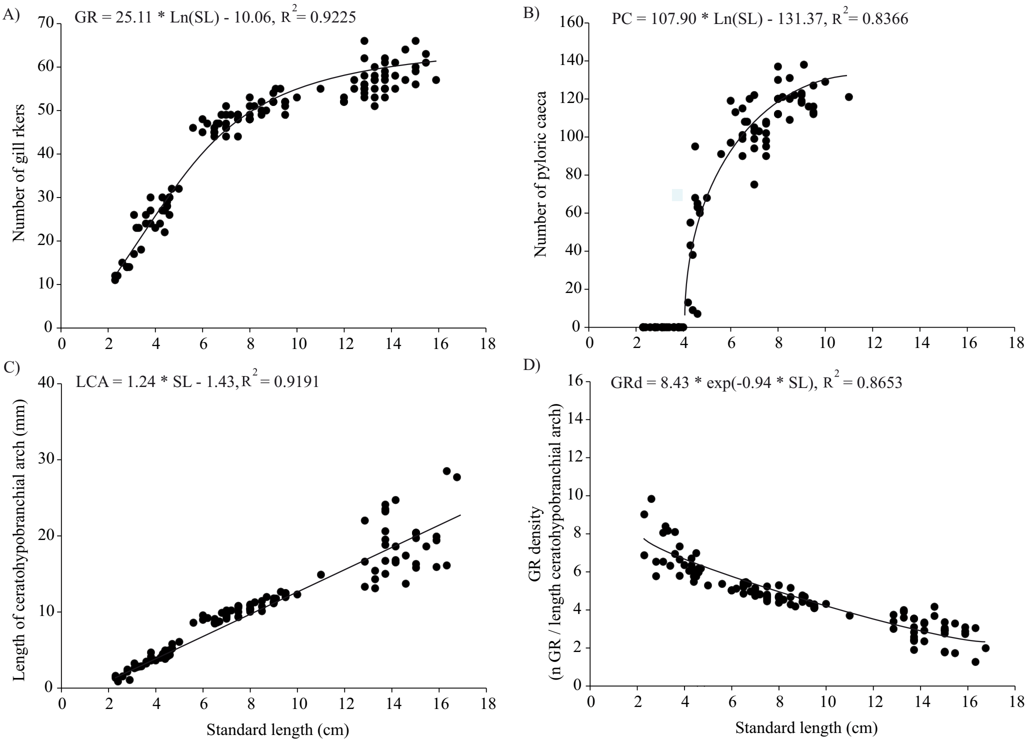

**Figure 6. Relationships between stardard length of sardine and different anatomical structures related with feeding: A) number of gill rakers GR; B) number of pyloric caeca PC; C) length of the ceratohypobranchial arch LCA (mm); D) density of gill rakers GRd (number of gill rakers / length ceratohypobranchial arch).**

### *3.4. Daily ration and consumption rates*

A daily feeding pattern was observed for sardine juveniles during summer 2007 (Figure 7a). Throughout the day stomach fullness index (SFI) values were rising until they reached a peak before sunset (sunrise time: 5:04 GMT; sunset time: 20:02 GMT). After that point SFI values declined during the night. For sardine juveniles during summer 2007, the feeding period was considered as the time between 5:12 and 16:56. In the same season, adults showed an increasing SFI from almost 0 at 8:00 until midnight (23:02), although the peak appeared at 11:07 (Figure 7b).

In winter (sunrise time: 8:17 GMT; sunset time: 17:41 GMT), juveniles had a feeding period of about 12 hours, from 4:34 to 16:15, when SFI started to decrease (Figure 7c), and adults did not show a clear pattern in their feeding periodicity and seemed to be continuously feeding, although they had an especially low SFI after sunset (20:12) (Figure 7d).

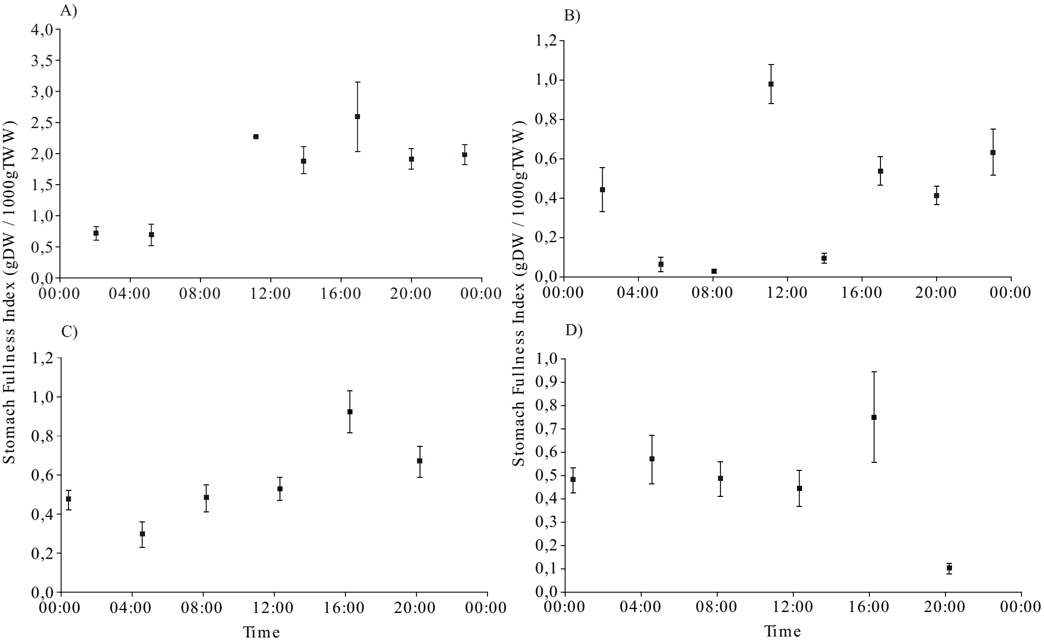

**Figure 7. Mean stomach fullness index ± 95% C.I.s plotted over time, as obtained in a sampling carried out in consecutive 24 hours. a) juveniles in summer; b) adults in summer; c) juveniles in winter; d) adults in winter. (In summer: sunrise time: 5:04 GMT; sunset time: 20:02 GMT. In winter: sunrise time: 8:17 GMT; sunset time: 17:41 GMT).**

The evacuation and consumption rates of adults and juveniles in both seasons, and calculated with both consumption models, are presented in Table 5.

**Table 5. Estimates of gastric evacuation Rmax ($h^{-1}$) and consumption rates C (DW 1000 $g^{-1}$ wet BW) for sardine adults and juveniles in summer and winter. Consumption values are also expressed as percent total weight (% TW). DW: dry weight; $C_E$: daily ration according to the Eggers model; $C_{E-P}$: daily ration according to the Elliott-Persson model.**

| | | | $C_E$ | | $C_{E-P}$ | |
|---|---|---|---|---|---|---|
| | | Rmax (±SE) | DW (±SE) | %TW | DW (±SE) | %TW |
| Adults | Summer | 0.259 (0.242) | 1.58 (0.06) | 1.21 | 1.77 (0.64) | 1.36 |
| | Winter | 0.508 (0.087) | 4.53 (0.09) | 3.48 | 4.88 (0.68) | 3.75 |
| Juveniles | Summer | 0.099 (0.076) | 3.94 (0.20) | 2.95 | 4.91 (1.55) | 3.78 |
| | Winter | 0.082 (0.042) | 1.48 (0.07) | 1.14 | 0.58 (0.31) | 0.45 |

## 4. Discussion

In order to depict how sardine in the Mediterranean sea interacts with the environment and to assess the way that prospective changes in the plankton community could affect the sardine populations, an accurate description of the trophic dynamics of the species during all its life stages, from larvae to adults, is essential.

In this work the stomach contents of the fishes were analyzed to obtain information of the preys they consumed in summer and winter. Then, based on the study of the development of some ontogenetical features of sardine, we also tried to elucidate at which body size sardine have already developed a completely functional filtering mechanism and can start efficiently shifting to a more phytoplanktonic diet.

Regarding our results, we saw that pyloric caeca do not appear until the metamorphosis begins (about 4 cm SL), but after that their complete development occurs very fast, and when sardine reached around 8 cm SL no more new pyloric caeca were formed. Although the existing pyloric caeca may continue growing in volume well after 8 cm SL, we can hypothesize that the digestive function of pyloric caeca (Buddington & Diamond, 1986) can be fully implemented as soon as sardine reach 8 cm SL.

Gill rakers appeared at the SL of 2.2 cm in our study, and similarly, in the classic works of Lee (1961) and Andreu (1969), that thoroughly analyzed the development of gill rakers in sardines from the western Mediterranean, 20 mm total length (TL) is signed as the point when gill rakers start growing in sardines. We also found that the increasing trend in the number of gill rakers becomes much lower when sardines reach 7 cm SL, meaning that filter-feeding of small particles (<750 µm is the threshold of prey size to be filtered, according to Garrido *et al.* (2007)) can be done with total efficacy from that size on. This is also in agreement with Nikolioudakis *et al.* (2012), in the Aegean sea, and with Scofield (1934), who studied *Sardinops sagax* in California, that claimed that sardines at the length of 70-100 mm TL are able to filter diatoms.

From our observations, we could notice that the length of the ceratohypobranchial arch increases linearly with the SL, while the number of gill rakers on that arch becomes stable at a certain SL, so the gap between gill rakers would increase with the SL of sardine. This could mean that the ability of filtration of the smallest particles would be reduced, but we believe that denticles could help to compensate the filtering capacity lost with wider inter-raker gaps (King & Macleod, 1976).

An aspect that requires further research is the daily ration. Although in our analysis we have seen a general pattern of diurnal feeding activity that extends until dusk, as observed also by Andreu (1969) in the Atlantic and by Nikolioudakis *et al.* (2011) in the Aegean sea, we can not assure that this is the normal behavior of sardines in the northwestern Mediterranean because at least 2 full 24-hour cycles, sampling every 3 h or less, are needed to assess with confidence the diel variations of feeding intensities (Tudela & Palomera, 1995). In addition, we did not find differences between models, even though the Elliot-Persson model is claimed to be adequate only when the frequency of sampling is every 3 hours or shorter (Elliot & Persson, 1978). Neither we found clear patterns in the consumption rates between sizes and seasons, although higher rates are normally expected in summer (Nikolioudakis *et al.*, 2011). However we did find that the evacuation rates were always higher in adults than in juveniles, likely meaning higher metabolic rates in larger individuals, but it is also an expected result, since instantaneous evacuation rates are affected by fish size (Elliot & Persson, 1978).

Nevertheless, high metabolic rates in sardine adults could make sense when larger sardines (from 7 cm SL on) use filter-feeding rather than particulate-feeding, as we demonstrated here, due to the higher energetic requirements of a continuous swimming activity, in contrast with smaller individuals, or other similar species as anchovy, which spend much of their time gliding, after a beat of tail (Lasker, 1970).

The existing discrepancies between the 2 classical works on European sardine diet, by Lebour (1921) and Demirhindi (1961), in the south coast of England and in Turkish waters, respectively, with the first saying that sardines after the metamorphosis shift to a diet based more on phytoplankton, and the later claiming an almost strictly zooplanktivorous diet in all ages of sardine, seem solved by the present and other recent researches. Bode *et al.* (2004) and Garrido *et al.* (2007), in Atlantic waters, and Nikolioudakis *et al.* (2012), in the Eastern Mediterranean, have suggested that filter-feeding is the main feeding behavior of adult sardines in the wild, given the high relative importance of prey <750 µm to sardine diet that they observed. The present work in the western Mediterranean confirms the findings of the other above-mentioned recent studies, showing that sardines above 7 cm SL can efficiently feed on phytoplankton. In fact, sardines above 4 cm SL in the western Mediterranean were eating mainly diatoms and decapod larvae in winter, exhibiting their ability to both filter and particulate-feeding. However, sardine larvae in this region, according to Morote *et al.* (2010) and to Costalago *et al.* (2012), ate basically copepods, so they did not seem to be able to feed on phytoplankton, likely due to the lack of the specific body structures to filter small particles.

Bulgakova (1996) explained that anchovy *Engraulis encrasicolus* could shift between filter and particulate-feeding depending on the concentrations of different prey items, and, regarding our results, the same could be said for sardine. Other authors (Bode *et al.*, 2004 and Garrido *et al.*, 2007) said that larger preys, such as copepods or decapod larvae, can also be important in sardine stomachs, particularly when there is lower abundance of other preys, suggesting that particulate-feeding might be also utilized in the wild to compensate for periods of low food availability (Margalef, 1960). According to this, in winter, when the abundances of both micro and mesozooplankton were higher, we observed that both juvenile and adult sardines relied more on diatoms than on any other prey type in terms of numerical quantities. In summer, on the other side, both juveniles and adults fed considerably on cladocerans. The selection of cladocerans, rather than copepods, in summer can be due to the greater ability of copepods in avoiding being captured by fishes (Strickler *et al.*, 2005).

Except larvae, that have been shown here and in other studies (Morote *et al.*, 2010, Costalago *et al.*, 2012) to be mandatory particulate feeders preying fundamentally on copepods, juvenile and adult sardines are opportunistic feeders, with a more heterogeneous diet than similar species such as anchovy (Tudela & Palomera, 1997, Costalago *et al.*, 2012). In addition, some authors have found correspondences between the plankton in the environment and in the stomachs (Varela *et al.*, 1990, Bode *et al.*, 2003), suggesting that sardines are essentially non-selective filter-feeders and their diets reflect the ambient plankton composition.

Although there is no diet overlap between juveniles, and probably adults, of sardine and anchovy (Costalago *et al*., submitted), it could happen, on the other hand, that larvae of both species would share the same alimentary resources if sea surface temperatures keep rising (Costalago *et al.*, 2011). Also, other species that have not been naturally common in the Gulf of Lions but have increased in the last years, like *Sardinella aurita* (Sabatés *et al.*, 2006) and *Sprattus sprattus* (GFCM, 2011, D. Roos, personal communication) are potentially intraguild competitors with sardine (Palomera *et al.*, 2007, Morote *et al.*, 2008), and could put further pressure on its population.

On the top of this, to our knowledge, cannibalism has not been reported in *S. pilchardus*, but intraspecific diet overlap between juveniles and adults is likely to occur, as confirmed by the similar feeding patterns observed here in all sardine individuals larger than 7 cm SL. This is a factor that naturally controls population growth, and thus should be taken into account in order to propose plans to manage the resource.

The analysis of the stomach contents classically has been a widely extended technique to study the ecology of fish (Hynes, 1950, Hyslop, 1980, Wootton, 1999). The numerical composition of preys in the stomach of fish provides information about the dietary behavior and can be highly useful for comparisons between similar species of predators (van der Lingen & Hutchings, 1998, Costalago *et al.*, submitted). However, numerical analysis of the preys in the stomachs of clupeid species can overestimate the contribution made by phytoplankton because of its small size and the low carbon:volume ratio of phytoplankton compared with zooplankton. For this reason, the assessment of the stomach contents of planktophagous fish should be better described by using methods based on the carbon fraction of ingested prey, as some authors have done in recent works (van der Lingen, 2002, Garrido *et al.*, 2008, Borme *et al.*, 2009) and also we did in the present study. We have compared the results of both methods (carbon and numerical contents) and proved that, in the case of adults of sardine, differences between the two analytical techniques exist. When using carbon contents, for example, the largest prey types (decapod larvae and copepods) contributed more than any other type, regardless its numerical importance, to the seasonal differences in diet, however, when using numerical percentages, the phytoplanktonic preys were the most relevant. Similarly, we found that dietary differences between juveniles and adults in both summer and winter, considering carbon contents, were due to decapod larvae and large copepods, or to cladocerans in summer, but considering the numerical composition of preys in the stomachs, those differences were due mainly to small copepods (Clauso-Paracalanidae) and phytoplankton, and also cladocerans in summer. We have seen that in juveniles the prey types that better described the diet were almost the same (Cladocera, decapod larvae and copepods) regarding both types of the stomach contents measures. We believe that this can be due to the fact that juveniles do not prey on phytoplankton during summer, feeding basically on cladocerans, while in adults in summer we found diatoms in 75 % of the stomachs and composing 8.24 % of the total number of preys, so these numbers would enhance the relevance of diatoms in the diet of adults respect to the diet of juveniles.

Also there were differences in the diet composition when comparing the percentages of number versus percentages in weight of preys; in this case, the numerical percentage gave more importance to cladocerans and diatoms, in summer and winter, respectively, but the weight percentage was much higher for decapod larvae than for any other prey type in both seasons. However, the IRI tended to give more importance to the preys numerically more dominant rather than to those with higher weights. In addition, results of SIMPER analysis showed larger mean distances in the diet compositions when using data from carbon content.

When comparing our results here, based on stomach contents, to those by Costalago *et al.* (2012), based on stable isotopes analysis and therefore providing a longer term insight of the diet prior to the capture, we observed that, regarding the isotopic analysis, appendicularians were always the most important prey for both juveniles and adults in both seasons, summer and winter, except for juveniles in summer that consumed mainly cladocerans. Contrastingly, in this work appendicularians were among the most important preys only when looking at the IRI in adults in winter. This demonstrates that some preys, like appendicularians, that are more easily digested, often can be underestimated in the stomach contents analysis of fish (Capitanio *et al.*, 2005).

Following the Ivlev's selectivity index, we found that in summer and winter the most positively selected preys by juveniles and adults (and also by larvae, according to Costalago *et al.* (2012)), and besides cladocerans in summer, were small copepods, such as Corycaeidae and Harpacticoidae (mainly *Microsetella* spp.). This result is especially relevant considering the importance of that kind of copepods in the pelagic food web (Turner, 2004), that serve, through small pelagic fishes, as a link between bacterial plankton and ecologically and economically important species that prey on small pelagic fishes (de Laender *et al.*, 2010).

Molinero *et al.* (2005) showed that high positive anomalies in water temperature in the northwestern Mediterranean can lead to a decrease in the population of copepods, with an obvious effect over the trophic dynamics of sardine in the region, that would be forced to rely more on primary producers as food. In addition, Conversi *et al.* (2010) claimed a likely increase of species of small copepods in a prospective warming Mediterranean sea, that would limit even more the expansion of phytoplankton species. Considering the energetic advantage of filtering for a well-adapted species on filtering food as sardine (van der Lingen *et al.*, 2006), limited availability of small particles of food could have negative consequences for sardine populations. Moreover, the likely future diminution of the cold period in the Mediterranean could provoke a reduction in the spawning season of sardine (Coll *et al.*, 2008a) and also competition between sardine and anchovy larvae could happen (Costalago *et al.*, 2011).

Sardine populations support a large community of species in higher trophic levels, some of them commercially important (Coll *et al.*, 2006, Preciado *et al.* 2008, Banaru *et al.*, 2012). Conservation of sardine and an adequate management of its fishery in the Gulf of Lions are therefore unavoidable premises to guarantee both socio-economic and ecological stability in the region. This work demonstrates that, apart from fisheries (Coll *et al.*, 2008b), sardine populations can also be strongly affected by alterations in the environment and the planktonic community.

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# Capítulo 2. Comparación de las dietas de juveniles de anchoa europea *Engraulis encrasicolus* y de sardina *Sardina pilchardus* in el Golfo de León en diferentes épocas.

**Resumen.** Se ha demostrado que la anchoa y la sardina son especies ecológicamente muy similares que comparten el mismo hábitat en el Mediterráneo y, en consecuencia, interaccionan entre ellas. Estas dos especies pelágicas simpátricas son planctívoras y consumen un amplio espectro de presas planctónicas durante el desarrollo de todas sus fases ontogénicas, con un potencial solapamiento de sus nichos ecológicos, aunque las interacciones alimenticias de las dos especies no se han estudiado aún en profundidad. En este trabajo comparamos los hábitos alimenticios de la fase juvenil de anchoa y de sardina durante distintas estaciones del año en el Mediterráneo noroccidental, por medio del análisis de sus contenidos estomacales y de sus características anatómicas involucradas en la alimentación. Esta investigación proporciona información acerca de cómo sus poblaciones pueden verse afectadas por los posibles cambios ambientales. Con este estudio hemos demostrado que la anchoa y la sardina en la región no presentan competencia por el alimento, y hemos descrito diferencias significativas entre las dos especies en cuanto a sus dietas, debidas a las diferencias en la morfología de sus estructuras relacionadas con la alimentación. En consecuencia, sus adaptaciones a distintos regímenes ambientales seguirán patrones diferentes.

# Comparison of the diets of European anchovy *Engraulis encrasicolus* and sardine *Sardina pilchardus* juveniles in the Gulf of Lions during different seasons.

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**ABSTRACT.** Anchovy and sardine in the Mediterranean are known to share the same habitat and, consequently, to interact among each other. These 2 sympatric pelagic species are planktivorous and consume a wide range of planktonic prey items during all their developmental stages, potentially overlapping their ecological niches, although the feeding interactions between the 2 species have been poorly investigated. Here we compare the dietary habits of the juvenile phases of anchovy and sardine during different seasons in the northwestern Mediterranean Sea through the analysis of their stomach contents and of their feeding-related anatomical characters. This research provides information about how their populations can be affected by environmental changes. With this study we have shown that anchovy and sardine do not present food competence, and we have described significant differences between the 2 species in their diets due to their different alimentary morphology. Thus, their adaptation to environmental shifts would follow different patterns.

KEY WORDS: small pelagic fish · environmental changes · trophic ecology · sympatry · nothwestern Mediterranean

## 1. Introduction

Most of the fish species in the ocean are likely to share the same habitat at least during a part of their life and, consequently, to interact among each others (Polunin & Pinnegar, 2008). Understanding the agents that influence the coexistence of species, such as trophic relationships, is the base for ecological studies.

Small pelagic fishes inhabit a relatively spatially homogenous environment if we compare it to, for example, the demersal environment, so the adaptive characteristics are also supposedly similar between species and therefore pelagic fishes are likely to share many specific traits in relation to their morphology, behavior and trophic and population dynamics (Checkley *et al*., 2009). Moreover, these species constitute the bulk of the fish biomass in many areas of the oceans and, being in a mid-trophic position in the food web, their ecological role in the ecosystem is crucial (Bakun, 1996, Cury *et al*., 2000).

Survival of fish juveniles greatly depends on their ability to capture and digest sufficient quantities of appropriate prey to avoid starvation and to ensure growth. Zooplankton seems to be the main source of food for small pelagic fish during all stages (Durbin, 1979, Blaxter & Hunter, 1982, Tudela & Palomera, 1997, Borme *et al*., 2009, Checkley *et al*., 2009, Morote *et al*., 2010, Costalago *et al*., 2012) and, regarding the “match–mismatch” hypothesis by Cushing (1990), the decoupling between fish peak production and their prey is one of the main sources of recruitment variability, so adverse changes in the planktonic ecosystem may result in a reduction in the number of fish that recruit into an existing population (Beaugrand *et al*., 2003).

In the Mediterranean Sea, European anchovy *Engraulis encrasicolus* and European sardine *Sardina pilchardus* are the most exploited species and have been broadly studied because their abundance and position in the food web makes them particularly important for the ecosystem (Palomera *et al*., 2007). These two sympatric pelagic species are planktivorous and consume a wide range of planktonic prey items during all their developmental stages (Costalago *et al*., 2012), potentially overlapping their ecological niches, although the feeding interactions between the two species have been poorly investigated.

It is also known that species-specific variations in the feeding habits of marine fishes are consistent with species-specific differences in the functional morphology of their feeding organs (Castillo-Rivera *et al*., 1996, Tanaka *et al*., 2006), and the same happens with stages-specific variations (Turingan *et al*., 2005). However, prevailing knowledge related to the feeding ecology of anchovy in the northwestern Mediterranean is mainly on adults and, despite the amount of works about their diet composition (Tudela & Palomera, 1997, Plounevez & Champalbert, 2000), little attention has been paid to the dependence of the feeding behavior on their morphology during the juvenile stage. In the case of sardine the lack of knowledge spreads to all its life stages, with only a few studies in the Mediterranean sea, besides Lee (1961): Rasoanarivo *et al*. (1991) and Morote *et al*. (2010), in the Gulf of Lions and in the Catalan sea, respectively, and both focused only on larvae smaller than 15 mm SL, Costalago *et al*. (2012), that studied sardine diet from larval to adult stages with stable isotopes in the Gulf of Lions and Nikolioudakis *et al*. (2012), that analyzed stomach contents of juvenile and adults in the Aegean sea,

The stomach contents of fishes provide direct information about their diet (Tanaka *et al*., 2006), and also the observation of the feeding related structures is useful to estimate the potential for diets similarity (Castillo-Rivera *et al*., 1996). The mechanisms that fishes employ for feeding are diverse, and can be shifted during the ontogenetic development of the individuals or depending on environmental conditions and presence and abundance of particular preys.

Although the spawning seasons of anchovy and sardine in the western Mediterranean are different -spring-summer and autumn-winter, respectively (Palomera *et al*., 2007)-, and both species take about one year to reach adulthood (Blaxter & Hunter, 1982), it is possible that juveniles of the two species co-occur in the same periods of the year, like summer, autumn and winter in the present case,. When this happens, a competition for food could be expected to take place even between juveniles.

The present contribution comprehensively examines and compares the dietary habits of the juvenile phases of *E. encrasicolus* and *S. pilchardus* during different seasons (summer, autumn and winter) in the Western Mediterranean Sea through the analysis of their stomach contents and of their feeding-related anatomical characters, such as gill rakers and pyloric caeca..

This research gives insights about the trophic ecology of the juveniles of the two most important pelagic fish species in the Mediterranean, and provides relevant information that will be useful to clarify how these two populations and their possible regime shifts, which are claimed to be trophodynamically mediated (van der Lingen *et al*., 2006), would be affected by environmental changes.

## 2. Materials and methods

### *2.1. Sample collection*

Sampling was conducted aboard the *N/O L'Europe* in the Gulf of Lions (northwestern Mediterranean) (Figure 1) during oceanographic campaigns in 3

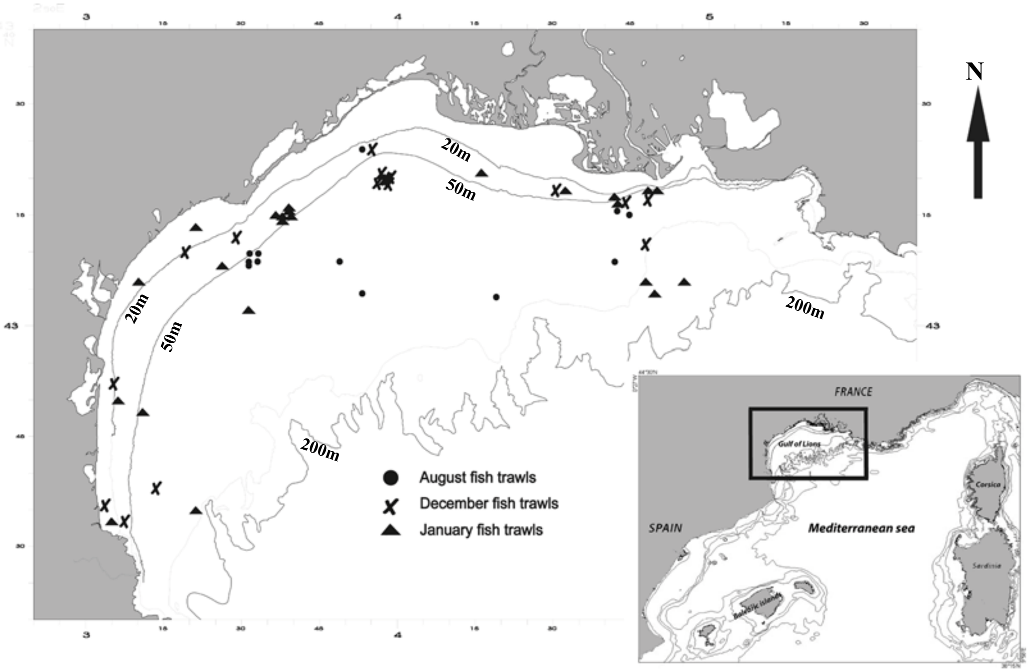

**Fig. 1. Study area (Gulf of Lions, NW Mediterranean), indicating fish sampling locations.**

different seasons: autumn (08-21 December 2007), summer (21-29 July 2008), and winter (11-27 January 2009).

Temperature (º C), salinity and fluorescence of the water column from sea surface to the bottom (up to 100 m depth) were measured with a Seabird 19 CTD at each station (16 sampling stations in summer, 15 in autumn and 13 in winter).

Plankton samples were collected by vertical tows performed with two different nets: a WP2 standard net (mesh size 200 µm; mouth opening diameter 58 cm) and a Calvet net (mesh size 53 µm; mouth opening diameter 25 cm). The volume of filtered water was estimated from the net-mouth area and the sampling depth. Immediately after the retrieval of the nets, plankton samples were sieved in succession through 200 µm and 50 µm, and 3000 µm and 200 µm, mesh to obtain two different size fractions (a 50-200 µm fraction, named hereafter microplankton, and a 200-3000 µm fraction, named hereafter mesozooplankton) from the Calvet net and the WP2 net respectively. All plankton samples were split with a Motoda plankton splitter (Motoda, 1959). One-half of each sample was fixed and preserved in a seawater-buffered formaldehyde solution (4% final concentration) for later determination of composition and abundance, whereas the other half was filtered through pre-dried, pre-weighed Whatman GF/C filters (25 mm Ø for microplankton and 47 mm Ø for mesozooplankton) for biomass estimation. The filters were stored onboard at -20º C.

Juveniles of both species were captured nearby the plankton stations with a pelagic trawling net equipped with a small-mesh cod-end (mesh length 5 mm, ISO 1107) and towed at an average speed of 3.6 knots over the shortest possible period (approximately 30 minutes) to try to avoid cod-end feeding and stressing of the fish. The samples were immediately frozen (-20º C) after sorting on board.

### *2.2. Laboratory procedures*

At laboratory, qualitative analysis of plankton was performed, and individuals were identified to the lowest taxonomical level possible under the stereomicroscope (Wild M12, at 100× magnification). The mesozooplankton samples were analyzed in aliquots representing about 10 % of the sample and repeated until counting at least 400 copepods in each; additional subsamples were also taken for any other abundant organism (i.e. cladocerans during summer). Microplankton samples were subsampled differently: 1 to 2 % of the original volume was analyzed to estimate the presence of nauplii, dinoflagellates, ciliates and diatoms; small copepods (mainly copepodites) were analyzed in volumes sufficient to count at least 400 individuals. Individuals of each identified taxon were counted and abundances (individuals $m^{-3}$) calculated. The filters with samples were dried at 60º C for 48 h to estimate the dry mass (mg $m^{-3}$) of the plankton fractions.

The genera *Paracalanus*, *Ctenocalanus*, *Clausocalanus* and *Parvocalanus* were classified as the 'Clauso-Paracalanidae' group.

The stomachs of 342 juvenile anchovies and of 136 juvenile sardines were dissected under the stereo-microscope and preserved individually in a buffered 4% formaldehyde–seawater solution. For the analysis of gut content, pools of the contents of up to 25 stomachs for each tow were made when available. The whole gut content was washed out onto a Petri dish and examined (at 100× magnification). All the preys were counted and identified up to the lowest possible taxonomical level and their widths and lengths were measured (in µm), if distinguishable, using the software ImageJ (Abramoff *et al*., 2004).

For the morphological analysis of fish, different measures were taken: body wet weight (BW) in grams, and standard and total body lengths (SL and TL, respectively) in millimeters. Among these fishes, 81 specimens of anchovy between 40-102 mm SL and 67 specimens of sardine between 40-110 mm SL were also randomly selected to count the number of pyloric caeca and to measure the characteristics of gill rakers, as in Tanaka *et al*. (2006), in the first branchial arch (lower or ceratohypobranchial branch) of the left side of the body; the number of gill rakers was counted, and the length of the gill rakers ($L_{GR}$) and the width of the gill raker spacings ($S_{GR}$) in mm were measured. The $L_{GR}$ was averaged from five long gill rakers in the middle of the gill arch and the $S_{GR}$ from five gill raker spacings at the basis of these rakers. Measurements were also made with the software ImageJ.

### *2.3. Data analysis*

Analysis of variance (ANOVA) tests were used to examine the differences in the hydrographic data among seasons (summer, autumn and winter). The assumptions of ANOVA were checked with a Shapiro-Wilk test for normality and a Levene test for homogeneity of variances. Differences between seasons regarding plankton biomass and abundance were estimated with a non parametric Kruskal-Wallis tests. Significance levels were adopted at $p < 0.001$ for hydrographic data analysis and at $p < 0.05$ for planktonic data analysis.

A SIMPER analysis was applied to numerical composition of preys in the stomachs in order to elucidate the most important prey species primarily providing the discrimination between the two species and between the three seasons. Only prey species with a percentage of contribution in the diet similarities >10 % are showed in the results. One-way analysis of similarity (ANOSIM) was used to test significance of the differences in the diet composition between seasons and between species.

The contribution of every prey item was calculated with the index of relative importance (*IRI*, Pinkas *et al*., 1971) based on the equation:

$$IRI = (\%W_i + \%N_i) * \%FO_i$$

where $W$ = carbon dry weight of prey type $i$ in µg, $N$ = number of individuals of prey $i$ in stomachs and $FO$ = frequency of occurrence of prey $i$ in stomachs. $W$ was obtained from estimations by Uye (1982), Saiz & Calbet (2007) and Borme *et al*. (2009).

Prey selectivity was estimated by means of Ivlev's diet selectivity index (*E*, Ivlev, 1961) for each case analyzed. The value of the index was calculated with the equation

$$E = (r_i - p_i) / (r_i + p_i)$$

where $r_i$ is the proportion of prey item $i$ in the fish stomach and $p_i$ is the proportion of prey item $i$ available from the marine environment.

Diet overlap index (*D*) of Schoener (1968) between the two species was calculated following the equation:

$$D = 1 - 0.5\ [\Sigma(pi - qi)]\ for\ i = 1,\ldots,n$$

where $p_i$ is the proportion of the item $i$ in the stomach of the species $p$, and $q_i$ is the proportion of the item $i$ in the stomach of the species $q$.

An analysis of covariance (ANCOVA) was also performed for comparison of the mean lengths of gill rakers ($L_{GR}$) and the spacing between gill rakers ($S_{GR}$) among species, using SL as covariate. This corrected the variation in the $L_{GR}$ and $S_{GR}$ attributable to the variation in SL. ANOVAs were used to test the significance of the slopes of the regressions between anatomical variables.

PRIMER software package (Version 6.1.9) (Clarke & Warwick, 2001) was used to perform both SIMPER and ANOSIM analysis, grouping prey species categories based on Bray-Curtis mean similarities. ANOVA and ANCOVA test were performed with SPSS v.19 statistical software.

## 3. Results

### *3.1. Oceanographic data and plankton composition of the environment*

The among-cruises comparison (Table 1) showed that there were no significant differences regarding both surface salinity and surface fluorescence, whereas surface temperature and average of the upper water column were significantly higher during summer than in the other 2 periods. Also, in winter average fluorescence was statistically higher, and average salinity was lower, than in the other seasons.

**Table 1. Mean temperature, salinity and fluorescence (log values) for each sampling period. F: F-statistic.**

| | **Summer** | **Autumn** | **Winter** | ***F*** |
|---|---|---|---|---|
| Surface Temp. (5 m) | 19.14 | 12.64 | 12.06 | 234.874*** |
| Surface Sal. (5 m) | 37.74 | 37.35 | 36.84 | 1.610 (ns) |
| Temp. Av. 0-50 m | 16.75 | 13.04 | 12.24 | 132.805*** |
| Salinity Av. 0-50 m | 37.94 | 37.92 | 37.38 | 4.976 *** |

***** p < 0.001**; ns: non significant

Information on total microplankton and mesozooplankton abundance (ind $m^{-3}$) and biomass (mg $m^{-3}$) during the surveys is summarized in Table 2. Abundances of both microplankton and mesozooplankton were significantly lower in autumn than in the other seasons, while no significant differences were found between summer and winter. Mesozooplankton biomass was also found significantly lower in autumn than during summer and winter. Microplankton biomass in autumn was significantly higher than in summer.

No statistical differences were found between other seasons regarding biomass of both micro and mesozooplankton.

The plankton community was mainly dominated by phytoplankton and copepods during the 3 seasons (Figure 2a). Excluding copepods and phytoplankton, the most relatively abundant groups were mollusca, in summer, and tintinnids, in autumn; and in winter similar percentages of molluscs, appendicularians and tintinnids were counted (Figure 2b). Other groups such as chaetognata, non-copepod crustaceans, cnidaria, doliolida, echinodermata, euphausids, ostracods, polychaeta and tintinnids were also present in the 3 seasons.

**Table 2. Mesozooplankton and microplankton stock in terms of abundance and biomass during the three periods (Min: minimum, Max: maximum, SD: standard deviation).**

| | | | Summer | Autumn | Winter |
|---|---|---|---|---|---|
| **Mesozooplankton** | **Total abundance (ind m$^{-3}$)** | Min | 3767.12 | 709.50 | 1211.68 |
| | | Max | 11881.19 | 9432.81 | 34522.29 |
| | | Mean | 8024.19 | 3721.51 | 14559.75 |
| | | SD | 2484.35 | 2818.81 | 12273.61 |
| | **Biomass (mg m$^{-3}$)** | Min | 14.20 | 1.93 | 7.62 |
| | | Max | 54.57 | 33.59 | 484.14 |
| | | Mean | 33.65 | 14.08 | 53.25 |
| | | SD | 10.34 | 10.65 | 96.64 |
| **Microplankton** | **Total abundance (ind m$^{-3}$)** | Min | 11186.63 | 1161.70 | 44731.10 |
| | | Max | 452339.33 | 9568.44 | 487819.55 |
| | | Mean | 107734.67 | 4406.42 | 299637.64 |
| | | SD | 138214.79 | 2716.53 | 194726.17 |
| | **Biomass (mg m$^{-3}$)** | Min | 9.10 | 1.30 | 7.81 |
| | | Max | 907.21 | 90.62 | 335.12 |
| | | Mean | 190.01 | 22.76 | 52.91 |
| | | SD | 277.53 | 24.18 | 72.62 |

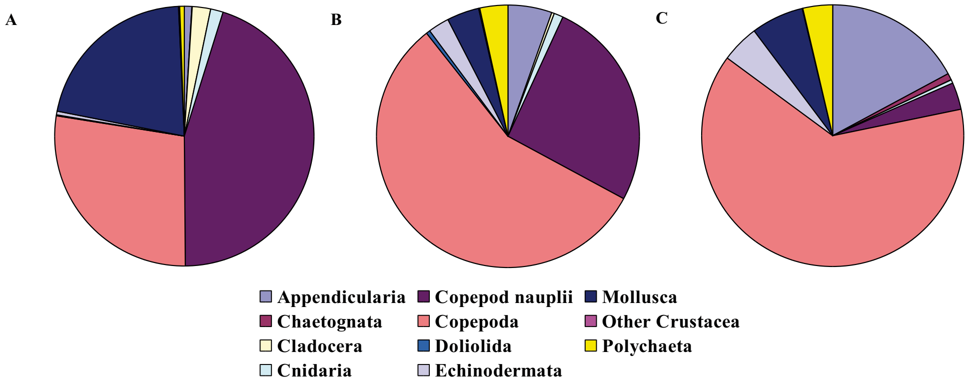

**Fig. 2 Composition of plankton community in the Gulf of Lions during (A) summer, (B) autumn and (C) winter showing the proportions of the four dietary functional groups defined for this study.**

### *3.1. Diet composition*

The stomachs of anchovies in summer contained *Centropages typicus*, Cladocera, Corycaeidae, *Oncaea* spp. and Clauso-Paracalanidae with similar percentages of occurrence in the stomachs (70-90 %), being also these prey types the most abundant in all the stomachs (Table 3). In autumn, the most common preys were *Microsetella rosea* and Corycaeidae, both appearing in 92 % of the analyzed anchovies' stomachs and, together with Clauso-Paracalanidae, were the most abundant preys (Table 3). In winter *Acartia* spp., Corycaeidae, *Oncaea* spp., Clauso-Paracalanidae and other unidentified copepods had the higher percentage of occurrence (83 %) in the stomachs of anchovies, but, in total, the most abundant prey type was appendicularia (62.56 %) (Table 3).

Sardines in summer had Corycaeidae copepods in 80 % of the stomachs, but the most abundant prey were cladocerans (84.30 % of total number of preys in all stomachs) (Table 3). In autumn *Acartia* spp., appendicularians, *C. typicus*, Corycaeidae, *M. rosea*, *Oncaea* spp., Clauso-Paracalanidae, *Temora* spp., and other calanoids appeared in all the stomachs, and the most abundant were Clauso-Paracalanidae (26.26 %) and appendicularians (49.28 %) (Table 3). In winter we found diatoms in all the stomachs as the most abundant prey in number (73.46 %), but also Clauso-Paracalanidae and unidentified copepods were relatively abundant (Table 3).

For anchovies, SIMPER analysis showed that the average contribution of the preys in the similarities of their seasonal diet composition was dominated in summer by 5 types of preys: Corycaeidae (19.06 %), Cladocera (18.15 %), *Oncaea* spp. (17.55 %), Clauso-Paracalanidae (17.25 %) and *Centropages typicus* (15.56 %); in autumn: *Microsetella rosea* (41.95 %), Corycaeidae (27 %) and Clauso-Paracalanidae (12.65 %); and in winter: Corycaeidae (20.62 %), Clauso-Paracalanidae (19.45 %), *Oncaea* spp. (19.00 %), unidentified copepods (17.76 %) and *M. rosea* (11.11 %).

For sardines, the preys that better contributed to describe the diets within each season were slightly different, regarding SIMPER analysis; in summer: Cladocera (38.48 %), Corycaeidae (26.95%), other crustaceans (15.74 %) and *M.*

*rosea* (12.26 %); in autumn: Appendicularia (24.15 %), Clauso-Paracalanidae (23.45 %), unidentified copepods (13.9%) and *M. rosea* (10.98%); and in winter: Diatoms (44.57 %) and unidentified copepods (16.5 %).

An ANOSIM analysis confirmed the similarities in the diet composition between the seasons in both species. Only anchovies in summer and in autumn had a statistically different diet composition among the 2 periods (significance level of sample statistic: 0.01 %).

The Index of Relative Importance (IRI, Table 3) confirmed the relevance of cladocerans in the diet of both species in summer, of appendicularians in the diet of anchovy in winter and of sardine in autumn, of diatoms in the diet of sardine in winter, and the prevalent relevance of copepods (mainly Corycaeidae, *Microsetella rosea*, *Oncaea* spp. and Clauso-Paracalanidae) in the diet of anchovy and sardine. Also other crustaceans (mainly decapod larvae), besides their low numerical abundance in the stomachs, had a high IRI in both anchovy and sardine due to their relatively elevated weight.

Ivlev's index of prey selectivity (Figure 3) showed that anchovy juveniles in summer ate preferably Corycaeidae and Clauso-Paracalanidae copepods and also had a positive selection of cladocerans; in autumn the copepods *Candacia* spp., Corycaeidae, *M. rosea*, *Temora* spp., and *Euterpina* spp., together with other unidentified crustaceans, were the most selected preys; in winter, calanoids, Corycaeidae, *M. rosea*, *Oncaea* spp. and other crustaceans, and also appendicularians, were the preferred preys. Sardine juveniles in summer selected mainly cladocerans and harpacticoid copepods; in autumn the selectivity index showed higher preferences for appendicularians and *M. rosea*; in winter phytoplankton (radiolarians and diatoms), appendicularians, Corycaeidae and other copepods were the most positively selected preys.

**Table 3.** Total stomach contents by weight (W), numerical abundance (N) of prey, frequency of occurrence (F) of prey in the stomachs and Index of Relative Importance (IRI)

| | Anchovy | | | | | | | | | | | | Sardine | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Summer | | | | Autumn | | | | Winter | | | | Summer | | | | Autumn | | | | Winter | | | |
| | W (%) | *N* (%) | *F* (%) | IRI (%) | W (%) | *N* (%) | *F* (%) | IRI (%) | W (%) | *N* (%) | *F* (%) | IRI (%) | W (%) | *N* (%) | *F* (%) | IRI (%) | W (%) | *N* (%) | *F* (%) | IRI (%) | W (%) | *N* (%) | *F* (%) | IRI (%) |
| *Acartia* spp. | 3.08 | 0.27 | 10 | 0.31 | 2.60 | 0.69 | 15 | 0.52 | 0.49 | 4.81 | 83 | 5.90 | 0.00 | 0.00 | 0.00 | 0.00 | 0.46 | 1.44 | 100 | 1.24 | 0.00 | 0.00 | 0 | 0.00 |
| Appendicularia | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.16 | 62.56 | 17 | 10.37 | 0.00 | 0.00 | 0 | 0.00 | 0.15 | 49.28 | 100 | 32.45 | 0.16 | 1.62 | 80 | 0.93 |
| *Calanus* spp. | 41.63 | 0.27 | 10 | 3.95 | 35.17 | 1.73 | 15 | 5.87 | 6.65 | 1.60 | 17 | 1.36 | 0.00 | 0.00 | 0 | 0.00 | 6.19 | 0.36 | 50 | 2.15 | 0.00 | 0.00 | 0 | 0.00 |
| *Candacia* spp. | 14.58 | 0.82 | 20 | 2.90 | 32.39 | 6.9 | 31 | 12.51 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 5.70 | 0.36 | 50 | 1.99 | 6.06 | 0.68 | 40 | 1.75 |
| *Centropages* spp. | 1.33 | 14.87 | 80 | 12.21 | 1.12 | 1.38 | 15 | 0.39 | 0.21 | 1.60 | 50 | 0.90 | 0.00 | 0.00 | 0 | 0.00 | 0.19 | 1.79 | 100 | 1.31 | 0.21 | 0.29 | 20 | 0.06 |
| Cladocerans | 7.28 | 30.85 | 80 | 28.73 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 1.21 | 84.30 | 60 | 51.47 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 |
| Corycaeidae | 1.49 | 7.16 | 80 | 6.52 | 2.55 | 15.91 | 92 | 17.63 | 0.24 | 6.42 | 83 | 5.50 | 0.50 | 4.09 | 80 | 3.69 | 0.45 | 1.08 | 100 | 1.00 | 0.47 | 0.88 | 20 | 0.18 |
| Diatoms | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 73.46 | 100 | 47.75 |
| *Euterpina* spp. | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.12 | 0.56 | 20 | 0.08 |
| Foraminiferans | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 1.75 | 100 | 1.14 |
| *Microsetella rosea* | 0.42 | 0.27 | 10 | 0.06 | 0.29 | 30.45 | 92 | 29.35 | 0.07 | 5.35 | 67 | 3.58 | 0.06 | 3.58 | 60 | 2.19 | 0.05 | 7.19 | 100 | 4.75 | 0.05 | 1.92 | 100 | 1.28 |
| Molluscs | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.22 | 0.09 | 20 | 0.04 |
| *Oithona* spp. | 0.58 | 0.00 | 0 | 0.00 | 0.49 | 0.69 | 15 | 18.22 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 |
| *Oncaea* spp. | 0.77 | 12.94 | 90 | 11.63 | 0.56 | 4.84 | 30 | 1.72 | 0.12 | 5.88 | 83 | 4.96 | 0.00 | 0.00 | 0 | 0.00 | 0.10 | 1.08 | 100 | 0.77 | 0.10 | 0.95 | 40 | 0.37 |
| Other crustaceans | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 88.42 | 3.21 | 67 | 60.57 | 92.26 | 0.34 | 40 | 37.16 | 82.32 | 0.36 | 50 | 27.13 | 87.52 | 0.96 | 60 | 34.51 |
| Paracalanidae | 1.91 | 26.17 | 70 | 18.51 | 1.62 | 19.72 | 62 | 13.58 | 0.31 | 7.48 | 83 | 6.44 | 0.32 | 1.02 | 40 | 0.54 | 0.28 | 26.26 | 100 | 17.42 | 0.30 | 3.23 | 80 | 1.83 |
| Polychaeta larvae | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.34 | 0.14 | 20 | 0.06 |
| *Temora* spp. | 5.58 | 3.85 | 60 | 5.33 | 4.71 | 3.46 | 31 | 2.60 | 0.89 | 0.53 | 17 | 0.24 | 0.93 | 1.36 | 40 | 0.92 | 0.83 | 1.08 | 100 | 1.25 | 0.88 | 0.07 | 20 | 0.12 |
| Tintinids | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.07 | 2.24 | 40 | 0.60 |
| Other Copepods | 14.58 | 1.93 | 50 | 7.77 | 18.48 | 14.18 | 46 | 15.59 | 2.33 | 4.81 | 83 | 5.90 | 3.64 | 1.02 | 40 | 1.87 | 3.25 | 9.71 | 100 | 8.51 | 3.46 | 10.78 | 100 | 9.26 |

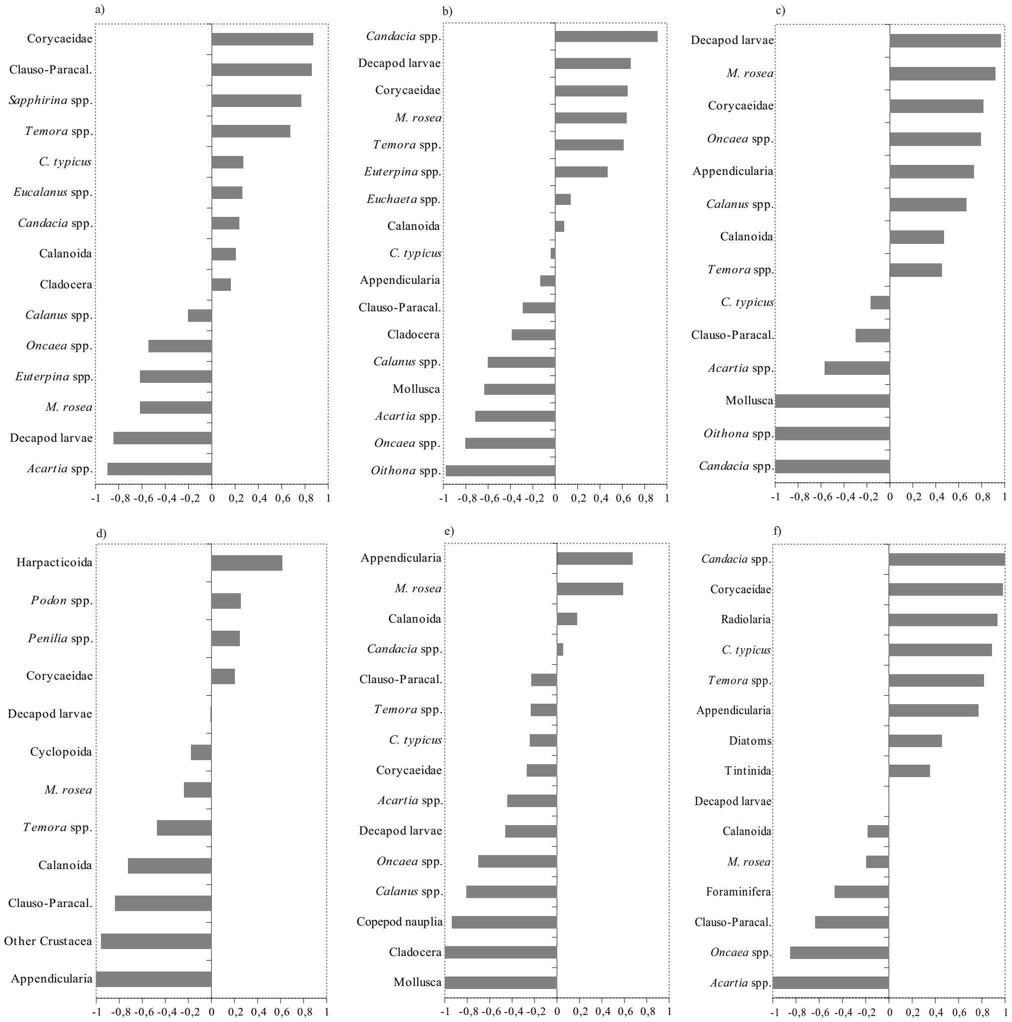

**Figure 3. Ivlev's dietary indexes for juveniles of: a) anchovy in summer; b) anchovy in autumn winter; c) anchovy in winter; d) sardine in summer; e) sardine in autumn; f) sardine in winter.**

The Schoener's index D, calculated for both species during the 3 different seasons, did not reflect diet overlap between sardine and anchovy juveniles in any case (summer: $D = 0.40$; autumn: $D = 0.33$; and winter: $D = 0.28$). The overlap is considered as significant when $D > 0.60$.

### *3.3. Development of feeding-related anatomical structures*

A Mann-Whitney U test set that there was no statistically significant difference ($p > 0.05$) in the standard length of both species (Table 4).

Significant differences ($p < 0.001$) between sardine and anchovy were found in others anatomical parameters (Table 4) after tested with a t-student test. Sardine presented significantly higher numbers of both gill rakers and pyloric caeca than anchovy and also significantly higher gill raker density (Table 4), which is the number of gill rakers per length of branchial arch.

**Table 4**. **Means ± Standard deviations of anatomical parameters of anchovy and sardine, showing whether or not there was statistical differences between species (Mann-Whitney test for standard length and t-student test for the rest).**

| | Anchovy | Sardine | F |
|---|---|---|---|
| Standard length (cm) | 5.53±2.74 | 6.03±2.24 | n.a. |
| Piloric caeca | 10.48±11.08 | 71.88±50.75 | 379.656*** |
| Gill rakers[1] | 25.12±10.99 | 36.80±15.66 | 30.956*** |
| Arch length[1] (mm) | 7.39±4.08 | 7.35±3.86 | 1.204 |
| Gill raker spacing (mm) | 0.22±0.08 | 0.18±0.04 | 63.583*** |
| Gill raker length (mm) | 2.41±1.73 | 2.64±1.51 | 6.391 |

***** p < 0.001**; [1] Ceratohypobranchial arch; n.a.: non applicable

The gill rakers (GR) number in anchovy became constant at SL > 5.30 cm, but in sardine the GR number maintained a significantly positive trend ($p < 0.05$) along all the sizes (Figure 4a).

Pyloric caeca in sardines started developing when fishes reach 4 cm SL, but in anchovies they appeared at a smaller size (3 cm SL). Once pyloric caeca appeared, their number in sardine was always significantly positively increasing ($p < 0.05$); on the contrary, in anchovies larger than 4cm SL the value of the slope of the regression was not significantly different to 0 (Figure 4b).

The mean gill rakers length ($L_{GR}$) and the mean gill rakers spacing ($S_{GR}$) were positively correlated with the SL for both species (Figures 4c and 4d, respectively). Anchovy had a significantly smaller linear regression slope than sardine regarding the gill rakers spacing ($p < 0.001$).

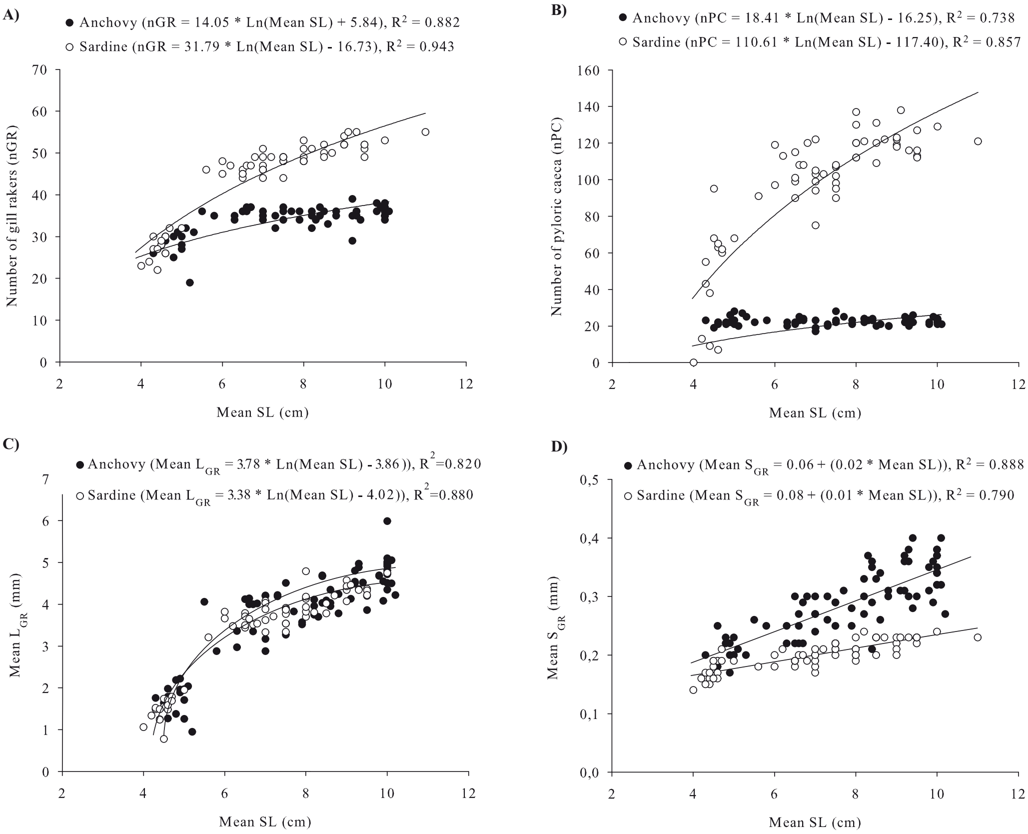

**Figure 4. Relationships between stardard length of anchovy and sardine juveniles and different anatomical structures related with their trophic behavior: A) number of gill rakers; B) number of pyloric caeca; C) mean length (mm) of the central gill rakers; D) mean gap (mm) between consecutive gill rakers, measured at the base of gill rakers.**

## 4. Discussion

In other areas than the Mediterranean Sea, several authors have studied the ecology of juveniles of these or similar species: Ciechomski (1966) in the southwest Atlantic (*Engraulis anchoita*), Watanabe & Saito (1998) and Takagi *et al*. (2009) in the northwest Pacific (*Sardinops melanostictus* and *E. japonicus*), Litz (2008) and Quiñónez-Velázquez *et al*. (2000) in the northeast Pacific (*E. mordax* and *S. sagax*), Bachiller (2008) in the north Iberian coast (*E. encrasicolus*) and van der Lingen *et al*. (2006) in the Benguela current system (*E. encra-*

*sicolus* and *S. sagax*). Some of these populations have been reported to display resource partitioning through diet segregation, as is expected to happen in most of the fish assemblages (Ross, 1986). However, to our knowledge, there were no studies to confirm the same fact between the Mediterranean small pelagic fish populations.

The present work was thus devoted to examine the trophic interactions between juveniles of both anchovy and sardine populations in the Mediterranean and to provide a perspective of their feeding behaviors based on their ontogenetic development.

In summer, anchovies and sardines avoided trophic overlap by feeding on different preys, mainly copepods and cladocerans, respectively. These 2 groups of preys were also among the most abundant in the summer plankton community. Ivlev's selectivity index showed that sardine selected basically cladocerans and small copepods (harpacticoids and Corycaeidae), while anchovy preferred other species of copepods.

In autumn, the diet overlap was also low due to the high selection of appendicularians and *Microsetella rosea* by sardine while anchovy selected predominantly other copepods and decapods.

During winter, sardine selected and fed very importantly on phytoplankton together with copepods like Corycaeidae, *Temora* spp. and *Centropages* spp., but anchovy preferred to eat appendicularians, small copepods (*M. rosea*, Corycaeidae and *Oncaea* spp.) and other crustaceans. We must remark that in this season, the Gulf of Lions presented the highest abundances of both microplankton and mesozooplankton, and the highest biomass of mesozooplankton, in comparison with autumn and summer.

Some experimental studies about feeding behavior of engraulids demonstrated that fishes tend to use filter feeding under higher food concentration, while shifting to particulate feeding when food concentration is relatively lower (Bulgakova, 1996). Our research demonstrated that the observations of these experimental studies can be confirmed also in the northwestern Mediterranean in the case of sardines, but the same can not be assured for anchovies, likely due to a less efficient anatomical adaptation to filter feeding than sardines. This is explained by our finding that even after the anchovies' food filter apparatus is already fully developed (i. e. when individuals reach around 5 cm SL) anchovy juveniles kept feeding predominantly on zooplankton rather than on phytoplankton.

As showed before, in both seasons summer and autumn, copepods are the main prey groups of anchovy, and at the same time a very important component of the plankton community. However, during winter appendicularians were by far the most abundant group in the stomachs of anchovies. This season also coincided with the highest relative abundance of appendicularians in the zooplankton community, only behind copepods. This could lead us to think that anchovy juveniles may be selecting their food to some extent depending on the availability of each potential prey group rather than, for example, on the palatability, as confirmed also by Costalago *et al*. (2012) and Morote *et al*. (2010). In addition, appendicularians are slightly more energetic than copepods (Davis & Myers, 1998), and energetically rich particles could be retained with their houses, so this could be another reason to be more selected by anchovy juveniles when available. Also, the fact that appendicularians are not able to swim as fast as copepods (Gorsky, 2005) may make them a more easily prey to catch.

However, preys such as appendicularians, might remain underestimated when observing stomach contents, likely because they are more easily digested, as noted in Costalago *et al*. (2012), that used stable isotopes to elucidate diets. Therefore, appendicularians were very important in the diets of both anchovy and sardine juveniles also in autumn.

The above described predatory behavior of anchovy, preying on relatively large and abundant plankters, also supports the theory that anchovy juveniles are particle feeders rather than filter feeders, which would be in correspondence with their anatomical characters. Fishes with many pyloric caeca tend to feed on smaller preys and eat more frequently (Groot, 1969, Darnell, 1970). The pyloric caeca are designed to hold foodstuffs for prolonged periods to ensure complete digestion (Sargent *et al*., 1979). The fact that sardines presented a significantly higher number of caeca could imply a greater need for digestive surface due to a more phytoplanktonic diet, because of the difficulty of digesting plant cells. No preys were observed within the pyloric caeca of the fishes analyzed in this study, likely meaning that the food contents had been already digested and showing that these organs are highly efficient.

In the case of sardine juveniles, although the number of GR always kept a positive relation with the SL, there is an inflexion point at 5.5 cm SL, where the slope started to level off. Both positive relation and SL at the inflexion point coincide with the pattern found in previous studies by Andreu (1953, 1960).

In addition, several clupeoids species have been reported to share the same morphological pattern between SL and the number of GR: *Brevoortia tyrannus* (June & Carlson, 1971), *Cetengraulis mysticetus* (Bayliff, 1963), *Clupea harengus* (Gibson, 1988), *Engraulis capensis* (King & McLeod, 1976), *E. japonica* (Shen, 1969) and *Sardinops caerulea* (Scofield, 1934).

One of the consequences of the continued addition of GR is that the gap between them increases at a slower rate than in those species whose GR number becomes relatively fixed, as shown in figure 5d. This could lead us to speculate that the two types of development characterize obligate phytoplanktivorous and facultative zooplanktivorous, respectively. Although behavioral observations to test such a speculation are scarce or not available, our results regarding the diet indicated that, at least during winter, sardine juveniles were mostly phytoplanktivorous, while anchovy juveniles preyed only on zooplankton. Supporting this idea, MacNeill & Brandt (1990) also stated that "gill rakers morphology should be more closely related to diet selectivity among filter-feeding planktivores than particulate feeders".

However, some authors defend that raker spacing may not be enough to fully determine the feeding behaviour of these fish species; for example, Angelescu (1982) and Bornusch & Lee (1992) suggested that in different engrauloids species the effective branchial retention capacity is affected by the development of fine structures tipped by denticles on the rakers. This aspect needs more accurate observations in the case of *E. encrasicolus* and *S. pilchardus*.

Another issue that should be more comprehensively studied in these two species is the mouth openness ability at the time of feeding, which is sometimes considered as a key factor determining the filtering raker interspaces (Gibson, 1988, Wright *et al*., 1983).

Additionally, Sanderson *et al*. (1991) described a slightly different function of the GR in the planktivore blackfish *Orthodon microlepidotus*. They observed that rakers acted as a barrier to water flow rather than as filter since water does not pass between these structures in the blackfish. Ingested prey would be carried by currents to the roof of the oral cavity, where they would be trapped by mucus before being swallowed. If this was true for *E. encrasicolus* and *S. pilchardus*, and given the relatively low variability of preys in their stomachs and the differences in their diets, it would mean that they are even more dietary selective than we could speculate by considering the GR as mere filtering structures.

According to our observations there was no general niche overlap between these 2 species at the juvenile stage, and therefore they did not compete for the same food resources. In natural populations the diet overlap is more common when food is abundant and is less evident when food is scarce (Schoener, 1974). However, given the difficulties to establish the food limitation thresholds for these species in their environment, assessing the trophic competition between marine small pelagic fish species needs more delved research.

With this study we have shown that anchovy and sardine do not present food competence and have described significant differences between the 2 species in their diets due to their different alimentary morphology. Thus, their adaptation to environmental shifts would follow different patterns.

For example, within a future scenario where plankton community would tend to be dominated by relatively small species of copepods (Conversi *et al.*, 2009, in the central Mediterranean), with an increase in the proportion of phytoplankton (Edwards, 2009, in the North sea) or even with a decrease in the proportion of copepods (Molinero *et al.*, 2005, in the northwestern Mediterranean) sardine populations could be favored over anchovy.

On the other hand, some studies affirm that forthcoming marine environmental changes will modify plankton community towards a dominance of non-siliceous phytoplankton species over diatoms (Gladan *et al.*, 2009, Lejeusne *et al.*, 2009), so sardines would find greater difficulties in feeding on diatoms, which are essential part of their diet during some periods of the year, as we have shown.

Although regime shifts seem to be trophodynamically mediated in other regions of the world where sardine (*Sardina* spp. or *Sardinops* spp.) and anchovy

(*Engraulis* spp.) species co-exist (Benguela current system, California current system, Kuroshio current system, Canary current system, Humboldt current system; reviews by Lluch-Belda *et al*., 1992, and van der Lingen *et al*., 2006), the same cannot be confirmed for the western Mediterranean populations. Moreover, if the anchovy spawning period in the Gulf of Lions, that normally happens in late-spring and summer, extends following the increasing sea surface temperature trends (Martín *et al*., 2011), sardine and anchovy larvae populations possibly would coincide in the future (Costalago *et al*., 2011) and, given their very similar diets at the larval stage (Costalago *et al*., 2012), could likely experience diet overlap.

This research reveals aspects of the trophic dynamics of the 2 most important pelagic fish species in the Mediterranean sea (Palomera *et al*., 2007) and could provide guidance for future ecological studies that are essential in such a variable environment if we want to improve the assessment and management of these small pelagic species.

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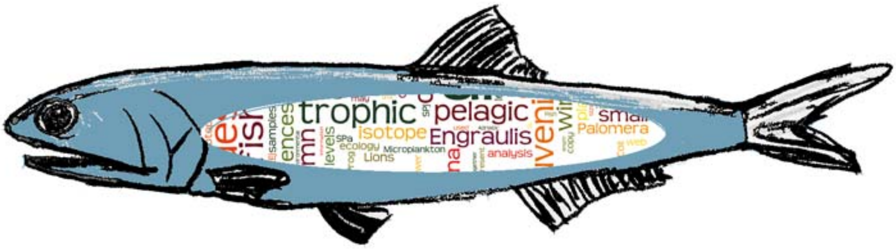
# Capítulo 3. CAMBIOS ONTOGENÉTICOS Y ESTACIONALES EN LOS HÁBITOS ALIMENTICIOS Y NIVELES TRÓFICOS DE DOS ESPECIES DE PECES PELÁGICOS PEQUEÑOS.

**Resumen.** Investigamos los cambios ontogénicos y estacionales en la ecologia trófica de dos especies de peces pelágicos pequeños, la anchoa europea, Engraulis encrasicolus, y la sardina europea, Sardina pilchardus, en el golfo de León (Mediterráneo noroccidental). Analizando los isótopos estables $\delta^{13}C$ y $\delta^{15}N$, determinamos la variación estacional de las fuentes de alimento y del nivel trófico de estas especies, y examinamos los regímenes alimenticios durante su desarrollo. Los resultados de estas investigaciones nos proporcionan estimas de la dieta de ambas especies. Comparamos los valores observados durante diferentes estaciones (verano, otoño e invierno) y en los diferentes estadios de desarrollo (larvas grandes, juveniles y adultos) en las dos especies, junto con los valores de los grupos potenciales de presas (microplancton, cladóceros, copépodos y apendicularias). El alimento preferente de las larvas fue el microplancton, aunque aparecieron diferencias en la dieta después de la metamorfosis. Los cladóceros fueron las presas preferidas cuando estaban disponibles (verano), y las apendicaularias fueron las presas más seleccionadas en otoño. Durante el invierno, las dietas parecían ser más homogéneas. La razón más probable para explicar las ligeras diferencias que hemos encontrado entre niveles tróficos son los distintos comportamientos alimenticios entre las larvas de las dos especies. Esta investigación demuestra que los estudios con isótopos estables pueden proveer de un método alternativo y/o complementario para determinar la dieta de los peces pelágicos pequeños en periodos largos y, además, proporcionan conocimientos exhaustivos del funcionamiento del ecosistema pelágico.



# Ontogenetic and seasonal changes in the feeding habits and trophic levels of two small pelagic fish species.

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**ABSTRACT.** We investigated ontogenetic and seasonal changes in the feeding ecology of 2 small pelagic fish species, the European anchovy *Engraulis encrasicolus* and the European pilchard *Sardina pilchardus* in the Gulf of Lions (NW Mediterranean). By analysing the stable isotopes δ13C and δ15N, we determined the seasonal variation in the food sources and in the trophic level of these species, and we examined dietary shifts during their development. The results of these investigations provided estimates of the diets of both species. We compared the values observed during different seasons (summer, autumn and winter) and at different developmental stages (late-larvae, juveniles and adults) for both species, together with the values of potential groups of prey (microplankton, cladocerans, copepods and appendicularians). Late-larvae preferred to feed on microplankton, although differences in the diet appeared after metamorphosis. Cladocerans were usually the preferred prey when available (summer), and appendicularians were the preferred prey in autumn. During the winter, the diets seemed to be more heterogeneous. Different feeding behaviours between the late-larvae of the 2 species were the most likely reason for the lightly different trophic levels found in the present study. This research demonstrates that studies with stable isotopes can furnish an alternative and/or complementary method for determining the diet of small pelagic fishes over extended periods and provides comprehensive knowledge of the functioning of the pelagic ecosystem.

KEY WORDS: *Engraulis encrasicolus* · *Sardina pilchardus* · Stable isotopes · NW Mediterranean · Trophic ecology · Microplankton · Copepods · Appendicularians · Cladocerans

## 1. Introduction

An understanding of the primary ecological processes in marine ecosystems, such as variation in food sources, trophic transfer through the food web and nutrient cycling, is fundamental for relating ecosystem functioning to management. In this context, the description of the trophic ecology of marine organisms is vital to determine the intrinsic factors that control their distribution, abundance, and, ultimately, their function within the ecosystem. Fishes have developed a wide variety of feeding-related structures (e.g. gill rakers, pyloric caeca, or teeth) and behaviours (e.g. filter feeding vs. particulate feeding) that may undergo modifications through ontogeny or season (Gerking, 1994), with diverse ecological implications. Many studies have addressed the trophic roles of both top and bottom species of the food web, i.e. marine predators and plankton communities, respectively (Hunter & Price, 1992, Rice, 1995, Pace *et al*., 1999), and, among these studies, some have focused on a group that is particularly important ecologically: the small pelagic fishes (Bakun, 1996, Cury *et al*., 2000, Palomera *et al*., 2007).

Small pelagic fish are essential elements of marine ecosystems owing to their significant biomass at intermediate levels of the food web (Cury *et al*., 2000, Palomera *et al*., 2007). Pelagic fish play a significant role in connecting the lower and upper trophic levels (Cury *et al*., 2000). Their massive populations, which exert a huge pressure on zooplankton and, at the same time, are the main food for many species, may vary greatly in size under intensive exploitation or following changes in productivity. Therefore, fluctuations in small pelagic populations owing to fishing or natural factors modify the structure and functioning of the marine ecosystem (e.g. Cury *et al*., 2000, Shannon *et al*., 2000, Daskalov, 2002). The significant abundance and success of pelagic fish in upwelling areas have been attributed to the flexibility of their feeding behaviour (van der Lingen *et al*., 2009). Pelagic fish have also been identified as important groups in many ecosystems (Libralato *et al*., 2006), e.g. in upwelling areas, where they exhibit a ‘wasp-waist’ flow control (Cury *et al.*, 2000). Therefore, the interactions among different populations of small pelagic fishes can also be strongly influenced by regime climatic shifts and can have strong impacts on fisheries (e.g. Klyashtorin, 1998, Rodríguez-Sanchez *et al*., 2002).

Low trophic-level species are directly influenced by the remarkable environmental differences between seasons (Calbet *et al*., 2001). The populations of small pelagic fish may be affected by any environmental change that influences the plankton community, which is the basis of the diet of these fishes. In fact, the seasonal unevenness of oceanographic parameters (i.e. salinity, fluorescence and, most importantly, temperature) and of river runoff has been shown to have important effects on the biology and viability of these fish populations (Lloret *et al*., 2001, 2004). The early life stages (i.e. larvae and juveniles) are especially sensitive to such effects (Govoni 2005, Ruiz *et al*., 2006, Costalago *et al*., 2011). Several species of small pelagic fish co-occur in the Mediterranean Sea.

Unquestionably, the European anchovy *Engraulis encrasicolus* and the European sardine *Sardina pilchardus* are the most relevant of these species in terms of both biomass and fishery catches (Palomera *et al*., 2007). These 2 small pelagic fish are also key species at mid-trophic levels in the Mediterranean Sea (Coll & Libralato, 2012). E. encrasicolus was found to be a key species in the North Adriatic (Coll *et al*., 2007, Barausse *et al*., 2009) and in the Aegean Sea (Tsagarakis *et al*., 2010). On the contrary, in the NW Mediterranean, S. pilchardus is among the leading keystone species and is also considered to exhibit 'wasp-waist' trophic control (Coll *et al*., 2006, Palomera *et al*., 2007, Navarro *et al*., 2011). Small pelagic fish are the most vulnerable to any environmental shift among fish species (Coll *et al*., 2008). At the same time, they are the main constituents of the diet of several pelagic, demersal and apical species (e.g. Coll *et al*., 2006, Palomera *et al*., 2007, Navarro *et al*., 2009).

A significant number of studies on the ecology of anchovy and sardine in the Mediterranean Sea have been conducted (see reviews by Palomera *et al*., 2007, Morello & Arneri, 2009), but relatively few of these studies address dietary composition. Previous papers that included dietary information focussed on larval stages of *Engraulis encrasicolus* and *Sardina pilchardus* (Conway *et al*., 1998, Tudela *et al*., 2002, Catalán *et al*., 2010, Morote *et al*. 2010), the adults of *E. encrasicolus* (Tudela & Palomera, 1997, Plounevez & Champalbert, 2000), or the juveniles and adults of *E. encrasicolus* (Borme *et al*., 2009). However, none of the previous works, that, in addition, have usually been limited in temporal resolution, included data about sardines except that of Morote *et al.* (2010), and they studied only larvae <1.6 cm in total length. A more comprehensive understanding of the trophic dynamics of assemblages of small pelagic fish from a seasonal and ontogenetic perspective is therefore essential to highlight the important role of these populations within the marine ecosystem. The present study intends to provide this necessary knowledge more comprehensively by gathering and analyzing data from different seasons and life stages of these 2 pelagic species for the first time in the Mediterranean.

All previous studies on the trophic ecology of these small pelagic fish species have been based on the direct analysis of stomach contents (Tudela & Palomera, 1997, Conway *et al*., 1998, Plounevez & Champalbert, 2000, Borme *et al*., 2009, Morote *et al*., 2010). This approach involves implicit methodological errors because it cannot accurately quantify the importance of prey items that are readily digested and because it does not identify the prey items that are actually assimilated following ingestion. The stable isotope approach can augment conventional means of dietary analysis because stable isotopes reflect time-integrated dietary records and present a perspective on trophic dynamics that involves a more substantial time period than the analysis of stomach contents can provide (Polunin & Pinnegar, 2008). Therefore, stable isotope analysis yields information that cannot always be obtained from direct observation and can support hypotheses about the developmental changes in the feeding strategies of a species and about species interactions because these hypotheses are based on data about assimilated food rather than ingested food. In fish, the stable isotope values of muscular tissue integrate dietary information between 40 and 80 d prior to sampling (Bode *et al*., 2007, Buchheister & Latour, 2010). Stable isotopes of nitrogen ($\delta^{15}N$) are indicators of trophic positions because consumers are predictably enriched in $\delta^{15}N$ relative to their food (Post, 2002, Vanderklift & Ponsard, 2003). Stable carbon isotope values ($\delta^{13}C$) give information on primary production and are useful for tracing the origin of the prey consumed (Vander Zanden *et al*., 1999, Pinnegar & Polunin, 2000). Furthermore, by combining stable isotope values for consumers and their prey, powerful isotopic mixing models can be applied to obtain estimates of the relative contribution of each potential prey item to the diet of the consumer (e.g. the stable isotope analysis in R using the [SIAR] isotopic mixing model; Parnell *et al*., 2010). These models add useful information to investigations of food selectivity and can be used to complement data from stomach content analysis (Peterson, 1999, Lin *et al*., 2007, Tripp-Valdez & Arreguín-Sánchez, 2009).

Flaherty & Ben-David (2010) pointed out that there are limitations in the use of isotopic mixing models because of the important differences in isotope values that can be found if there is spatial heterogeneity, so that habitat-derived variation in consumers' isotopes would be mistaken as diet variation in resource isotope values. Since populations of *E. encrasicolus* and *S. pilchardus* in the Gulf of Lions are widespread and homogenously distributed over all the continental shelf and therefore feeding on the same available resources, we could assume that this limitation would not be of great influence in our case.

The present study uses $\delta^{13}C$ and $\delta^{15}N$ values as indicators of the trophic relationships between Engraulis encrasicolus and Sardina pilchardus for different age groups (late-larvae, juveniles and adults) during different seasons (summer, autumn and winter) and therefore under different environmental conditions. The study investigates seasonal and ontogenetic changes in the food sources and trophic levels of these species, and it examines possible dietary shifts during development. These analyses yield estimates of the diet compositions of both species. All of these findings are important because they can supply the knowledge needed to fill current information gaps existing in both experimental and direct observational studies and allow improved management of fish stocks.

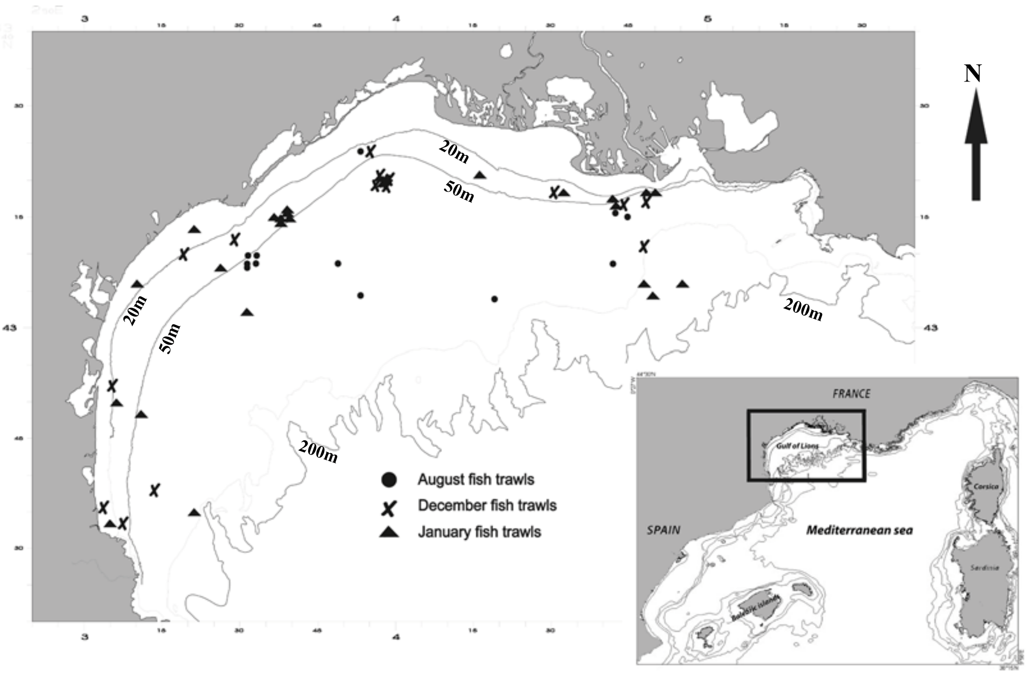

**Fig. 1. Study area (Gulf of Lions, NW Mediterranean), indicating fish sampling locations.**

## 2. Materials and methods

### *2.1. Study area and sample collection*

The present study was conducted in the Gulf of Lions (Fig. 1), one of the most productive areas of the NW Mediterranean (Salat, 1996). In terms of biomass, it is also the most important area of the Mediterranean for small pelagic fish species (Barangé *et al*., 2009). During 3 different oceanographic cruises on board the *N/O 'L'Europe* (IFREMER, France), we collected late-larvae, juveniles and adults of *Engraulis encrasicolus* and *Sardina pilchardus*. The standard length ranges considered to classify the individuals within these groups were: for late-larvae 2.0 to 3.5 cm for *E. encrasicolus* and 2.5 to 4.0 cm for *S. pilchardus*, for juveniles 3.6 to 8.5 cm for *E. encrasicolus* and 4.1 to 10.5 cm for *S. pilchardus* and for adults 8.6 to 12 cm for *E. encrasicolus* and 10.6 to 14 cm for *S. pilchar-*

*dus*. All individuals were considered as adults when they reached the minimum length at first maturity observed during the cruises. The first cruise was conducted during autumn (12 to 21 December 2007), the second cruise was conducted during summer (21 to 29 July 2008) and the third was conducted during winter (11 to 27 January 2009). All specimens were caught with a pelagic trawling net equipped with a small-mesh codend (mesh length: 5 mm; ISO 1107) and towed at an average speed of 3.6 knots over a 30 to 40 min period. The samples were immediately frozen (-20° C) after sorting on board.

During each season, plankton samples were collected at the same sites where the pelagic trawls were made. Sampling was done at 16 plankton stations in summer, 15 in autumn and 13 in winter using a standard WP2 net with a mesh size of 200 µm and a scaled-down WP2 net with a mesh size of 53 µm. The WP2 net sample was sieved through a 3000 µm plankton mesh to obtain the 200 to 3000 µm mesozooplankton fraction and the scaled-down WP2 net was sieved through a 200 µm plankton mesh to obtain the 53 to 200 µm microplankton fraction. All plankton samples were split with a Motoda plankton splitter (Motoda, 1959). One-half of each sample was preserved in buffered 4 % formaldehyde- seawater solution for subsequent qualitative analyses of plankton community composition, whereas the other half was frozen (-20° C) on board for biomass measurements and stable isotopic determination. Qualitative analysis of plankton was performed in the laboratory, and individuals were identified to the lowest taxonomical level possible under a stereomicroscope (Leica MZ12) with a magnification of up to 100×. The meso-zooplankton samples were analysed in aliquots representing about 10 % of the sample and repeated until counting at least 400 copepods in each; additional subsamples were also taken for any other abundant organism (i.e. cladocerans during summer). Microplankton samples were subsampled differently: 1 to 2 % of the original volume was analysed to estimate the presence of nauplii, dinoflagellates, ciliates and diatoms; small copepods (mainly copepodites) were analysed in volumes sufficient to count at least 400 individuals. The number of individuals of each identified taxon and abundances (ind $m^{-3}$) were calculated.

### *2.2. Stable isotope analysis*

A portion of dorsal muscle (without skin) was extracted from each individual (late-larvae, juveniles and adults). Muscle has been defined as the most appropriate tissue to analyse stable isotope in fish (Sweeting *et al*. 2005). Plankton samples from each season were defrosted in the laboratory, pooled together and sorted into different potential prey groups (microplankton, copepods and appendicularians in all the seasons, and also cladocerans in summer) which were selected according to previous studies of stomach contents (Morote *et al*., 2010 for larvae, D. Costalago unpubl. data for juveniles, Plounevez & Champalbert, 2000 for adults).

All fish and plankton samples were freeze-dried, powdered, and 0.9 to 1.0 mg of each sample was packed into tin capsules. The samples were then oxidised with CuO and $CO_3O_4$/Ag at approximately 900° C in a Flash EA 1112 Elemental Analyser coupled with a pyrolyser TC-EA and a gas bench through a Conflo III Finnigan MAT interface. $NO_X$ was reduced with Cu at 680° C. The combustion products $N_2$ and $CO_2$ were introduced into a Delta C Finnigan MAT mass spectrometer through an $MgClO_4$ drying column. The isotope ratio mass spectrometry facility at the Serveis Científico-Tècnics of the University of Barcelona (Spain) applies international standards, generally run for every 12 samples: IAEA CH7

(87 % of C), IAEA CH6 (42 % of C) and USGS 24 (100 % of C) for $\delta^{13}$C and IAEA N1 and IAEA N2 (with 21 % of N) and IAEA NO3 (13.8 % of N) for $\delta^{15}$N.

The $\delta^{13}$C values were corrected for the effect of lipids both in fish and prey samples following Logan *et al*. (2008). This procedure reduces the time and uncertainty associated with lipid extraction procedures, and it improves the estimates of dietary proportions derived from stable-isotope mixing models (Phillips & Gregg, 2001, Logan *et al*., 2008).

### *2.3. Isotopic mixing model*

To estimate the diet composition at each age (late-larvae, juveniles and adults) during each season (summer, autumn and winter) we applied a Bayesian model in SIAR 4.1.1 (Stable Isotope Analysis in R 2.12.2). This model runs under the free software R (R Development Core Team, 2009). The model allows the inclusion of sources of uncertainty. In particular, the variability in the isotope signatures (mean and standard deviation) of prey species can be incorporated into the model (Parnell *et al*., 2010). SIAR uses Markov-chain Monte Carlo modelling, takes data on animal stable isotopes and fits a Bayesian model of the diet habits based on a Gaussian likelihood function with a Dirichlet prior mixture distribution for the mean.

The model also assumes that each target value (i.e. the stable isotope data for each individual) comes from a Gaussian distribution with an unknown mean and standard deviation. The structure of the mean is a weighted combination of the food sources' isotopic values. The standard deviation depends on the uncertainty in the fractionation corrections and the natural variability among target individuals within a defined group. We used the isotopic discrimination of 1.01

± 0.17 ‰ for C and 3.56 ± 0.17 ‰ for N as the average of discrimination factors estimated for muscle analyses of different marine fish species provided in Caut *et al*. (2009).

### 2.4. Trophic level

To estimate the trophic levels (TL) of the different individuals we used the equation:

$$TL_{consumer} = TL_{basal} + (\delta^{15}N_{consumer} - \delta^{15}N_{prey}) / \Delta\delta^{15}N$$

where $TL_{consumer}$ refers to the mean TL of each individual fish. $\delta^{15}N_{prey}$ and $\delta^{15}N_{consumer}$ were, respectively, the isotopic values of microplankton and individual fish obtained in the present study (in each season). We applied a basal trophic level ($TL_{basal}$) of 1.5, assuming that the microplankton (mostly composed by phytoplankton) showed a trophic level between 1 of primary producers and 2 of micro- and mesozooplankton (Coll *et al*., 2006). The values of the isotopic discrimination factor for N ($\Delta\delta^{15}N$) were the same used for the isotopic mixing model (estimated from Caut *et al*., 2009).

### 2.5. Selectivity index

The output data of the SIAR model, together with the relative composition of the functional groups of plankton in the environment, were used to estimate the Ivlev diet selectivity index (Ivlev, 1961, Krebs, 1989) for each case analyzed. The value of the index was calculated with the equation:

$$(r_i - p_i) / (r_i + p_i)$$

where $r_i$ is the proportion of prey item *i* calculated from the SIAR model and $p_i$ is the proportion of prey item *i* available from the marine environment.

### *2.6. Statistical analysis*

ANOVA tests were used to examine the differences in both $\delta^{13}C$ and $\delta^{15}N$ values among species and ages in each season (summer, autumn and winter). Post hoc comparisons for observed means were performed with a Tukey test. The assumptions of ANOVA were checked with a Kolmogorov-Smirnov test for normality and a Levene test for homogeneity of variances. All analyses were performed with SPSS v.19. A significance level of $p < 0.05$ was used for all tests unless otherwise stated.

## **3. Results**

### *3.1. Plankton composition*

The microplankton samples were composed primarily of phytoplankton (mainly diatoms; to a lesser extent, dinoflagellates, such as *Ceratium* spp. and *Peridinium* spp.; and tintinnids), together with high numbers of copepod nauplii during the summer and winter and small copepods, especially *Oncaea* spp., throughout the year and, during the winter, *Paracalanus parvus*. Mesozooplankton was dominated by copepods (mainly calanoids). The plankton community also included a relatively less important number of appendicularians during the autumn and winter (Fig. 2). Cladocerans occurred during summer but not during other seasons (Fig. 2).

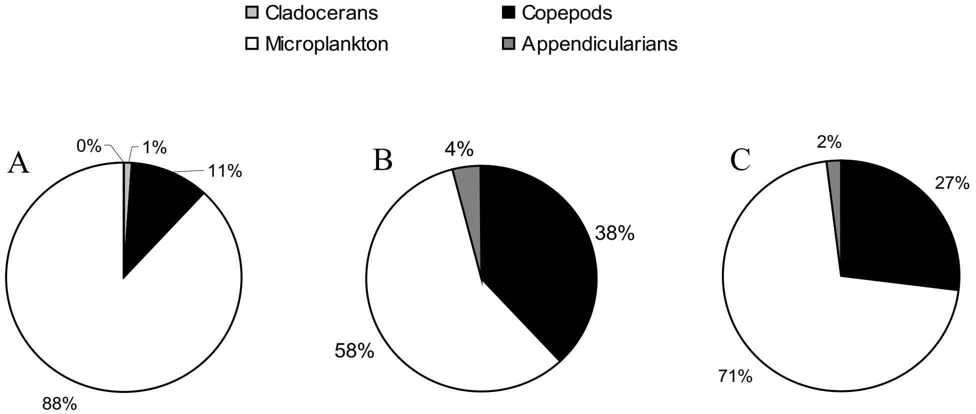

**Fig. 2 Composition of plankton community in the Gulf of Lions during (A) summer, (B) autumn and (C) winter showing the proportions of the four dietary functional groups defined for this study.**

### *3.2. Isotopic differences*

*Sardina pilchardus* showed lower $\delta^{15}N$ values in summer and the highest $\delta^{15}N$ values in autumn, except for late-larvae (winter), which had significantly lower $\delta^{15}N$ values than in any other age group and season (Table 1, Fig. 3). Regarding *S. pilchardus* $\delta^{13}C$ values (juveniles and adults), there were no significant differences ($p = 0.25$) between autumn and winter seasons (Table 2), while summer $\delta^{13}C$ values were statistically different from the other 2 seasons (Tables 1 & 2, Fig. 3). *S. pilchardus* late-larvae (winter) also showed significantly lower values of $\delta^{13}C$ compared to adults and juveniles (Table 2). *S. pilchardus* values of $\delta^{13}C$ and $\delta^{15}N$ did not differ in autumn between adults and juveniles ($p = 0.870$ for $\delta^{15}N$, $p = 0.213$ for $\delta^{13}C$; Table 1). For both *S. pilchardus* age groups (juveniles and adults) analysed together, $\delta^{13}C$ appeared to differ between the summer and other seasons, and $\delta^{15}N$ differed between winter and the other seasons (Table 1, Fig. 3).

*Engraulis encrasicolus* $\delta^{15}N$ isotopic values in both autumn and winter displayed marked differences between adults and juveniles (Table 1, Fig. 3). *E. encrasicolus* late-larvae (summer) had significantly higher values of $\delta^{15}N$ than juveniles (Table 1, Fig. 3). No differences in $\delta^{15}N$ values were observed among seasons for adult *E. encrasicolus*, whereas $\delta^{15}N$ values were statistically different for juveniles during autumn than in other seasons. When adults and juveniles were considered together, significant differences in $\delta^{15}N$ values were found among the 3 periods (Table 1, Fig. 3). *E. encrasicolus* $\delta^{13}C$ values exhibited substantial differences during the summer for both juveniles and adults. *E. encrasicolus* late-larvae (summer) had higher $\delta^{13}C$ values than adults.

**Table 1. Sample size (n), mean ± standard deviation of standard length (SL), stable isotope values ($\delta^{15}N$ and $\delta^{13}C$), and trophic level (TL) of anchovy and sardine of different age groups (adult, juvenile and late-larva) during summer, autumn and winter in the Gulf of Lions.**

| | n | SL(cm) | $\delta^{13}C$ (‰) | $\delta^{15}N$ (‰) | TL |
|---|---|---|---|---|---|
| **ANCHOVY** | | | | | |
| **Summer** | | | | | |
| Adult | 15 | 11.57 ± 0.44 | -19.56 ± 0.16 | 8.23 ± 0.34 | 2.42 ± 0.09 |
| Juvenile | 15 | 6.37 ± 0.85 | -18.26 ± 0.41 | 7.78 ± 0.41 | 2.27 ± 0.12 |
| Late-larva | 15 | 2.15 ± 0.16 | -18.38 ± 0.11 | 8.14 ± 0.32 | 2.39 ± 0.09 |
| **Autumn** | | | | | |
| Adult | 14 | 10.58 ± 0.18 | -17.83 ± 0.32 | 8.16 ± 0.48 | 2.90 ± 0.13 |
| Juvenile | 15 | 8.11 ± 0.49 | -17.67 ± 0.29 | 8.53 ± 0.45 | 3.05 ± 0.12 |
| **Winter** | | | | | |
| Adult | 7 | 8.80 ± 0.23 | -17.42 ± 0.22 | 8.07 ± 0 33 | 2.63 ± 0.09 |
| Juvenile | 15 | 7.57 ± 0.17 | -17.38 ± 0.24 | 7.54 ± 0.53 | 2.45 ± 0.15 |
| **SARDINE** | | | | | |
| **Summer** | | | | | |
| Adult | 17 | 13.62 ± 0.43 | -19.32 ± 1.16 | 8.52 ± 0.43 | 2.53 ± 0.12 |
| Juvenile | 15 | 7.02 ± 0.20 | -17.51 ± 0.21 | 7.97 ± 0.29 | 2.33 ± 0.08 |
| **Autumn** | | | | | |
| Adult | 15 | 12.91 ± 0.87 | -17.68 ± 0.71 | 8.91 ± 0.48 | 3.22 ± 0.13 |
| Juvenile | 15 | 9.11 ± 0.93 | -17.97 ± 0.52 | 8.95 ± 0.84 | 3.24 ± 0.23 |
| **Winter** | | | | | |
| Adult | 15 | 11.35 ± 0.91 | -17.64 ± 0.37 | 8.69 ± 0.41 | 2.87 ± 0.12 |
| Juvenile | 14 | 9.74 ± 0.52 | -17.77 ± 0.41 | 8.88 ± 0.53 | 2.95 ± 0.14 |
| Late-larva | 15 | 3.12 ± 0.11 | -18.10 ± 0.21 | 6.74 ± 0.42 | 2.19 ± 0.12 |

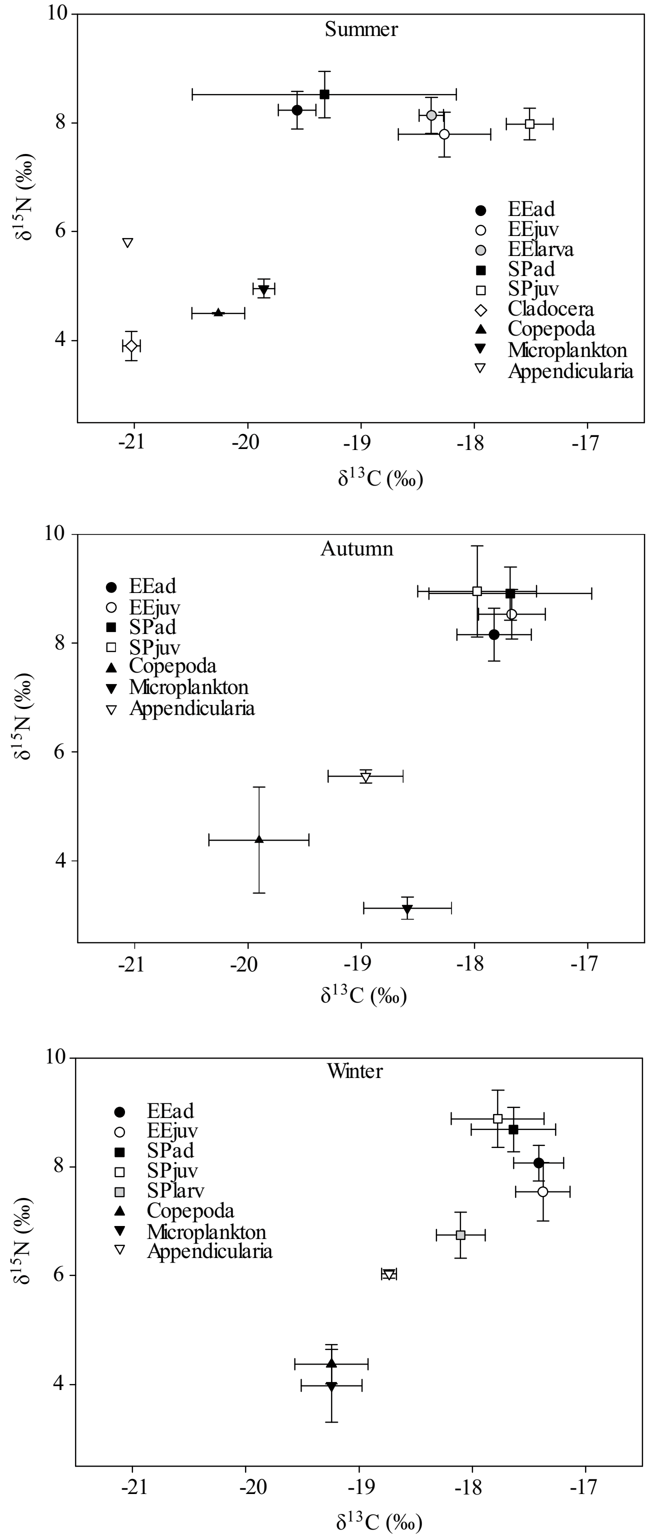

**Fig. 3. Engraulis encrasicolus, Sardina pilchardus. δ15N and δ13C values (mean ± SD) of *E. encrasicolus* late-larvae (EEl), juveniles (EEj) and adults (EEa), and *S. pilchardus* late-larvae (SPl), juveniles (SPj) and adults (SPa) during summer, autumn and winter. Reference values for the main prey groups (see Fig. 2) in each season are also given.**

The comparisons between the 2 species showed that, during the summer, the $\delta^{15}N$ values of *Sardina pilchardus* and *Engraulis encrasicolus* adults differed significantly. During the autumn, only differences between adults' $\delta^{15}N$ values were significant between species. During the winter, however, both the adults and juveniles exhibited significant species differences in $\delta^{15}N$ values. The $\delta^{13}C$ values of the juveniles also differed between the species in winter and summer (Tables 1 & 2, Fig. 3). A comparison of the late-larvae (*E. encrasicolus* in summer and *S. pilchardus* in winter) found statistically significant differences in the values of both $\delta^{13}C$ and $\delta^{15}N$ (Table 2).

Concerning types of prey, appendicularians showed higher $\delta^{15}N$ values than other types in all seasons (Table 3, Fig. 3). Excluding summer, microplankton presented the lowest $\delta^{15}N$ values. The $\delta^{13}C$ values are generally variable between seasons (Fig. 3).

**Table 2. Summary of the ANOVA results for inter-seasonal (summer, autumn and winter) variation in stable isotopes over species and age of anchovy and sardine in the Gulf of Lions. Abbreviations for age combinations in Tukey post hoc test summaries are: L = late-larvae; J = juvenile; A = adults; pairs of means differing significantly (p = 0.05) by Tukey test are linked with an 'x'. df = degrees of freedom.**

| | Effect | *F* | df | p | Post hoc |
|---|---|---|---|---|---|
| Summer | | | | | |
| $\delta^{15}N$ | Species | 6.55 | 1,75 | <0.001 | |
| | Age | 14.83 | 1,75 | <0.001 | A x L |
| | Species x Age | 0.25 | 2,75 | 0.62 | |
| $\delta^{13}C$ | Species | 10.87 | 1,75 | 0.002 | |
| | Age | 57.54 | 2,75 | <0.001 | A x L, J x L, A |
| | Species x Age | 2.89 | 1,75 | 0.11 | |
| Autumn | | | | | |
| $\delta^{15}N$ | Species | 14.71 | 1,58 | <0.001 | |
| | Age | 1.85 | 1,58 | 0.18 | |
| | Species x Age | 1.19 | 1,58 | 0.28 | |
| $\delta^{13}C$ | Species | 0.38 | 1,58 | 0.54 | |
| | Age | 0.27 | 1,58 | 0.61 | |
| | Species x Age | 2.98 | 1,58 | 0.89 | |
| Winter | | | | | |
| $\delta^{15}N$ | Species | 51.58 | 1,65 | <0.001 | |
| | Age | 96.81 | 2,65 | <0.001 | L x J, A |
| | Species x Age | 7.03 | 1,65 | 0.07 | |
| $\delta^{13}C$ | Species | 11.45 | 1,65 | <0.001 | |
| | Age | 8.29 | 2,65 | <0.001 | L x J, A |
| | Species x Age | 0.93 | 1,65 | 0.34 | |

**Table 3. Sample size (n), mean ± standard deviation of stable isotope values ($\delta^{15}N$ and $\delta^{13}C$) of prey groups during summer, autumn and winter in the Gulf of Lions.**

| | n | $\delta^{13}C$ (‰) | $\delta^{15}N$ (‰) |
|---|---|---|---|
| **Summer** | | | |
| Cladocera | 3 | -21.03 ± 0.08 | 3.90 ± 0.27 |
| Copepods | 3 | -20.26 ± 0.23 | 4.50 ± 0.01 |
| Microplankton | 3 | -19.85 ± 0.09 | 4.95 ± 0.17 |
| Appendicularia | 1 | -21.06 | 5.82 |
| **Autumn** | | | |
| Copepods | 3 | -19.90 ± 0.44 | 4.38 ± 0.97 |
| Microplankton | 4 | -18.59 ± 0.38 | 3.13 ± 0.20 |
| Appendicularia | 2 | -18.96 ± 0.33 | 5.55 ± 0.12 |
| **Winter** | | | |
| Copepods | 3 | -19.24 ± 0.32 | 4.37 ± 0.36 |
| Microplankton | 5 | -19.24 ± 0.27 | 3.98 ± 0.67 |
| Appendicularia | 3 | -18.74 ± 0.06 | 6.03 ± 0.08 |

### *3.3. Dietary differences*

The diets estimated with the SIAR model (isotopic values in Tables 1 & 3) indicated that during the summer, *Engraulis encrasicolus* adults fed on cladocerans (37.8 %) and appendicularians (24.7 %), whereas juveniles fed primarily on cladocerans (33.8 %) and copepods (35.5 %). The juveniles also fed on microplankton (27.1 %). The late-larvae fed primarily on microplankton (50.2 %) and also on copepods (35 %) (Fig. 4). During the summer, *Sardina pilchardus* adults had a heterogeneous diet, with appendicularians (29.2 %) as the main prey. The diet of *S. pilchardus* juveniles was based on cladocerans (36.8 %), copepods (33.5 %) and microplankton (25.1 %) (Fig. 4).

During the autumn, the diets of adults were similar to the diets of juveniles for both *Sardina pilchardus* and *Engraulis encrasicolus*. The main prey types for *S. pilchardus* were appendicularians (88 % in adults and 82.8 % in juveniles). Appendicularians were also the primary prey item for *E. encrasicolus* (59.4 % in adults and 75.4 % in juveniles) (Fig. 4).

*Sardina pilchardus* adults and juveniles in winter also showed a diet based on appendicularians (49 % in adults and 58.1 % in juveniles), but the primary prey item of late-larvae was microplankton (56.1 %). In the same season *Engraulis encrasicolus* juveniles fed primarily on microplankton (69 %), whereas adult *E. encrasicolus* fed primarily on copepods and appendicularians in similar amounts (40.7 and 40.8 %, respectively) (Fig. 4).

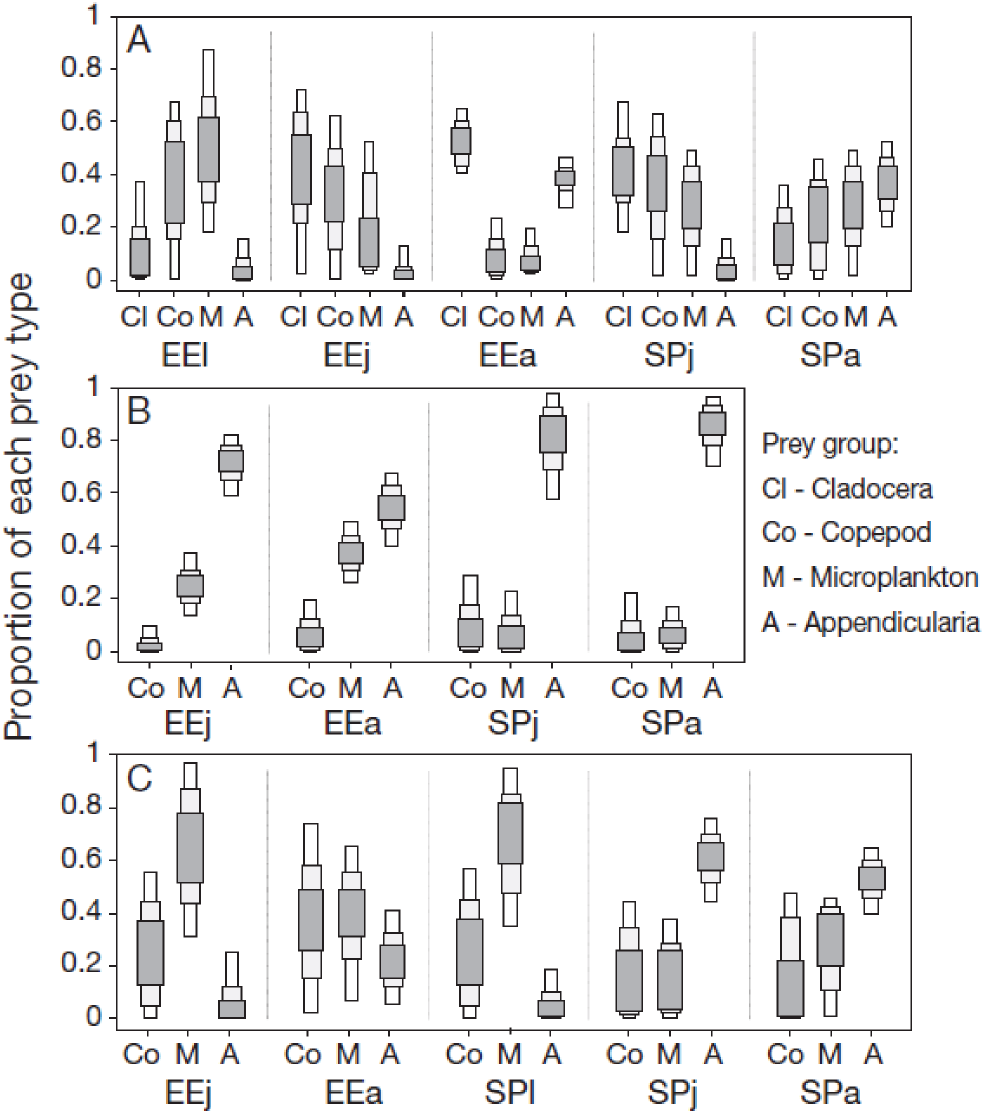

**Fig. 4. Engraulis encrasicolus, Sardina pilchardus. Results of stable isotope analysis in R (95, 75 and 50% credibility intervals) showing estimated prey contributions to the diet of anchovy late-larvae (EEl), juveniles (EEj) and adults (EEa), and sardine late-larvae (SPl), juveniles (SPj) and adults (SPa) from the Gulf of Lions (northwestern Mediterranean) during (A) summer, (B) autumn and (C) winter.**

### 3.4. Prey selectivity

Ivlev's selectivity index showed that during the summer, both cladocerans and appendicularians were the most heavily selected prey (Fig. 5). Positive selection was also exhibited for copepods, while microplankton was negatively selected in all cases (Fig. 5). During autumn, appendicularians were generally preferred. An apparent neutral selection was shown for copepods, and negative selection was shown for microplankton (Fig. 5). During the winter, copepods, followed by appendicularians, were highly selected in nearly all cases. The only exception was *Sardina pilchardus* late-larvae, which did not select appendicularians. Microplankton was not positively selected in any case (Fig. 5).

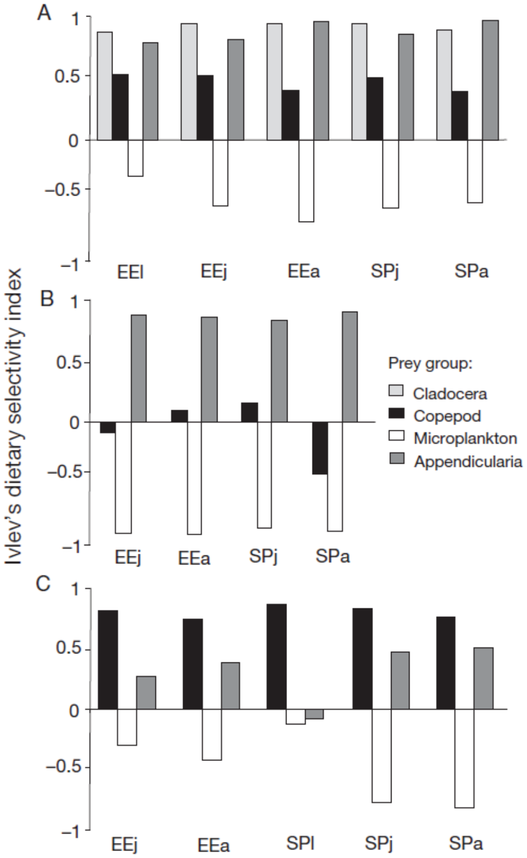

**Fig. 5. Ivlev's dietary selectivity index for E. encrasicolus late-larvae (EEl), juveniles (EEj) and adults (EEa), and S. pilchardus latelarvae (SPl), juveniles (SPj) and adults (SPa) during (A) summer, (B) autumn and (C) winter.**

## 4. Discussion

Our study determined the trophic dynamics of anchovy *Engraulis encrasicolus* and sardine *Sardina pilchardus* during different seasons and at different life stages. The results of the study emphasised the feeding plasticity of these species in the Gulf of Lions, as already observed in the Adriatic Sea (Borme *et al*., 2009). The results showed that the late-larvae of both species feed more abundantly on microplankton than on any other prey and that a remarkable difference in the diet occurred after metamorphosis. If cladocerans were available (i.e. during the summer), they were usually the preferred prey. The high selectivity for appendicularians in autumn seemed to explain the high trophic levels found for this season because appendicularians are the prey group with the highest $\delta^{15}N$ values. These high $\delta^{15}N$ values of appendicularians could be due to the retention of zooplankton organisms of high $\delta^{15}N$ values, such as carnivorous copepods or even small larvae, on their houses (Deibel & Lee, 1992); this is in accordance with the data of Hobson *et al*. (2002), who obtained a higher value of stable-nitrogen isotope and a higher trophic level in appendicularians than in mixed zooplankton samples.

The difference in trophic levels between *Engraulis encrasicolus* and *Sardina pilchardus* late-larvae resulted from their distinct feeding behaviour. E. encrasicolus late-larvae, which showed values of $\delta^{15}N$ similar to those in the other stages, generally fed on copepods and microplankton, a diet similar to that of *S. pilchardus* late-larvae. However, despite the high proportion of these types of prey in the sea during summer, *E. encrasicolus* late-larvae have been found to feed preferentially on appendicularians and cladocerans if these food items are available. Morote *et al*. (2010) found that *E. encrasicolus* larvae <15 mm standard length (SL) fed primarily on copepods and cladocerans (see also Tudela *et* *al*., 2002), whereas *S. pilchardus* ate a relatively high number of protists along with copepod nauplii. Other studies of *S. pilchardus larvae* >13 mm SL reported a diet based on phytoplankton (Rasoanarivo *et al*., 1991). Given the mean SL of the larvae examined in the current study, it is probable that they did not retain the isotopic signal from parental feeding activity (Pepin & Dower, 2007). In view of these considerations and the results cited, it seems reasonable to suppose that *S. pilchardus* late-larvae, whose diet is more herbivorous, would exhibit a lower trophic level than that of *E. encrasicolus* late-larvae. This difference is even clearer from the selectivity indices, which indicate that E. encrasicolus late-larvae tend to feed on prey with higher $\delta^{15}$N values rather than on the microplankton that represent a primary constituent of the diet of *S. pilchardus* late-larvae.

The observed differences between ages during the winter and summer for *Engraulis encrasicolus* and *Sardina pilchardus* reflected a change in the diet across the ontogenetic development of the fishes. We did not find differences during autumn, because no late-larvae of any species were collected during that period. These findings suggested the hypothesis that the diet shift occurred primarily at the time of metamorphosis (Lindsay *et al*., 1998), whereas juveniles and adults maintained similar diets. However, *E. encrasicolus* during summer exhibited clear differences between juveniles and the other 2 stages. Lindsay *et al*. (1998) also found a drastic change in $\delta^{15}$N in Japanese anchovy *E. japonicus* as individuals grew from 15 to 30 mm SL and another change between 30 and 70 mm SL. These size ranges are almost coincident with the sizes of the juveniles in our study (6.37 ± 0.85 cm of SL). Therefore, dietary changes may also occur after metamorphosis.

The most frequently cited explanation for the ontogenetic dietary shift refers to the development of the feeding apparatus of the fishes (June & Carlson, 1971, King & Macleod, 1976, MacNeill & Brandt, 1990, Gerking, 1994). According to these authors, larvae become able to filter-feed when the development of their gill rakers is complete. Thus, differences in the diet are related to the minimum prey sizes that are efficiently retained by the feeding apparatus.

In contrast, Tanaka *et al*. (2006) analysed the stomach contents and gill-raker morphology of 3 species of planktivorous pelagic fishes. These authors found that the differences in the diets of these species were explained by differences in feeding behaviour (filter-feeding vs. particulate feeding) rather than by differences in morphology. This conclusion supports our results for juveniles and adults of *E. encrasicolus* during summer. These fish, caught at different locations and different times, are considered to have a fully developed filtering apparatus. Consequently, the differences in the diet indicated here by stable isotopes could depend on shifts in feeding habits mediated by food density. For example, the fish could shift between filter-feeding and particulate feeding, depending on the concentrations of different prey items (Bulgakova, 1996).

Previous studies based on stomach contents analysis argued that *Engraulis encrasicolus* in the Gulf of Lion is mainly zooplanktivorous (Tudela & Palomera, 1997, Plounevez & Champalbert, 2000, Morote *et al*., 2010, Costalago *et al*., 2011), whereas *Sardina pilchardus* also feeds significantly on microheterotrophs (Rasoanarivo *et al*., 1991, Morote *et al*., 2010). These observations, together with our results, led us to hypothesise that both *E. encrasicolus* and *S. pilchardus* are omnivorous all through their life cycles. However, our results demonstrated a slightly higher trophic level for *S. pilchardus* in all seasons and stages, except late-larvae, a similar pattern to that described for the Atlantic coast of the Iberian Peninsula (Bode *et al*., 2007). This discrepancy was explained by van der Lingen (1998) and Bode *et al.* (2006), who demonstrated that sardines primarily obtain protein nitrogen from zooplankton rather than from phytoplankton. This argument is also supported by the observation that herbivores generally have higher $\delta^{15}$N variability (Mill *et al*., 2007). Moreover, the differences between the present study and those by Coll *et al*. (2006) and Navarro *et al*. (2011), both focused on the Catalan Sea, could be considered a consequence of the diet data used by those authors. In particular, they based their results on anchovy diet data reported by Tudela & Palomera (1997) and on sardine diet data from the eastern Mediterranean (Demirhindi, 1961), resulting in a higher trophic level in *E. encrasicolus* than in sardine (3.05 and 2.97, respectively). Alternatively, some differences in the diets of *E. encrasicolus* and *S. pilchardus* between areas may also have influenced these results.

A comparison of the trophic levels found in our study with values from upwelling areas shows that adults of both anchovy and sardine in the Gulf of Lions normally exhibited trophic levels similar to the trophic levels found for homologous species in regions with upwelling. Bode *et al*. (2007) found trophic levels of 3.5 for *Sardina pilchardus* and of 3.4 for *Engraulis encrasicolus* for the northern Iberian Atlantic coast. Miller *et al*. (2010) found trophic levels of 2.9 for *S. sagax* and of 3.1 for *E. mordax* for the California Current. Moreover, the trophic level of 2.9 derived for *S. sagax* in the southern Benguela Current is lower than the value for *S. pilchardus* in the Gulf of Lions (van der Lingen & Miller, 2011). In this context, Miller *et al*. (2011) showed that fish from less productive areas exhibited relatively higher trophic levels than those from more productive areas. Therefore, the food web of areas with relatively low average primary production, like the Gulf of Lions, is more linear than the food web in zones with upwelling (e.g. the Galician coast, where the trophic structure is more intricate and ramified) (Agostini & Bakun, 2002). A more linear food web implies that individuals, in this case small pelagic fish in the Mediterranean sea, are more dependent on the adjacent lower trophic level than those small pelagic fish from more highly productive areas (Miller *et al*., 2011), a pattern that could be interpreted as a bottom-up ecosystem structure in the Mediterranean. However, in view of the high biomass of the mid-trophic level small pelagic fish in the northwestern Mediterranean, this structure is actually closer to a ‘wasp-waist’ system, as found in the Adriatic Sea (Coll *et al*., 2007).

The trophic dynamics of zooplankton and small pelagic fish occupy the most significant position within marine pelagic food webs (Shannon *et al*., 2009). In the Gulf of Lions, a substantial amount of seasonal variability affecting the lower trophic levels has been widely reported (Molinero *et al*., 2005). These temporal fluctuations reflect an important feature of the area: the effect of the Rhone River. The Rhone is the primary source of the runoff entering the Mediterranean. The mean annual flow of the river is 1700 $m^3\ s^{-1}$, and its catchment area is 98000 $km^2$ (Darnaude *et al*., 2004). In fact, correlations between river discharge and marine pelagic fish abundance in the northwestern Mediterranean have been extensively studied (García & Palomera, 1996, Lloret *et al*., 2001, 2004) and are generally explained by the enhancement of planktonic production produced by the input of nutrients from rivers. We found significant differences in the $\delta^{13}C$ signatures of fishes among seasons in all cases. These results demonstrate the importance of seasonal variability for the structure of the food web.

Lindsay *et al*. (1998) also found that, in central Japan, rivers may supply the coastal trophic web with different $\delta^{15}N$ signatures depending on the season. In agreement with this result, our study showed that this heavy nitrogen isotope was generally more abundant in both food sources and fish samples during autumn. The difference may reflect the higher amount of rainfall during autumn in this region. Moreover, Odum (1985) suggested that the augmented contributions of flows to detritus could serve as a marker of disruption in energy transport from lower to higher trophic levels, conferring higher signature to $\delta^{15}$N than expected with a less direct transmission to predators. With this in mind, it can be argued that nutrients derived from the Rhone River in autumn tend to have heavier N. Correspondingly, we showed that $\delta^{15}$N values in summer were generally lower for the 2 fish species than in the other 2 studied seasons, probably because summer is the driest season and the river discharges are lower.

In conclusion, the present study shows that adults of *Engraulis encrasicolus* and *Sardina pilchardus* generally prey over larger plankton than juveniles and late-larvae. We also illustrate the importance of appendicularians in the diet of both species, especially when cladocerans are not available, and prove that stable isotope analysis is an essential tool for complement dietary studies based on direct observations of stomach contents, because appendicularians are likely easily digested (Capitanio *et al*., 2005) and could have been underestimated in some cases (Capitanio *et al*., 1997, Costalago, unpublished data). Moreover, based on our output data from the SIAR model and the data we gathered from the plankton samples, we also showed an innovative manner of calculating dietary selectivity through Ivlev´s selectivity index. To our knowledge, this approach has never previously been attempted, and we believe that, with limitations and future improvements, it can be useful for drawing a more comprehensive and accurate picture of trophic dynamics.

Assessing the isotopic values of different types of prey in different seasons is therefore essential in understanding whether isotopic seasonal variations of pelagic fish are caused by changes in diet or by variations of basal isotopic levels due to environmental oscillations. Anthropogenic alterations in the ecosystem, such as overfishing, eutrophication, or climate change often entail alterations in the trophic structure of the communities; therefore, the above-discussed findings may be useful in future management scenarios that would take into account an ecosystem approach to fisheries in the northwestern Mediterranean.

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# Capítulo 4. Conocimiento ecológico para la gestión pesquera: Condición y crecimiento de larvas grandes de anchoa durante diferentes estaciones del año en el Mediterráneo noroccidental.

**Resumen.** Para una gestión adecuada de la pesquería de anchoa Engraulis encrasicolus en el Mediterráneo se requieren varias aproximaciones ecológicas. Con esta idea, se realizaron varias campañas oceanográficas para estudiar la ecología de larvas y juveniles de esta especie, la cual se sabe que, en el Golfo de León, se reproduce durante el periodo más caluroso del año (de mayo a septiembre). Se hizo un especial esfuerzo en el estudio de las larvas más grandes (de 15 mm de longitud total hasta la metamorfosis), ya que otros autores se habían centrado en el estudio de larvas por debajo de esta talla. Inesperadamente, aparecieron larvas durante el mes de diciembre de 2007, cuyo rango de tallas debía corresponder a una puesta posterior al periodo de puesta indicado anteriormente. Con el fin de obtener información sobre la probabilidad de supervivencia de los dos grupos de larvas (capturadas en verano y a final del otoño) se estudiaron posibles diferencias en la condición nutricional de estas larvas comparando índices de composición lipídica y estimando tasas de crecimiento a partir de medidas de los otolitos. El análisis de los ácidos grasos, utilizados como trazadores de relaciones tróficas, indicó que estas larvas comen principalmente zooplancton. La condición nutricional de las larvas de ambos periodos era muy similar. En cambio, las tasas de crecimiento eran mayores para las larvas de verano, probablemente debido a la mayor temperatura media de la estacióm estival. Nuestros resultados son especialmente relevantes en un contexto ecológico en el que el aumento de la temperatura media del agua superficial en el Mediterráneo occidental podría favorecer la extensión del periodo de puesta de la anchoa hasta finales de otoño.

# Ecological understanding for fishery management: condition and growth of anchovy late larvae during different seasons in the northwestern Mediterranean.

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**ABSTRACT.** The fishery of the European anchovy *Engraulis encrasicolus* in the Mediterranean needs several ecological approaches to be properlymanaged. As such, several surveys were carried out to study the ecology of larvae and juveniles of this species, which reproduces during the warmest period of the year (May through September) in the Gulf of Lions. In particular, we studied the late larvae (15 mm total length until metamorphosis), especially as other authors have focused on larvae below that size. Unexpectedly, we also collected anchovy late larvae during the December 2007 survey, whose range in size corresponded to a later spawning period than previously reported. Differences in the nutritional condition of these larvae were assessed by comparing indices of lipid composition and estimating growth rates from otolith measurements to provide information on the probability of survival between the two groups. The analysis of fatty acids, used as tracers of trophic relationships, indicates that these larvae fedmainly on zooplankton. Nutritional conditions of summer and late autumn larvae were very similar. In contrast, growth rates were higher for August larvae, probably due to the different temperatures in the two seasons. Our results are especially relevant in an ecological context where the increasing meanwater temperatures in the western Mediterranean could favor the extension of the anchovy spawning period up to late-Autumn months.

KEY WORDS: *Engraulis encrasicolus* · late-larvae · northwestern Mediterranean · condition · growth

## 1. Introduction

The European anchovy *Engraulis encrasicolus* is a highly valued fishery resource in the Western Mediterranean Sea (García *et al*., 1996, Pertierra *et al*., 1996, Barange *et al*., 2009). Several studies have been carried out regarding diverse aspects of the biology and the ecology of the species in this area to improve the available tools for its fishery management (Palomera *et al*., 2007).

The Northwestern Mediterranean is one of the most productive areas in this sea due to the cyclonic current that flows southwards over the slope of the Gulf of Lions, carrying a significant nutrient load from the Rhône River (Salat, 1996). The Gulf of Lions also displays remarkable environmental differences between seasons, which directly influence low trophic level species (Calbet *et al*., 2001). The anchovy population could then be easily compromised by any sort of alteration that additionally impinges on these organisms, especially during the early development stages (i.e. eggs, larvae and juveniles) when they are particularly sensitive to any change (Palomera *et al*., 2007).

The anchovy spawning period in the Gulf of Lions extends from April to late September, with a peak in late June (Palomera, 1992). Therefore late larvae are expected to be found mainly during the summer, while juveniles should emerge in the autumn and winter.

During the SARDONE project, devoted to improve the management strategies of the European anchovy, two cruises were undertaken in August and December of 2007 in the Gulf of Lions in order to collect anchovy late larvae and juveniles, respectively. Unexpectedly, and in spite of the allegedly unfavorable conditions, an important number of anchovy late larvae were caught in December (a mean of 26.75 larvae tow$^{-1}$, against 34.20 larvae tow$^{-1}$ in summer), well after the reported end of the spawning period.

An understanding of the growth and feeding ecology of the species is important given that this knowledge is essential to understand how the population temporally and spatially develops in the environment. The relationship between this population and the plankton community is not a straightforward subject but some approaches have been attempted (Isari *et al*., 2008, Morais *et al*., 2010), especially those concerning how the zooplankton affects the population strength. Indeed, the growth rates and the nutritional condition of a population of several species, including European anchovy, have been key subjects for the study of the recruitment strength in many works (Butler, 1991, Ward *et al*., 2006, Palomera *et al*., 2007, Hidalgo *et al*., 2008, Islam & Tanaka, 2009, La Mesa *et al*., 2009).

In the present case we analyze for the first time in the Mediterranean the nutritional condition of anchovy late larvae (19-35 mm) in the field during both summer and late-Autumn seasons via lipid composition studies, and we also study their diet through the fatty acids found both in larvae and in zooplankton, which is the basic prey of anchovy at all the development stages (Plounevez &

Champalbert, 1999, Pasquaud *et al*., 2008, Bacha & Amara, 2009, Borme *et al*., 2009, Catalán *et al*., 2010, Morote *et al*., 2010). Growth rates were also studied for both larvae populations to determine whether the different environmental conditions affect the early stage development of the anchovy. This work will thus help to explain to what extent the general conditions of an unexpected December late larvae population differs from the August late larvae and whether these two populations have similar viability.

## 2. Materials and methods

### *2.1. Sample collection*

Samples were collected during two cruises carried out in the Gulf of Lions (Northwestern Mediterranean sea; figure 1) in 2007 on board the *N/O L'Europe* (IFREMER, France). The first cruise (PELMED07) was conducted in the summer from the 28th of July to the 9th of August 2007 and the second cruise (JUVALION07) was carried out in late autumn, from the 8th to the 21st of December 2007.

Temperature and salinity of the water column from sea surface to 50 m depth were measured via a Seabird 19 CTD at each station. Also data of sea surface temperature in September to December since 1982 to early 2011 of Gulf of Lions area from NOAA were acquired to study possible trends.

Zooplankton samples for an analysis of biomass and plankton composition were collected using a standard WP2 net with a mesh size of 200 µm and sieved through a 3000 µm plankton mesh to obtain the 200-3000 µm mesozooplankton fraction, and by means of a scaled-down version WP2 net, with a mesh size of

53 µm and a mouth diameter of 25 cm, and sieved through a 200 µm plankton mesh to obtain the 53-200 µm fraction of microplankton. All zooplankton samples were split up with a Motoda plankton splitter (Motoda, 1959) and one-half was preserved in formalin to carry out subsequent qualitative analysis, while the other half was frozen on board for biomass measurements and lipids analysis. As the purpose of comparatively assess the nutritional condition of the two different temporal larvae populations came up during the December cruise, plankton samples for lipids analysis were not collected during the first cruise.

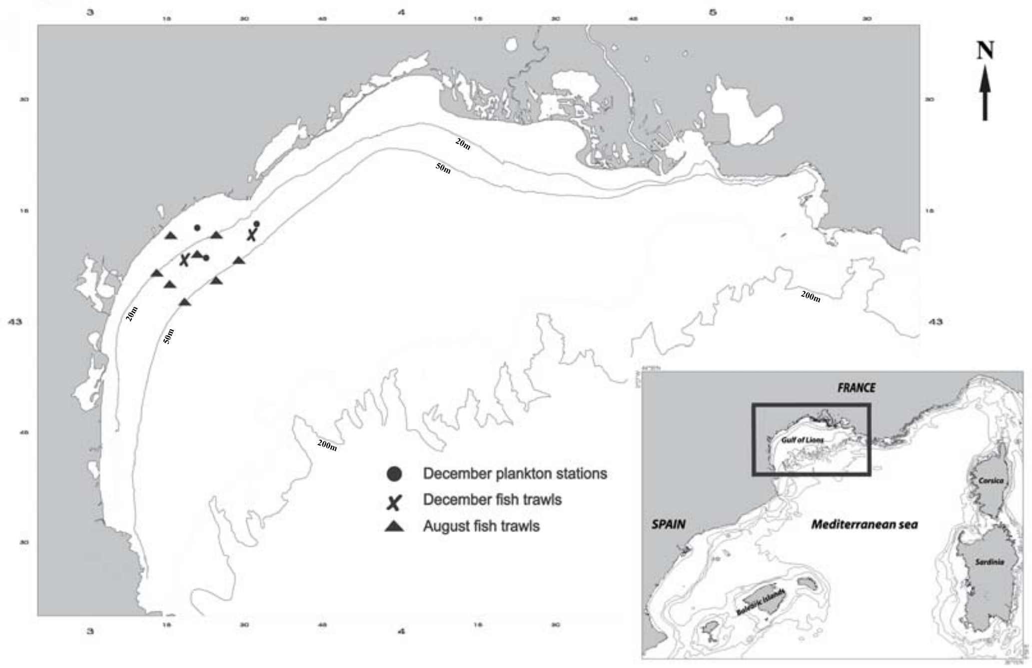

**Fig. 1. Map of the study area, with the positions of plankton stations and trawls in August and December 2007.**

Late larvae of anchovy were caught with a pelagic trawling net, towed at an average speed of 3.6 knots over a 30-40 min time span. This trawling time might seem too long to obtain larvae in proper conditions for biochemical analysis; however, the alteration of lipids composition in muscular tissue of fish larvae is small within a time up to 3 h after death (Lochmann *et al*., 1996).

Samples were immediately frozen in liquid nitrogen after sorting on board and transferred into a -80° C freezer just after the arrival in the laboratory..

### *2.2. Lipid and fatty acid analysis*

Wet weight and standard length of each larva were measured in the laboratory to the nearest 10 µg and 0.1 mm, respectively, before removing the head for otoliths analysis and the gut, as recommended by Lochmann *et al*. (1996). The empiric relationship between larval wet and dry weights was calculated via linear regression from other larvae of the same cruises and size ranges.

Microplankton and mesozooplankton samples from each station were pooled together before proceeding with the fatty acids extraction.

Lipid extraction was performed according to the method of Folch *et al*. (1957). Lipid content was measured following the protocol of Olsen & Henderson (1989) via high-performance thin-layer chromatography (HPTLC), which was followed by quantitative densitometry in visible light with a Bio-Rad Gel Doc XR densitometer, using Quantity One 4.6.2 software.

The nutritional condition of the *E. encrasicolus* late larvae was evaluated by comparing the triacylglycerol/cholesterol (TAG / CHOL) index (Håkanson, 1993) and the ratio between the percentage of total lipids and the dry mass (Norton *et al*., 2001). Fulton's condition index (FCI) was calculated with wet weight (W, in g) and standard length (SL, in mm) data, following the equation:

$$FCI = W \times 100 / SL^3$$

Fatty acids extraction and trans-methylation was accomplished following the protocol of Christie (1989) as modified by Li & Watkins (2001).

Four out of seven samples of larvae from August 2007, together with five larvae samples and three zooplankton samples from December 2007, were feasible to read by the gas-chromatographer.

Gas chromatographic (GC) analysis of fatty acid methyl esters (FAMEs) were then performed using a Thermofisher Scientific GC8060 gas-chromatograph coupled with a MD800 mass-spectrometer. The apparatus was fitted with a BPX-70 capillary column (30 m x 0.25 mm i.d. x 0.25 µm). Helium was used as carrier gas, with a speed of 1 ml min$^{-1}$. The programmed oven temperature was 60° C (1 min) to 260° C (10 min) with an increment of 8° C min$^{-1}$. The injector temperature was set at 270° C and the injector split was set at 35 ml min$^{-1}$. Mass-spectrometry was conducted with an ion source temperature of 200° C and an interphase temperature at 260° C. Ionization was performed by electron impact at 70 eV, and the weight range analyzed was 50-550 Da.

FAMEs were identified by comparing their retention times with those of the standard mixture, Supelco 37 Component FAME mix. The quantification of the identified FAMEs was calculated through GC peak areas integration.

The diet of late larvae was evaluated according to the indices based on fatty acids relations 16:1(ω-7) / 16:0, 18:1(ω-9) / 18:1(ω-7) and EPA / DHA, and on PUFA / SFA relation (St. John & Lund., 1996; Auel *et al*., 2002; Rossi *et al*., 2006).

### *2.3. Growth analysis*

A total of 61 larvae from August and 44 larvae from December, ranging in size from 19-31 mm (SL) and from 20-27 mm (SL), respectively, were used for a growth analysis. Both sagitta otoliths were extracted from the head of the anchovy larvae under a Leica dissection microscope (Wild M12) equipped with polarizing filters and mounted in Crystalbond 509 Amber on labeled glass slides. The otolith growth analysis was undertaken at 100× magnification under transmitted light with a microscope (Zeiss Axiospoth) coupled to a digital video recorder, while otolith nucleus was analyzed at 1000× magnification. Otolith radius (OR) and increment width (IW) (µm) were measured to the nearest 0.1 µm using Image-Pro Plus 5.0. The increments were measured along the longest radius, from the middle of one D-zone to the middle of the next D-zone. Following the results of Aldanondo *et al*. (2008), for the same species in the Bay of Biscay and under experimental conditions, increments were assumed to be daily (DI) being the first increment laid down at hatching. Until the paper of Aldanondo *et al*. (2008), previous works on European anchovy growth had assumed that the first increment deposition took place at the beginning of exogenous feeding, i.e. two days after hatching, as proposed by Palomera *et al*. (1988) in the first paper on anchovy larval otoliths. All otoliths were read twice by two different persons, and only if the DI differed by 1 daily increment were they accepted.

Taking into account the narrow range of lengths that we are analyzing, we have assumed linear growth in agreement with the results of La Mesa *et al*. (2009), that is the first paper on anchovy that analyzes the growth at the metamorphic period, as is the case of our samples. Accordingly, the individual growth rate (IGR, mm $d^{-1}$) from the time of hatching until the time of capture was then calculated by using the equation proposed by Takahashi & Watanabe (2005).

$$IGR = (SL - SL_0) / Age$$

where $SL$ is the measured larvae standard length corrected by using Theilacker's method (Theilacker, 1980), $SL_0$ is the larvae standard length at hatching, estimated to be 2.5 mm according to laboratory studies on the studied species

(Regner, 1985), and *Age* = DI.

### *2.4. Data analysis*

Seasonal differences between water oceanographic parameters as well as Fulton’s condition index, lipids total content, lipid classes and proportion of fatty acids in larvae and plankton were assessed by means of Mann-Whitney non-parametric tests for independent samples (Dytham, 2003), and for oceanographic data ANOVA tests were performed.

Fatty acids percentage compositions were pairwise compared between larvae of both cruises, and between larvae and zooplankton collected during the December 2007 cruise by using the former test. Similarities in the fatty acid composition between samples were measured by Euclidean distances (Legendre and Legendre, 1998). A non-metric multidimensional scaling (nMDS) was carried out on the samples similarity matrix to visually describe overall patterns.

Statistical analyses were carried over with software STATISTICA 6.0 by Statsoft, Inc., and PRIMER-E 6 software. Significance level for all tests was adopted at $p < 0.05$.

## **3. Results**

### *3.1. Oceanographic data and zooplankton composition*

The mean temperature within the water column (0-50 m) and the mean surface temperature (0-5 m) were significantly higher ($p < 0.0001$) in August (mean ± standard deviation values of 19.14º C ± 1.32 for surface temperature and 16.75º

C ± 1.02 for average temperature) compared to December (mean ± standard deviation values of 12.64º C ± 0.91 for surface temperature and 13.04º C ± 0.66 for average temperature), while no significant differences were observed for salinity between the two cruises (mean ± standard deviation values of 37.74 ± 0.20 and 37.35 ± 1.48 for surface salinity and 37.94 ± 0.06 and 37.93 ± 0.40 for average salinity in August and December, respectively).

Data of temperature acquired from NOAA (Reynolds *et al.*, 2002) were monthly averaged in the Gulf of Lions (area comprised between 2.5º W to 6.5º W and 41.3º N to 45.5º N), from September 1981 to December 2010, showing a positive trend in the sea surface temperature during the last 4 months of every year (16.5º in September 1981 to 17.2º C in December 2010; $SST = 14.97 + 0.54 * 10^{-4} * Serial\_date$; where Serial_date is the number of days since the January 1st of 1900).

The non-parametric Mann-Whitney *U* test revealed that there were significant differences for microplankton biomass between August and December cruises ($p < 0.05$), with higher values recorded during the summer (mean ± standard deviation values of 201.59 ± 283.25 mg $m^{-3}$ and 22.76 ± 24.18 mg $m^{-3}$, respectively).

The same pattern was observed for the mesozooplankton, where pairwise multiple comparisons (*t*-test) revealed that August 2007 samples had significantly higher biomass ($p < 0.05$) compared to those collected in December 2007 (mean ± standard deviation values of 33.65 ± 10.34 mg $m^{-3}$ and 14.07 ± 10.65 mg $m^{-3}$, respectively).

The analysis of both microplankton and mesozooplankton composition revealed a dominance of copepods, especially calanoids and cyclopoids, within the community during the two seasons (Fig. 2). Nevertheless, in August cladocerans were also important, while they were not recorded in December cruise (Fig. 3).

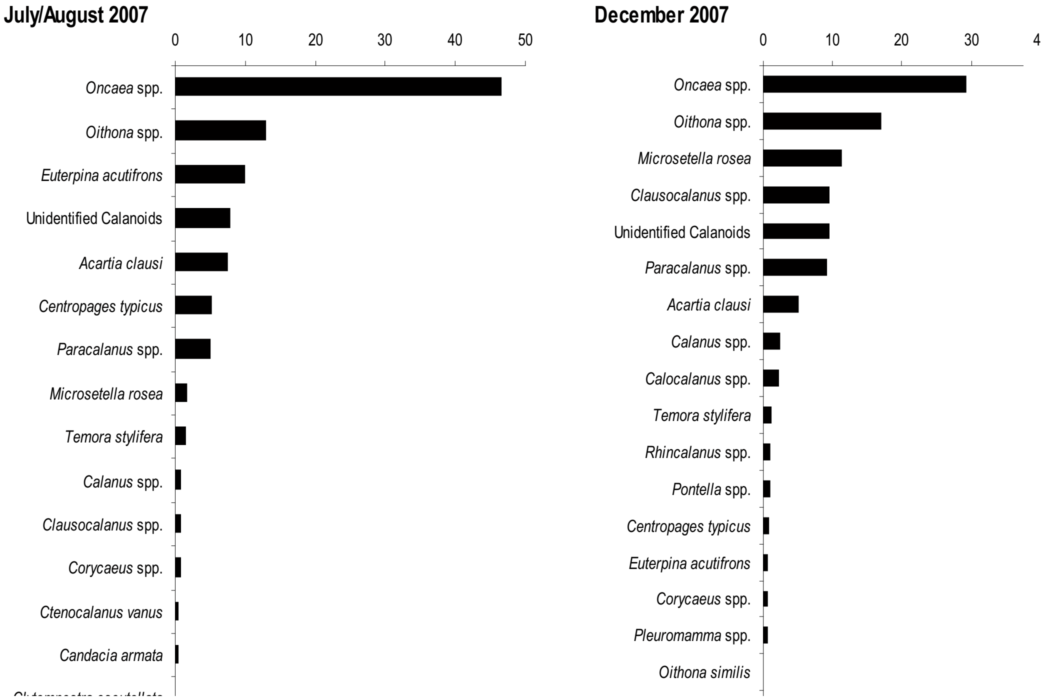

**Fig. 2. Relative abundances of the main copepods species within micro and mesozooplankton samples of both August and December 2007 campaigns.**

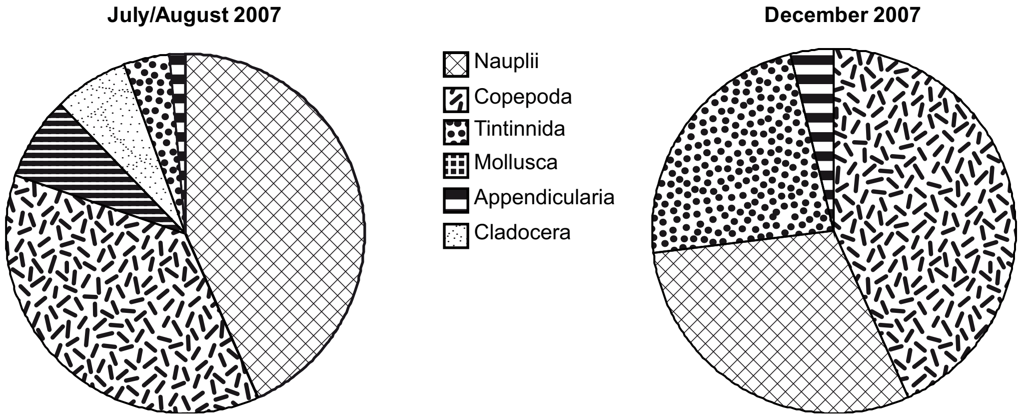

**Fig. 3. Pie charts illustrating the mean percentage composition of the main plankton groups in August and December 2007 cruises.**

### *3.2. Growth rate*

Otolith growth was significantly different between the two periods with the otoliths of the December larvae being smaller compared to those of the August larvae of the same age (t-test, $p < 0.001$) (Fig. 4). For both groups, increment width increased continuously, although for larvae caught in December, that ranged on age from 33 to 54 days, the largest increment width reached was half that of the maximum increment width in the August samples, that ranged from 22 to 44 days (4 vs. 8 µm $day^{-1}$, respectively).

This pattern was matched by mean individual growth rates (IGR) from hatching until the time of capture, that ranged between 0.50-0.93 mm $d^{-1}$ (mean ± standard deviation values of 0.74 ± 0.09 mm $d^{-1}$) for August larvae and 0.43-0.74 mm $d^{-1}$ (mean ± standard deviation values of 0.59 ± 0.07 mm $d^{-1}$) for December larvae, indicating that IGR was significantly higher in the warmer period (t-test, $p < 0.001$).

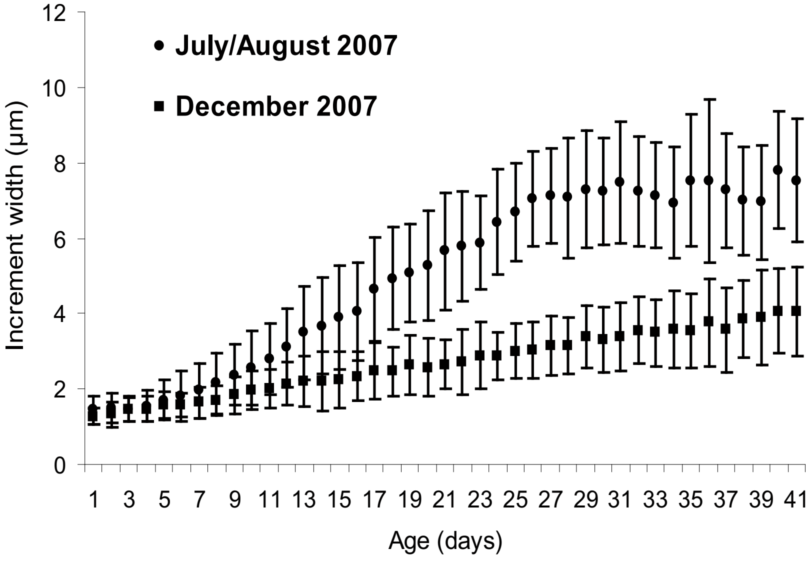

**Fig. 4. Anchovy larvae mean increment width by estimated age (days) for larvae caught in August and December 2007. (Error bars: standard deviation).**

### 3.3. Nutritional condition

A single linear regression between larvae dry mass and wet weight was estimated for both cruises (Fig. 5) because the one-way ANCOVA did not show significant differences between August and December ($p > 0.05$), considering wet weight as covariant.

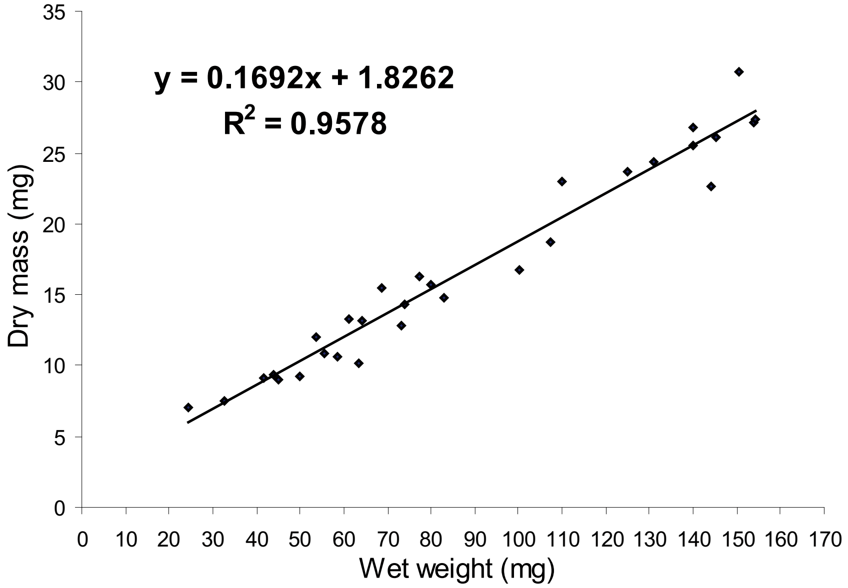

**Fig. 5. Relationship between dry mass (DM) and wet weight (WW) of anchovy late larvae. Linear regression fitted by DM = 0.1692*WW + 1.8262 ($r^2$ = 0.9578).**

Subsequently, the empirical equation obtained from that relationship was applied to estimate the larvae dry mass (mean ± standard deviation values of 23.0 ± 7.0 and 18.0 ± 7.0 mg / larva for August and December late larvae, respectively) as well as in further analysis regarding the dry mass of lipid contents in the samples.

Mean standard lengths did not significantly differ between the two cruises (mean ± standard deviation values of 27.3 ± 3.0 mm and 27.0 ± 3.4 mm for August 2007 and December 2007, respectively).

On the contrary, Fulton's condition index of larvae was significantly higher ($p < 0.05$) in the August individuals (mean ± standard deviation values of 0.598 ± 0.131 and 0.489 ± 0.153 in August and December, respectively).

Pools of 4-6 larvae each from August and December cruises were processed for lipid extraction.

Lipids content in larvae did not show any statistical differences between the two seasons ($p = 0.123$), and no significance was found regarding triacylglycerol ($p = 1.000$), cholesterol ($p = 0.705$), free fatty acid ($p = 0.570$) or polar lipid content ($p = 1.000$) within the anchovy samples (Table 1).

**Table 1. Total lipid content, lipid class values and TAG/CHOL index (µg*larva$^{-1}$), presented as Mean ± SD.**

| | August 2007 (N = 7 pools of 4-6 larvae each) | December 2007 (N = 5 pools of 4-5 larvae each) |
|---|---|---|
| Total lipid content | 770.4 ± 275.1 | 664.5 ± 92.7 |
| % lipid / dry weight | 4.2 ± 0.8 | 4.4 ± 0.2 |
| Neutral lipids: | | |
| Triacylglycerol (TAG) | 922.2 ± 379.4 | 873.8 ± 242.2 |
| Cholesterol | 1414.4 ± 476.6 | 1227.8 ± 249.9 |
| Free fatty acid | 1406.0 ± 425.6 | 1228.2 ± 221.7 |
| Steryl esther | – | – |
| Polar lipids | 230.6 ± 65.9 | 223.1 ± 37.8 |
| TAG / CHOL index | 0.64 ± 0.06 | 0.70 ± 0.09 |

The TAG / CHOL ratio was determined to range between 0.53-0.72 for August larvae and 0.60 – 0.82 for December larvae, and it did not exhibit significant differences between cruises as well ($p = 0.186$).

### *3.4. Fatty acids analysis*

Of the 23 fatty acids identified, 16:0, eicosapentaenoic acid or EPA (20:5($\omega$-3)) and docosahexanoic acid or DHA (22:6($\omega$-3)) made up 58-75 % of total fatty acids in the zooplankton and larvae samples (Table 2), with DHA and 16:0 being the most common fatty acids found in both August and December larvae and within the zooplankton. The other abundant fatty acids found were 14:0, 18:0, 16:1(ω-7), 18:1(ω-9) and 18:1(ω-7).

The proportion of PUFA was higher than any other type of FFAA among larvae of both cruises, while SFA were the most abundant in the zooplankton.

A multi-dimensional scaling (MDS), with a stress coefficient <0.01, shows the similarity in fatty acid composition among the larvae of August and December and the zooplankton of December. Three groups can be differentiated on the plot, specifically a group comprised of 4 out of 5 samples of the December anchovy larvae, the 4 samples of August larvae, and the 3 samples of zooplankton (Fig. 6). ANOSIM test confirmed the presence of significant differences in the multivariate fatty acid composition between all three groups ($R^2 = 0.83$ at $p < 0.0001$).

Table 3 shows the mean values of the fatty acid indices estimated from the data obtained in this work, apart from those related to the summer zooplankton, which were acquired from (Rossi *et al.*, 2006). It is important to notice that the composition of the zooplankton during the summer of 2007 was equal to that described by Rossi *et al*. (2006).

The indices 18:1(ω-9) / 18:1(ω-7) and PUFA / SFA indicate the degree of carnivory in late anchovy larvae and zooplankton. No statistically significant difference was observed among the larvae of the two cruises or among the December larvae and the December zooplankton, although the overall values of these indices were relatively high (Auel *et al.*, 2002). The indices 16:1(ω-7) / 16:0 and EPA / DHA, which estimate the importance of diatoms in the diet of larvae, show significant differences among August and December larvae, with both indices being higher in August.

There is also a statistically significant difference between December larvae and zooplankton, with the larvae having a lower ratio than the zooplankton.

**Table 2. Fatty acids composition of anchovy late larvae and zooplankton, presented as Mean % ± SD.**

| | *Engraulis encrasicolus* | | Zooplankton |
|---|---|---|---|
| | August 2007 (N = 4) | December 2007 (N = 5) | December 2007 (N = 3) |
| Fatty acids | | | |
| 14:0[a,b] | 5.06 ± 0.39 | 1.71 ± 0.32 | 8.94 ± 2.10 |
| 15:0[a,b] | 0.61 ± 0.02 | 0.49 ± 0.06 | 0.88 ± 0.37 |
| 16:0 | 23.39 ± 1.47 | 25.63 ± 7.17 | 24.31 ± 4.83 |
| 17:0[a] | 0.51 ± 0.02 | 0.69 ± 0.03 | 0.67 ± 0.19 |
| 18:0[b] | 4.22 ± 0.52 | 4.32 ± 0.12 | 5.73 ± 0.48 |
| 20:0[a] | 0.06 ± 0.01 | 0.04 ± 0.003 | 0.11 ± 0.02 |
| 22:0 | 0.05 ± 0.01 | 0.04 ± 0.01 | – |
| 24:0[a] | 0.14 ± 0.03 | 0.08 ± 0.02 | 0.07 ± 0.00 |
| Total saturated | 34.0 | 33.0 | 40.7 |
| 15:1 | 0.04 ± 0.01 | 0.04 ± 0.01 | 0.11 ± 0.00 |
| 16:1 (ω-7)[a,b] | 3.66 ± 0.38 | 1.42 ± 0.09 | 6.47 ± 1.74 |
| 18:1 (ω-9)[a,b] | 5.16 ± 0.33 | 5.57 ± 0.13 | 6.82 ± 1.48 |
| 18:1 (ω-7) | 1.91 ± 0.28 | 1.92 ± 0.16 | 1.84 ± 0.36 |
| 20:1 (ω-9) | 0.20 ± 0.15 | 0.13 ± 0.01 | 0.42 ± 0.10 |
| 22:1 (ω-9) | – | – | 0.08 ± 0.00 |
| 24:1 (ω-9)[a] | 0.12 ± 0.03 | 0.27 ± 0.05 | 0.08 ± 0.00 |
| Total monounsaturated | 11.1 | 9.3 | 15.8 |
| 18:2 (ω-6)[a] | 1.19 ± 0.11 | 0.62 ± 0.12 | 0.83 ± 0.29 |
| 18:3 (ω-6) | 0.55 ± 0.09 | 0.40 ± 0.05 | 0.64 ± 0.08 |
| 18:3 (ω-3)[a,b] | 0.85 ± 0.15 | 1.18 ± 0.15 | 1.89 ± 0.05 |
| 20:3 (ω-6) | 0.04 ± 0.00 | 0.03 ± 0.01 | – |
| 20:3 (ω-3) | 0.05 ± 0.01 | 0.07 ± 0.01 | – |
| 20:4 (ω-6) | 0.48 ± 0.06 | 0.55 ± 0.09 | 0.31 ± 0.05 |
| 20:5 (ω-3) | 11.29 ± 0.64 | 10.79 ± 0.85 | 16.57 ± 6.34 |
| 22:6 (ω-3)[b] | 34.25 ± 2.96 | 39.28 ± 4.47 | 17.35 ± 6.01 |
| Total polyunsaturated | 48.7 | 52.9 | 37.6 |
| Others | 6.2 | 4.8 | 5.9 |
| Total | 100 | 100 | 100 |

a: Statistical difference between August and December 2007 larvae, $p < 0.05$.
b: Statistical difference between December 2007 larvae and zooplankton, $p < 0.05$.

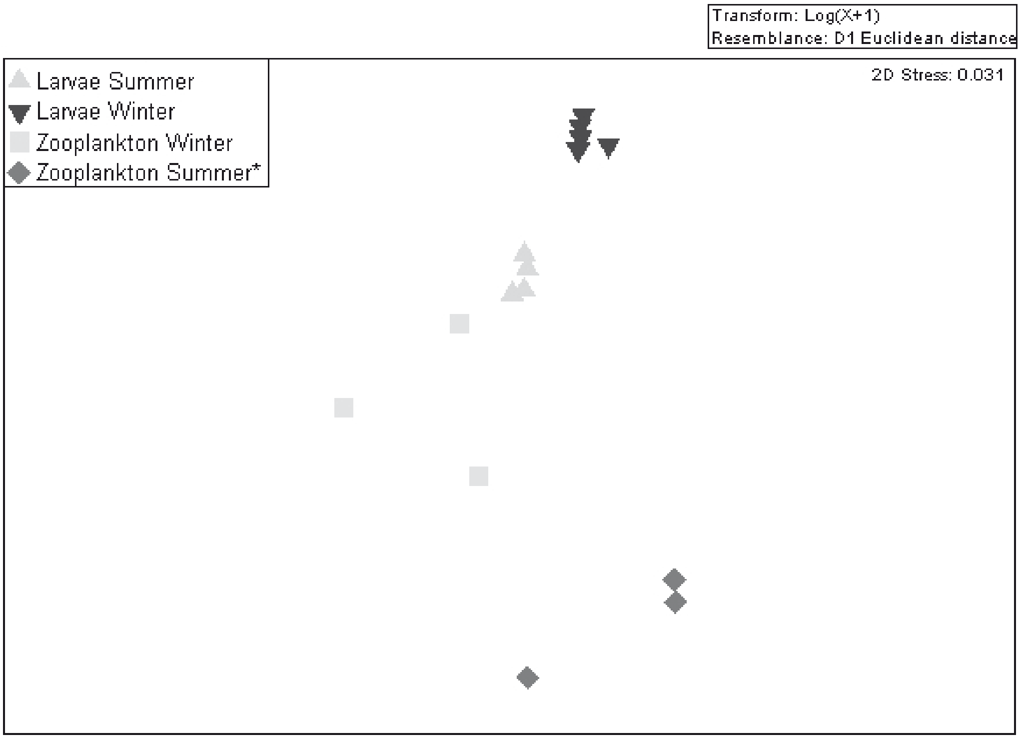

**Fig. 6. Non-metric multi-dimensional scaling (nMDS) plot of similarities in the fatty acid composition of anchovy late larvae and zooplankton. *Data of zooplankton summer from Rossi *et al*. (2006).**

**Table 3. Fatty acids trophic indices in August and December 2007 anchovies larvae and December 2007 zooplancton, presented as mean ± SD. (*) June 2000 zooplankton taken as reference level from Rossi *et al.*, 2006.**

| | *Engraulis encrasicolus* | | Zooplankton | |
|---|---|---|---|---|
| | August 2007 | December 2007 | June 2000* | December 2007 |
| 16:1(ω-7) / 16:0 | 0.16 ± 0.01 | 0.06 ± 0.01 | 0.96 | 0.27 ± 0.08 |
| 20:5 (ω-3) / 22:6 (ω-3) | 0.33 ± 0.01 | 0.27 ± 0.02 | 0.53 | 0.96 ± 0.42 |
| C16 / C18 | 1.79 ± 0.38 | 1.94 ± 0.55 | 1.70 | 1.76 ± 0.30 |
| 18:1(ω-9) / 18:1 (ω-7) | 2.70 ± 0.30 | 2.89 ± 0.23 | 3.15 | 3.71 ± 0.62 |
| PUFA / SFA | 1.43 ± 0.21 | 1.60 ± 0.42 | 0.65 | 0.92 ± 0.42 |

## 4. Discussion

*Engraulis encrasicolus* in the Northwestern Mediterranean has been intensively exploited (Palomera *et al*., 2007); therefore, alterations of any factor (e. g. temperature, salinity, currents, predation, food availability and overexploitation) affecting early stages (i.e. eggs, larvae and juveniles) of engraulids could have vital importance for the strength of recruitment and thus for the future of the population, due to their high larval growth and mortality rates (Houde, 1989, Takahashi & Watanabe, 2005, Ruiz *et al*., 2006).

Evidence showing that European anchovy larvae feed on plankton (Conway *et al*., 1999, Tudela *et al*., 2002, Bacha & Amara, 2009, Morote *et al.*, 2010) lead us to assume that changes in the plankton community affect the feeding habits of larvae, thus influencing their nutritional condition and, possibly, their survival (Fuiman and Cowan Jr., 2003).

Zooplankton biomass and taxonomic composition showed clear differences between August and December 2007, with the summer being the period when a higher presence of these organisms was recorded. Nevertheless, neither the TAG / CHOL index (Fraser *et al*., 1987) nor the lipids percentage nor the polar lipids content (Norton *et al*., 2001) indicates differences between seasons regarding the nutritional condition of anchovy late larvae. Håkanson (1989, 1993) pointed out that TAG / CHOL values below 0.2-0.3 indicate a poor nutritional condition, thus according to our results that showed TAG / CHOL average values around 0.64-0.70 in both seasons, it can be stated that both larvae in August and December presented a satisfactory nutritional condition. For this reason the observed differences in food availability cannot be assumed to affect the biochemical condition of both larvae populations.

Fulton's index showed statistically significant differences between August and December larvae concerning physical condition, showing that weight at size is higher in summer larvae compared to those collected in late autumn. The apparent discrepancy between biochemical nutritional condition and physical condition data can be explained by considering that morphometric condition indices take longer to show the effects of food intake(Catalán *et al*., 2007). In addition, taking into account that information from both FCI and growth rates show effects of a previous period compared to biochemical indices, it is not surprising that results of FCI agree with those of growth analysis. As the condition of our larvae needs to be evaluated for a short period of time, we consider that Fulton's index should not be taken into account to assess the nutritional condition in this study. The absence of differences between biochemical conditions of anchovy larvae observed in our study could be explained to a certain extent by the observed seasonal differences in sea water temperature. Moreover temperature is recognized to substantially influence the metabolic rates in marine organisms (McLaren, 1963, Ikeda, 1985) and in the specific zooplankton composition. Indeed, during the summer, larvae must compensate for the higher metabolic rate imposed by the higher temperature, with an energetically richer diet. Conversely, larvae could simply eat more. However, this assumption could be insufficient to compensate for the energy expense since higher prey capture requires a higher energetic cost.

In summer, despite the higher zooplankton biomass, a significant presence of cladocerans was recorded (Fig. 3). Cladocerans are considered of less energetic value than copepods (Boldt & Haldorson, 2002), in agreement with previous findings of Champalbert (1996) and Calbet *et al*. (2001). Moreover, stomach contents analysis carried out on juvenile stages of anchovy of the investigated area show a high preference for cladocerans in summer (SARDONE project, 2010; also see Chapter 2). Although we cannot be sure that the studied late larvae feed on the exact same prey as juveniles do, feeding preferences of larvae are based on zooplankton rather than on any other group (Tudela *et al*., 2002, Borme *et al*., 2009, Catalán *et al*., 2010, Morote *et al*., 2010) due to their inability to catch smaller preys without a proper development of gill rakers (van der Lingen *et al*., 2006). Therefore it is very likely that anchovy late larvae are also frequently feeding on cladocerans during the summer, as it happens in the Adriatic Sea (Borme *et al*., 2009).

On the other hand, the December larvae population is supposed to feed predominantly on copepods, which are higher energetic zooplankters. The differences in metabolic rates as well as in the energy content of diet could explain the similar nutritional condition observed in the seasonal pools of late larvae.

Masuda (2003) demonstrated that a lack of docosahexanoic acid could produce an ineffective feeding behavior, and even a higher mortality rate within a population. The finding that DHA was present and showed similar concentrations in both populations further confirm the occurrence of similar nutritional conditions in anchovy late larvae in both cruises. This result could also be explained by the fact that, in summer, lower DHA concentration was expected because a high UVB radiation can negatively affect DHA production by algae and August is the month with the highest solar radiation in the Western Mediterranean. In addition, Kainz *et al*. (2004) stated that the cladoceran community shows lower DHA concentrations. The higher plankton biomass available during August would help to compensate for the DHA deficit of larvae and result in equal conditions compared to winter.

Several studies regarding the effect that essential fatty acids (EFAs) have on fish development have being devised for reared larvae (Morais *et al*., 2007), but only a few examine natural populations at sea (Reuss & Poulsen, 2002). The present study intended to ascertain the role that FFAA play as trophic markers between anchovy late larvae and plankton in the Gulf of Lions, and to determine the main planktonic groups that are part of the diet of the larvae.

Anchovy fatty acid composition suffers seasonal variation (Zlatanos & Laskaridis, 2007). In particular, saturated fatty acids (SFAs) 16:0, 20:0, 22:0 and 24:0 can be more easily synthesized by all aquatic organisms (Dalsgaard *et al.*, 2003), while PUFAs, which are the first preference for fish lipases (Lie & Lambertsen, 1985) and must be obtained from the diet, are present in low concentration in oligotrophic seas (Fahl & Kattner, 1993). In addition, PUFAs are critical for survival of marine organisms (Brett & Müller-Navarra, 1997). For these reasons, trophic indices used in this study were based basically on unsaturated fatty acids.

The higher proportion of PUFAs compared to other types obtained in this study can be easily explained when taking into account the accumulation of these FFAA with age in anchovy larvae (Rossi *et al.*, 2006).

The absolute values of the indices 16:1(ω-7) / 16:0 and EPA / DHA are comparatively low (Auel *et al.*, 2002), and confirm a clear tendency towards a non-diatom diet (Table 3). The low value of the C16 / C18 index corroborates the mentioned low prefe-rence for diatoms.

Although limited, diatom consumption appears significantly higher during summer. This should be expected because there is a spring bloom of diatoms in this area (Leblanc *et al.*, 2003). As shown by the low overall values of the above mentioned indices and by some other works (e.g. Conway *et al.*, 1999, Tudela *et al.*, 2002, Islam & Tanaka, 2009, Morote *et al.*, 2010) anchovy larvae normally do not feed on phytoplankton. Thus, it is likely that any trace of phytoplankton in the larvae comes either indirectly from the consumed zooplankton or from accidentally ingested phytoplankton.

On the contrary, our find of moderately high levels of 18:1(ω-9) and long-chain monounsaturated fatty acids points out that calanoid copepods are important prey for anchovy late larvae (Werner & Auel, 2005), as showed in other studies with the carnivory indices 18:1(ω-9) / 18-1(ω-7) and PUFA / SFA (Cripps & Atkinson, 2000, Garrido *et al.*, 2008).

To date, knowledge of European anchovy reproductive behavior in the western Mediterranean indicates that the spawning period begins in the spring, when the water starts to heat up and reaches 14 to 15º C, and finishes in late-September, when water temperature starts to drop (Palomera, 1992).

However, the growth rates estimated in this work, together with otoliths analysis and age determination, indicate that anchovy larvae gathered in December 2007 were hatched approximately between the end of October and mid- November, which is well after the end of the spawning period previously known for this species (Palomera, 1992; Palomera *et al.*, 2007). Thus, a prolongation of the spawning period took place that year, likely brought about by a process of sea surface heating. During the last decades, mean temperatures in the Western Mediterranean have been rising (Salat & Pascual, 2002, Reynolds *et al.*, 2002). If the autumn-winter anchovy larvae population is becoming a norm in the biology of the species, we could likely assume that the spawning period of *E. encrasicolus* is being extended, favoring a wider spawning period and perhaps the survival of these late larvae of European anchovy in colder months.

Our analysis regarding the growth of both August and December cohorts shows that growth rates were significantly different among cohorts, being higher in August. This difference could be due to water temperature or to food availability (Takahashi & Watanabe, 2005, Aldanondo *et al.*, 2008). Mean temperature during August 2007 was 19º C, while during December cruise it was 12º C, a difference that could cause statistically different growth rates, yet still in agreement with the similar nutritional condition recorded in this study, as explained above in terms of different energetic expenditure.

Feeding activities have a positive correlation to water temperature (Houde, 1989), so lower growth rates in December may be due to a reduction in food intake and metabolic rates, rather than to food shortage (Takahashi & Watanabe, 2005), as the good nutritional condition observed in this period seems to confirm. Moreover, the Gulf of Lions is a rich environment in terms of food availability, favouring a proper nutritional condition in anchovy larvae, as exposed by García *et al*. (1998).

The noted lack of significant differences both in the TAG / CHOL index and in the polar lipids content between seasons in the anchovy larvae would imply a major relevance of temperature in the differences found in growth rates. These differences are also confirmed by Takasuka & Aoki (2006), who found a direct relationship between temperature and growth rate in Japanese anchovy larvae.

The present study does not indicate that either of the populations of larvae has a greater probability of survival than the other. Further research concerning the recruitment success of these late larvae populations is needed.

Some studies suggest that mortality of anchovy larvae of the Mediterranean is inversely related to growth rates (Allain *et al*., 2003, Palomera *et al*., 2007, La Mesa *et al.*, 2009), supported by the idea that larvae with slower growth rate remain as larvae longer ("stage-duration" mechanism, see Chambers & Leggett (1987)) and extends the exposure to predation. In this sense, we think that the "bigger is better" hypothesis, as described by Leggett & Deblois (1994), is a paradigm that fits to our results.

Takasuka *et al*. (2003) also found that larvae of Japanese anchovy with lower growth rates were proportionally more abundant within predators' stomachs than in the sea, inferring that were more vulnerable to predation

If this holds true, anchovy larvae found in December 2007 would be more vulnerable to predation and have less probability to reach the adult stage

However, further studies regarding the biology of predators that feed on anchovy larvae should be carried out, in order to estimate how their feeding activity could affect the probability of mortality of anchovy larvae.

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# Anexos

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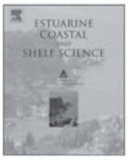

# Ecological understanding for fishery management: Condition and growth of anchovy late larvae during different seasons in the Northwestern Mediterranean

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## ABSTRACT

The fishery of the European anchovy *Engraulis encrasicolus* in the Mediterranean needs several ecological approaches to be properly managed. As such, several surveys were carried out to study the ecology of larvae and juveniles of this species, which reproduces during the warmest period of the year (May through September) in the Gulf of Lions. In particular, we studied the late larvae (15 mm total length until metamorphosis), especially as other authors have focused on larvae below that size. Unexpectedly, we also collected anchovy late larvae during the December 2007 survey, whose range in size corresponded to a later spawning period than previously reported. Differences in the nutritional condition of these larvae were assessed by comparing indices of lipid composition and estimating growth rates from otolith measurements to provide information on the probability of survival between the two groups. The analysis of fatty acids, used as tracers of trophic relationships, indicates that these larvae fed mainly on zooplankton. Nutritional conditions of summer and late autumn larvae were very similar. In contrast, growth rates were higher for August larvae, probably due to the different temperatures in the two seasons. Our results are especially relevant in an ecological context where the increasing mean water temperatures in the Western Mediterranean could favor the extension of the anchovy spawning period up to late-Autumn months.

## 1. Introduction

The European anchovy *Engraulis encrasicolus* is a highly valued fishery resource in the Western Mediterranean Sea (García and Palomera, 1996; Pertierra and Lleonart, 1996; Barange et al., 2009). Several studies have been carried out regarding diverse aspects of the biology and the ecology of the species in this area to improve the available tools for its fishery management (Palomera et al., 2007). The Northwestern Mediterranean is one of the most productive areas in this sea due to the cyclonic current that flows southwards over the slope of the Gulf of Lions, carrying a significant nutrient load from the Rhône River (Salat, 1996). The Gulf of Lions also displays notable environmental differences between seasons, which directly influence low trophic level species (Calbet et al., 2001). The anchovy population could then be easily compromised by any sort of alteration that additionally impinges on these organisms, especially during the early development stages (i.e. eggs, larvae and juveniles) when they are particularly sensitive to any change (Palomera et al., 2007). The anchovy spawning period in the Gulf of Lions extends from April to late September, with a peak in late June (Palomera, 1992). Therefore late larvae are expected to be found mainly during the summer, while juveniles should emerge in the autumn and winter.

During the SARDONE project, devoted to improve the management strategies of the European anchovy, two cruises were undertaken in August and December of 2007 in the Gulf of Lions in order to collect anchovy late larvae and juveniles, respectively. Unexpectedly, and in spite of the allegedly unfavorable conditions, a notable amount of anchovy late larvae were caught in December (a mean of 26.75 larvae/tow, against 34.20 larvae/tow in summer), well after the reported end of the spawning period.

An understanding of the growth and feeding ecology of the species is important given that this knowledge is essential to understand how the population temporally and spatially develops in the environment. The relationship between this population and the plankton community is not straightforward but some approaches have been attempted (Isari et al., 2008; Morais et al., 2010), especially those concerning how the zooplankton affects the population strength. Indeed, the growth rates and the nutritional condition of a population of several species, including European anchovy, have been key subjects for the study of the recruitment strength (Butler, 1991; Ward et al., 2006; Palomera et al., 2007; Hidalgo et al., 2008; Islam and Tanaka, 2009; La Mesa et al., 2009).

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In the present case we analyze for the first time in the Mediterranean the nutritional condition of anchovy late larvae (19–35 mm) in the field during both summer and late-Autumn seasons via lipid composition studies, and we also study their diet through the fatty acids found both in larvae and in zooplankton, which is the basic prey of anchovy at all the development stages (Plounevez and Champalbert, 1999; Pasquaud et al., 2008; Bacha and Amara, 2009; Borme et al., 2009; Catalán et al., 2010; Morote et al., 2010). Growth rates were also studied for both larvae populations to determine whether the different environmental conditions affect the early stage development of the anchovy. This work will thus help to determine to what extent the general conditions of an unexpected December late larvae population differs from the August late larvae and whether these two populations have similar viability.

## 2. Materials and methods

### 2.1. Sample collection

Samples were collected during two cruises carried out in the Gulf of Lions (Northwestern Mediterranean sea; Fig. 1) in 2007 on board the *R/V L'Europe* (IFREMER, France). The first cruise (PELMED07) was conducted in the summer from the 28th of July to the 9th of August 2007 and the second cruise (JUVALION07) was carried out in late autumn, from the 8th to the 21st of December 2007. Temperature and salinity of the water column from sea surface to 50 m depth were measured via a Seabird 19 CTD at each station. Also data of sea surface temperature in September to December since 1982 to early 2011 of Gulf of Lions area from NOAA were acquired to study possible trends.

Zooplankton samples for an analysis of biomass and plankton composition were collected using a standard WP2 net with a mesh size of 200 μm and sieved through a 3000 μm plankton mesh to obtain the 200–3000 μm mesozooplankton fraction, and by means of a scaled-down version WP2 net, with a mesh size of 53 μm and a mouth diameter of 25 cm, and sieved through a 200 μm plankton mesh to obtain the 53–200 μm fraction of microplankton. All zooplankton samples were split with a Motoda plankton splitter (Motoda, 1959) and one-half was preserved in formalin to carry out subsequent qualitative analysis, while the other half was frozen on board for biomass measurements and lipid analysis. As the need to comparatively assess the nutritional condition of the two different temporal larvae populations only arose during the December cruise, plankton samples for lipids analysis were not collected during the first cruise.

Late larvae of anchovy were caught with a pelagic trawling net, towed at an average speed of 3.6 knots over a 30–40 min time span. This trawling time might seem too long to obtain larvae in proper conditions for biochemical analysis; however, the alteration of lipid composition in muscular tissue of fish larvae is small within a time up to 3 h after death (Lochmann et al., 1996).

Samples were immediately frozen in liquid nitrogen after sorting on board and transferred into a −80 °C freezer just after the arrival in the laboratory.

### 2.2. Lipid and fatty acid analysis

Wet weight and standard length of each larva were measured in the laboratory to the nearest 10 μg and 0.1 mm, respectively, before removing the head for otoliths analysis and the gut, as

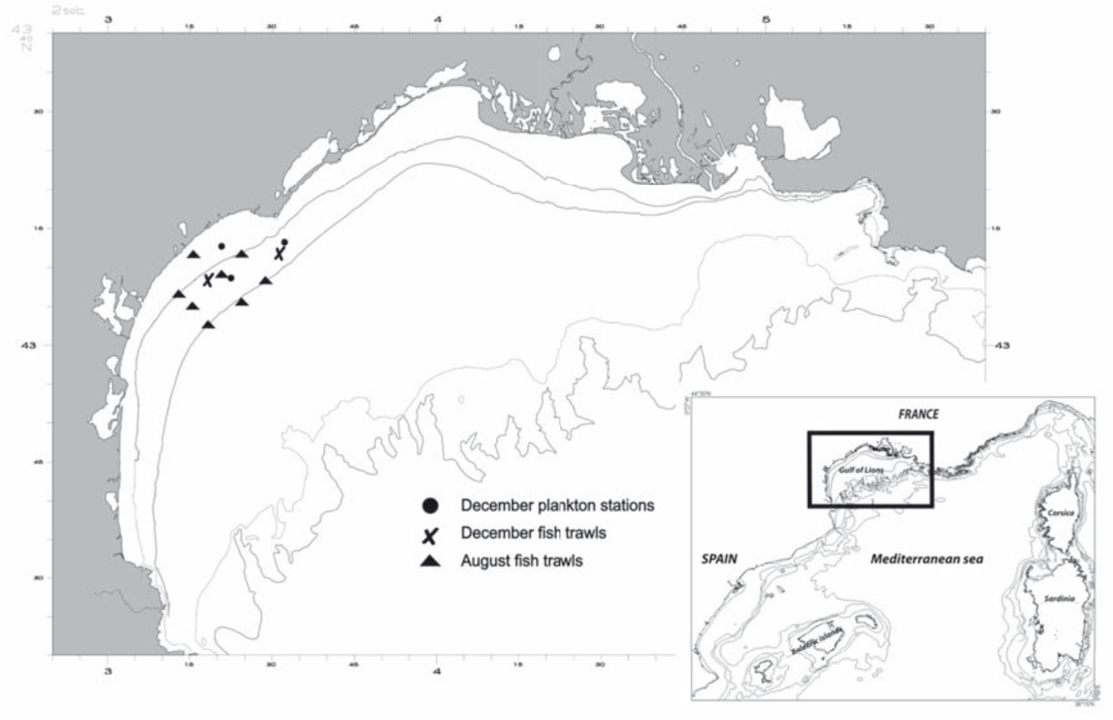

**Fig. 1.** Map of the study area, with the positions of plankton stations and trawls in August and December 2007.

recommended by Lochmann et al. (1996). The empirical relationship between larval wet and dry weights was calculated via linear regression from other larvae of the same cruises and size ranges. Microplankton and mesozooplankton samples from each station were pooled together before proceeding with the fatty acid extraction.

Lipid extraction was performed according to the method of Folch et al. (1957). Lipid content was measured following the protocol of Olsen and Henderson (1989) via high-performance thin-layer chromatography (HPTLC), which was followed by quantitative densitometry in visible light with a Bio-Rad Gel Doc XR densitometer, using Quantity One 4.6.2 software.

The nutritional condition of the *Engraulis encrasicolus* late larvae was evaluated by comparing the triacylglycerol/cholesterol (TAG/CHOL) index (Håkanson, 1993) and the ratio between the percentage of total lipids and the dry mass (Norton et al., 2001). Fulton's condition index (FCI) was calculated with wet weight (W, in g) and standard length (SL, in mm) data, following the equation:

$$\mathrm{FCI} = \mathrm{W} \times 100/\mathrm{SL}^3$$

Fatty acids extraction and trans-methylation was accomplished following the protocol of Christie (1989) as modified by Li and Watkins (2001). Four out of seven samples of larvae from August 2007, together with five larvae samples and three zooplankton samples from December 2007, were suitable for analysis by gas-chromatography.

Gas chromatographic (GC) analysis of fatty acid methyl esters (FAMEs) was then performed using a Thermofisher Scientific GC8060 gas-chromatograph coupled with a MD800 mass-spectrometer. The apparatus was fitted with a BPX-70 capillary column (30 m × 0.25 mm i.d. × 0.25 μm). Helium was used as carrier gas, with a speed of 1 ml/min. The programmed oven temperature was 60 °C (1 min) to 260 °C (10 min) with an increment of 8 °C $\mathrm{min}^{-1}$. The injector temperature was set at 270 °C and the injector split was set at 35 ml $\mathrm{min}^{-1}$. Mass-spectrometry was conducted with an ion source temperature of 200 °C and an interphase temperature at 260 °C. Ionization was performed by electron impact at 70 eV, and the weight range analyzed was 50–550 Da.

FAMEs were identified by comparing their retention times with those of the standard mixture, Supelco 37 Component FAME mix. The quantification of the identified FAMEs was calculated through GC peak areas integration.

The diet of late larvae was evaluated according to the indices based on fatty acid relations 16:1(ω-7)/16:0, 18:1(ω-9)/18:1(ω-7) and EPA/DHA, and on PUFA/SFA relation (St. John and Lund, 1996; Auel et al., 2002; Rossi et al., 2006).

### 2.3. Growth analysis

A total of 61 larvae from August and 44 larvae from December, ranging in size from 19 to 31 mm (SL) and from 20 to 27 mm (SL), respectively, were used for a growth analysis. Both sagittal otoliths were extracted from the head of the anchovy larvae under a Leica dissection microscope (Wild M12) equipped with polarizing filters and mounted in Crystalbond 509 Amber on labeled glass slides. The otolith growth analysis was undertaken at 100× magnification under transmitted light with a microscope (Zeiss Axiospoth) coupled to a digital video recorder, while the otolith nucleus was analyzed at 1000× magnification. Otolith radius (OR) and increment width (IW) (μm) were measured to the nearest 0.1 μm using Image-Pro Plus 5.0. The increments were measured along the longest radius, from the middle of one D-zone to the middle of the next D-zone. Following the results of Aldanondo et al. (2008), for the same species in the Bay of Biscay and under experimental conditions, increments were assumed to be daily (DI) being the first increment laid down at hatching. Prior to Aldanondo et al. (2008), studies on European anchovy growth had assumed that the first increment deposition took place at the beginning of exogenous feeding, i.e. two days after hatching, as proposed by Palomera et al. (1988) in the first paper on anchovy larval otoliths. All otoliths were read twice by two different persons, and only if the DI differed by 1 daily increment were they accepted.

Taking into account the narrow range of lengths that we are analyzing, we have assumed linear growth in agreement with the results of La Mesa et al. (2009), the first study of anchovy that analyzes the growth at the metamorphic period, as is the case of our samples. Accordingly, the individual growth rate (IGR, mm $\mathrm{d}^{-1}$) from the time of hatching until the time of capture was then calculated by using the equation proposed by Takahashi and Watanabe (2005).

$$\mathrm{IGR} = (\mathrm{SL} - \mathrm{SL}_0)/\mathrm{Age}$$

where SL is the measured larvae standard length corrected by using the method of Theilacker (1980), $\mathrm{SL}_0$ is the larvae standard length at hatching, estimated to be 2.5 mm according to laboratory studies on the studied species (Regner, 1985), and Age = DI.

### 2.4. Data analysis

Seasonal differences between oceanographic parameters as well as Fulton's condition index, lipid total content, lipid classes and proportion of fatty acids in larvae and plankton were assessed by means of Mann–Whitney non-parametric tests for independent samples (Dytham, 2003), and for oceanographic data ANOVA tests were performed.

Fatty acid percentage compositions were pairwise compared between larvae of both cruises, and between larvae and zooplankton collected during the December 2007 cruise using the former test. Similarities in the fatty acid composition between samples were measured by Euclidean distances (Legendre and Legendre, 1998). A non-metric multi-dimensional scaling (nMDS) was carried out on the samples similarity matrix to visually describe overall patterns. Statistical analyses were carried out using STATISTICA 6.0 by Statsoft, Inc., and PRIMER-E 6 software. Significance level for all tests was adopted at $p < 0.05$.

## 3. Results

### 3.1. Oceanographic data and zooplankton composition

The mean temperature within the water column (0–50 m) and the mean surface temperature (0–5 m) were significantly higher ($p < 0.0001$) in August (mean ± standard deviation values of 19.14 °C ± 1.32 for surface temperature and 16.75 °C ± 1.02 for average temperature) compared to December (mean ± standard deviation values of 12.64 °C ± 0.91 for surface temperature and 13.04 °C 0.66 for average temperature), while no significant differences were observed for salinity between the two cruises (mean ± standard deviation values of 37.74 ± 0.20 and 37.35 ± 1.48 for surface salinity and 37.94 0.06 and 37.93 ± 0.40 for average salinity in August and December, respectively).

Data of temperature acquired from NOAA (Reynolds et al., 2002) were monthly averaged in the Gulf of Lions (area comprised between 2.5° W to 6.5° W and 41.3° N to 45.5° N), from September 1981 to December 2010, showing a positive trend in sea surface temperature during the last 4 months of every year (16.5° in September 1981 to 17.2 °C in December 2010; $\mathrm{SST} = 14.97 + 0.54 \times 10^{-4} * \mathrm{Serial\_date}$; where Serial_date is the number of days since the January 1st of 1900).

There were significant differences for microplankton biomass between August and December cruises (non-parametric Mann–Whitney *U* test, $p < 0.05$), with higher values recorded during the summer (mean ± standard deviation values of $201.59 \pm 283.25$ mg $m^{-3}$ and $22.76 \pm 24.18$ mg $m^{-3}$, respectively). The same pattern was observed for the mesozooplankton, where pairwise multiple comparisons (*t*-test) revealed that August 2007 samples had significantly higher biomass ($p < 0.05$) compared to those collected in December 2007 (mean ± standard deviation values of $33.65 \pm 10.34$ mg $m^{-3}$ and $14.07 \pm 10.65$ mg $m^{-3}$, respectively). The analysis of both microplankton and mesozooplankton composition revealed a dominance of copepods, especially calanoids and cyclopoids, within the community during the two seasons (Fig. 2). Nevertheless, in August cladocerans were also important, while they were not recorded in December cruise (Fig. 3).

### 3.2. Growth rate

Otolith growth was significantly different between the two periods with the otoliths of the December larvae being smaller compared to those of the August larvae of the same age (*t*-test, $p < 0.001$) (Fig. 4). For both groups, increment width increased continuously, although for larvae caught in December, that ranged on age from 33 to 54 days, the largest increment width reached was half that of the maximum increment width in the August samples, that ranged from 22 to 44 days (4 vs. 8 μm $day^{-1}$, respectively). This pattern was matched by mean individual growth rates (IGR) from hatching until the time of capture, that ranged between 0.50 and 0.93 mm $d^{-1}$ (mean ± standard deviation values of $0.74 \pm 0.09$ mm $d^{-1}$) for August larvae and 0.43–0.74 mm $d^{-1}$ (mean ± standard deviation values of $0.59 \pm 0.07$ mm $d^{-1}$) for December larvae, indicating that IGR was significantly higher in the warmer period (*t*-test, $p < 0.001$).

### 3.3. Nutritional condition

A single linear regression between larvae dry mass and wet weight was estimated for both cruises (Fig. 5) as the one-way ANCOVA did not show significant differences between August and December ($p > 0.05$), considering wet weight as covariant. Subsequently, the empirical equation obtained from that relationship was applied to estimate the larvae dry mass (mean ± standard deviation values of $23.0 \pm 7.0$ and $18.0 \pm 7.0$ mg/larva for August and December late larvae, respectively) as well as in further analysis regarding the dry mass of lipid contents in the samples.

Mean standard lengths did not significantly differ between the two cruises (mean ± standard deviation values of $27.3 \pm 3.0$ mm and $27.0 \pm 3.4$ mm for August 2007 and December 2007, respectively). On the contrary, Fulton's condition index of larvae was significantly higher ($p < 0.05$) in the August individuals (mean ± standard deviation values of $0.598 \pm 0.131$ and $0.489 \pm 0.153$ in August and December, respectively).

Pools of 4–6 larvae each from August and December cruises were processed for lipid extraction. Lipid content in larvae did not show any statistical differences between the two seasons ($p = 0.123$), and no significance was found regarding triacylglycerol ($p = 1.000$), cholesterol ($p = 0.705$), free fatty acid ($p = 0.570$) or polar lipid content ($p = 1.000$) within the anchovy samples

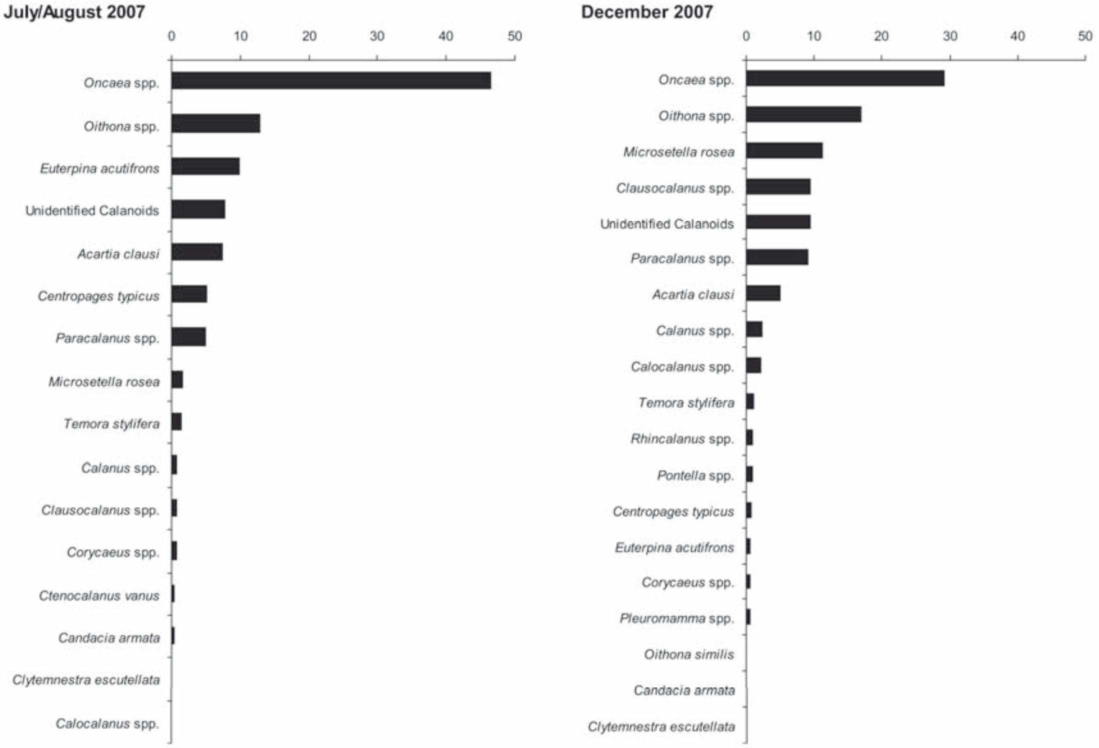

**Fig. 2.** Relative abundances of the main copepods species within micro and mesozooplankton samples of both August and December 2007 campaigns.

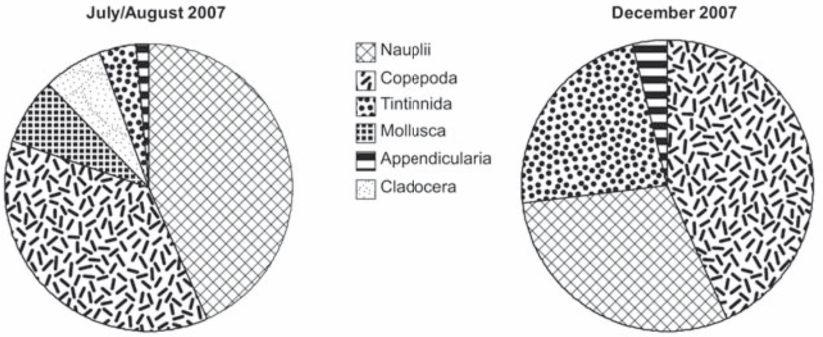

**Fig. 3.** Pie charts illustrating the mean percentage composition of the main plankton groups in August and December 2007 cruises.

(Table 1). The TAG/CHOL ratio was determined to range between 0.53 and 0.72 for August larvae and 0.60–0.82 for December larvae, and it also did not exhibit significant differences between cruises ($p = 0.186$).

### 3.4. Fatty acids analysis

Of the 23 fatty acids identified, 16:0, eicosapentaenoic acid or EPA (20:5(ω-3)) and docosahexanoic acid or DHA (22:6(ω-3)) made up 58–75% of total fatty acids in the zooplankton and larvae samples (Table 2), with DHA and 16:0 being the most common fatty acids found in both August and December larvae and within the zooplankton. The other abundant fatty acids found were 14:0, 18:0, 16:1(ω-7), 18:1(ω-9) and 18:1(ω-7). The proportion of PUFA was higher than any other type of FFAA among larvae of both cruises, while SFA were the most abundant in the zooplankton.

A multi-dimensional scaling (MDS) ordination, with a stress coefficient <0.01, shows the similarity in fatty acid composition among the larvae of August and December and the zooplankton of December. Three groups can be differentiated on the plot, specifically a group comprised of 4 out of 5 samples of the December anchovy larvae, the 4 samples of August larvae, and the 3 samples of zooplankton (Fig. 6). The ANOSIM test confirmed the presence of significant differences in the multivariate fatty acid composition between all three groups ($R^2 = 0.83$ at $p < 0.0001$).

Table 3 shows the mean values of the fatty acid indices estimated from the data obtained in this work, apart from those related to the summer zooplankton, which were acquired from (Rossi et al., 2006). It is of note that the composition of the zooplankton during the summer of 2007 was equal to that described by Rossi et al. (2006).

The indices 18:1(ω-9)/18:1(ω-7) and PUFA/SFA indicate the degree of carnivory in late anchovy larvae and zooplankton. No statistically significant difference was observed among the larvae of the two cruises or among the December larvae and the December zooplankton, although the overall values of these indices were relatively high (Auel et al., 2002).

The indices 16:1(ω-7)/16:0 and EPA/DHA, which estimate the importance of diatoms in the diet of larvae, show significant differences between August and December larvae, with both indices being higher in August. There is also a statistically significant difference between December larvae and zooplankton, with the larvae having a lower ratio than the zooplankton.

## 4. Discussion

*Engraulis encrasicolus* in the Northwestern Mediterranean has been intensively exploited (Palomera et al., 2007) and so alterations of any factor (e. g. temperature, salinity, currents, predation, food availability and overexploitation) affecting early stages (i.e. eggs, larvae and juveniles) of engraulids could be important for the strength of recruitment and thus for the future of the population, due to their high larval growth and mortality rates (Houde, 1989; Takahashi and Watanabe, 2005; Ruiz et al., 2006).

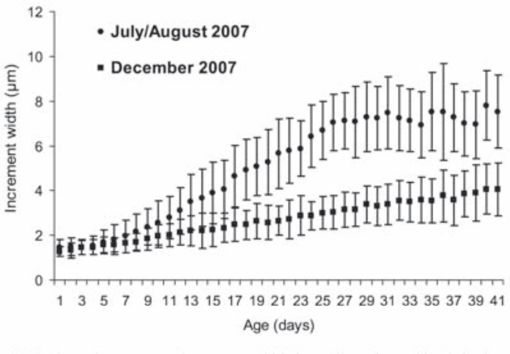

**Fig. 4.** Anchovy larvae mean increment width by estimated age (days) for larvae caught in August and December 2007. (Error bars: standard deviation).

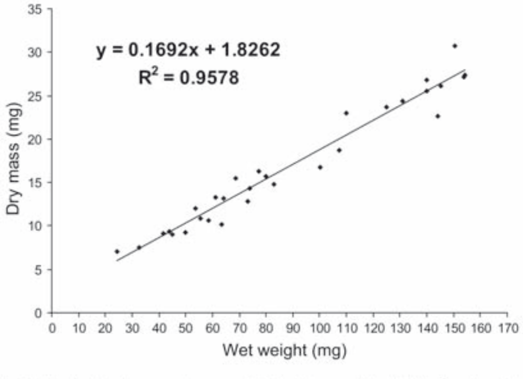

**Fig. 5.** Relationship between dry mass (DM) and wet weight (WW) of anchovy late larvae. Linear regression fitted by $DM = 0.1692*WW + 1.8262$ ($r^2 = 0.9578$).

**Table 1**
Total lipid content, lipid class values and TAG/CHOL index (μg*larva-1), presented as Mean ± SD.

| | August 2007 ($N = 7$ pools of 4–6 larvae each) | December 2007 ($N = 5$ pools of 4–5 larvae each) |
|---|---|---|
| Total lipid content | 770.4 ± 275.1 | 664.5 ± 92.7 |
| % lipid/dry weight | 4.2 ± 0.8 | 4.4 ± 0.2 |
| *Neutral lipids* | | |
| Triacylglycerol (TAG) | 922.2 ± 379.4 | 873.8 ± 242.2 |
| Cholesterol | 1414.4 ± 476.6 | 1227.8 ± 249.9 |
| Free fatty acid | 1406.0 ± 425.6 | 1228.2 ± 221.7 |
| Steryl esther | – | – |
| Polar lipids | 230.6 ± 65.9 | 223.1 ± 37.8 |
| TAG/CHOL index | 0.64 ± 0.06 | 0.70 ± 0.09 |

Evidence showing that European anchovy larvae feed on plankton (Conway et al., 1999; Tudela et al., 2002; Bacha and Amara, 2009; Morote et al., 2010) lead us to assume that changes in the plankton community affect the feeding habits of larvae, thus influencing their nutritional condition and, possibly, their survival (Fuiman and Cowan, 2003).

Zooplankton biomass and taxonomic composition showed clear differences between August and December 2007, with the summer being the period when a higher presence of these organisms was recorded. Nevertheless, neither the TAG/CHOL index (Fraser et al., 1987) nor the lipid percentage or the polar lipids content (Norton et al., 2001) indicate differences between seasons regarding the nutritional condition of anchovy late larvae. Håkanson (1989, 1993) pointed out that TAG/CHOL values below 0.2–0.3 indicate a poor nutritional condition, thus according to our results that showed TAG/CHOL average values around 0.64–0.70 in both seasons, it can be stated that both larvae in August and December presented a satisfactory nutritional condition. For this reason the observed differences in food availability cannot be assumed to affect the biochemical condition of both larvae populations.

**Table 2**
Fatty acids composition of anchovy late larvae and zooplankton, presented as mean % ± SD.

| | Engraulis encrasicolus | | Zooplankton |
|---|---|---|---|
| | August 2007 ($N = 4$) | December 2007 ($N = 5$) | December 2007 ($N = 3$) |
| *Fatty acids* | | | |
| 14:0$^{a}$,$^{b}$ | 5.06 ± 0.39 | 1.71 ± 0.32 | 8.94 ± 2.10 |
| 15:0$^{a}$,$^{b}$ | 0.61 ± 0.02 | 0.49 ± 0.06 | 0.88 ± 0.37 |
| 16:0 | 23.39 ± 1.47 | 25.63 ± 7.17 | 24.31 ± 4.83 |
| 17:0$^{a}$ | 0.51 ± 0.02 | 0.69 ± 0.03 | 0.67 ± 0.19 |
| 18:0$^{b}$ | 4.22 ± 0.52 | 4.32 ± 0.12 | 5.73 ± 0.48 |
| 20:0$^{a}$ | 0.06 ± 0.01 | 0.04 ± 0.003 | 0.11 ± 0.02 |
| 22:0 | 0.05 ± 0.01 | 0.04 ± 0.01 | – |
| 24:0$^{a}$ | 0.14 ± 0.03 | 0.08 ± 0.02 | 0.07 ± 0.00 |
| *Total saturated* | 34.0 | 33.0 | 40.7 |
| 15:1 | 0.04 ± 0.01 | 0.04 ± 0.01 | 0.11 ± 0.00 |
| 16:1 (ω-7)$^{a,b}$ | 3.66 ± 0.38 | 1.42 ± 0.09 | 6.47 ± 1.74 |
| 18:1 (ω-9)$^{a,b}$ | 5.16 ± 0.33 | 5.57 ± 0.13 | 6.82 ± 1.48 |
| 18:1 (ω-7) | 1.91 ± 0.28 | 1.92 ± 0.16 | 1.84 ± 0.36 |
| 20:1 (ω-9) | 0.20 ± 0.15 | 0.13 ± 0.01 | 0.42 ± 0.10 |
| 22:1 (ω-9) | – | – | 0.08 ± 0.00 |
| 24:1 (ω-9)$^{a}$ | 0.12 ± 0.03 | 0.27 ± 0.05 | 0.08 ± 0.00 |
| *Total monounsaturated* | 11.1 | 9.3 | 15.8 |
| 18:2 (ω-6)$^{a}$ | 1.19 ± 0.11 | 0.62 ± 0.12 | 0.83 ± 0.29 |
| 18:3 (ω-6) | 0.55 ± 0.09 | 0.40 ± 0.05 | 0.64 ± 0.08 |
| 18:3 (ω-3)$^{a,b}$ | 0.85 ± 0.15 | 1.18 ± 0.15 | 1.89 ± 0.05 |
| 20:3 (ω-6) | 0.04 ± 0.00 | 0.03 ± 0.01 | – |
| 20:3 (ω-3) | 0.05 ± 0.01 | 0.07 ± 0.01 | – |
| 20:4 (ω-6) | 0.48 ± 0.06 | 0.55 ± 0.09 | 0.31 ± 0.05 |
| 20:5 (ω-3) | 11.29 ± 0.64 | 10.79 ± 0.85 | 16.57 ± 6.34 |
| 22:6 (ω-3)$^{b}$ | 34.25 ± 2.96 | 39.28 ± 4.47 | 17.35 ± 6.01 |
| *Total polyunsaturated* | 48.7 | 52.9 | 37.6 |
| Others | 6.2 | 4.8 | 5.9 |
| Total | 100 | 100 | 100 |

[a] Statistical difference between August and December 2007 larvae, $p < 0.05$.
[b] Statistical difference between December 2007 larvae and zooplankton, $p < 0.05$.

**Fig. 6.** Non-metric multi-dimensional scaling (nMDS) plot of similarities in the fatty acid composition of anchovy late larvae and zooplankton. *Data of zooplankton summer from Rossi et al. (2006).

Fulton's index showed statistically significant differences between August and December larvae concerning physical condition, showing that weight at size is higher in summer larvae compared to those collected in late autumn. The apparent discrepancy between biochemical nutritional condition and physical condition data can be explained by considering that morphometric condition indices take longer to show the effects of food intake (Catalán et al., 2007). In addition, taking into account that information from both FCI and growth rates show effects of a previous period compared to biochemical indices, it is not surprising that results of FCI agree with those of growth analysis. As the condition of our larvae needs to be evaluated for a short period of time, we consider that Fulton's index should not be taken into account to assess the nutritional condition in this study. The absence of differences between biochemical conditions of anchovy larvae observed in our study could be explained to a certain extent by the observed seasonal differences in sea water temperature. Moreover temperature is recognized to substantially influence the metabolic rates in marine organisms (McLaren, 1963; Ikeda, 1985) and in the specific zooplankton composition. Indeed, during the summer, larvae must compensate for the higher metabolic rate imposed by the higher temperature, with an energetically richer diet. Conversely, larvae could simply eat more. However, this assumption could be insufficient to compensate for the energy expense since higher prey capture requires a higher energetic cost.

In summer, despite the higher zooplankton biomass, a significant presence of cladocerans was recorded (Fig. 3). Cladocerans are considered of less energetic value than copepods (Boldt and Haldorson, 2002), in agreement with previous findings of

**Table 3**
Fatty acids trophic indices in August and December 2007 anchovies larvae and December 2007 zooplankton, presented as mean ± SD.

| | Engraulis encrasicolus | | Zooplankton | |
|---|---|---|---|---|
| | August 2007 | December 2007 | June 2000$^{a}$ | December 2007 |
| 16:1(ω-7)/16:0 | 0.16 ± 0.01 | 0.06 ± 0.01 | 0.96 | 0.27 ± 0.08 |
| 20:5 (ω-3)/22:6 (ω-3) | 0.33 ± 0.01 | 0.27 ± 0.02 | 0.53 | 0.96 ± 0.42 |
| C16/C18 | 1.79 ± 0.38 | 1.94 ± 0.55 | 1.70 | 1.76 ± 0.30 |
| 18:1(ω-9)/18:1 (ω-7) | 2.70 ± 0.30 | 2.89 ± 0.23 | 3.15 | 3.71 ± 0.62 |
| PUFA/SFA | 1.43 ± 0.21 | 1.60 ± 0.42 | 0.65 | 0.92 ± 0.42 |

[a] June 2000 zooplankton taken as reference level from Rossi et al. (2006).

Champalbert (1996) and Calbet et al. (2001). Moreover, stomach contents analysis carried out on juvenile stages of anchovy of the investigated area show a high preference for cladocerans in summer (SARDONE project, 2010). Although we cannot be sure that the studied late larvae feed on the exact same prey as juveniles, feeding preferences of larvae are based on zooplankton rather than on any other group (Tudela et al., 2002; Borme et al., 2009; Catalán et al., 2010; Morote et al., 2010) due to their inability to catch smaller prey without a proper development of gill rakers (van der Lingen et al., 2006). Therefore it is very likely that anchovy late larvae are also frequently feeding on cladocerans during the summer, as occurs in the Adriatic Sea (Borme et al., 2009). On the other hand, the December larvae population is supposed to feed predominantly on copepods, which are higher energetic zooplankters. The differences in metabolic rates as well as in the energy content of diet could explain the similar nutritional condition observed in the seasonal pools of late larvae.

Masuda (2003) demonstrated that a lack of docosahexanoic acid could produce an ineffective feeding behavior, and even a higher mortality rate within a population. The finding that DHA was present and showed similar concentrations in both populations further confirm the occurrence of similar nutritional conditions in anchovy late larvae in both cruises. This may also be explained by the fact that, in summer, lower DHA concentration was expected because a high UVB radiation can negatively affect DHA production by algae and August is the month with the highest solar radiation in the Western Mediterranean. In addition, Kainz et al. (2004) stated that the cladoceran community shows lower DHA concentrations. The higher plankton biomass available during August would help to compensate for the DHA deficit of larvae and result in equal conditions compared to winter.

Several studies regarding the effect of essential fatty acids (EFAs) on fish development have been devised for reared larvae (Morais et al., 2007), but only a few examine natural populations at sea (Reuss and Poulsen, 2002). The present study intended to ascertain the role that FFAA play as trophic markers between anchovy late larvae and plankton in the Gulf of Lions, and to determine the main planktonic groups that are part of the diet of the larvae. Anchovy fatty acid composition suffers seasonal variation (Zlatanos and Laskaridis, 2007). In particular, saturated fatty acids (SFAs) 16:0, 20:0, 22:0 and 24:0 can be more easily synthesized by all aquatic organisms (Dalsgaard et al., 2003), while PUFAs, which are the first preference for fish lipases (Lie and Lambertsen, 1985) and must be obtained from the diet, are present in low concentration in oligotrophic seas (Fahl and Kattner, 1993). In addition, PUFAs are essential for the survival of marine organisms (Brett and Müller-Navarra, 1997). For these reasons, trophic indices used in this study were based basically on unsaturated fatty acids.

The higher proportion of PUFAs compared to other types obtained in this study can be easily explained when taking into account the accumulation of these FFAA with age in anchovy larvae (Rossi et al., 2006). The absolute values of the indices 16:1(ω-7)/16:0 and EPA/DHA are comparatively low (Auel et al., 2002), and confirm a clear tendency towards a non-diatom diet (Table 3). The low value of the C16/C18 index corroborates the inferred low preference for diatoms.

Although limited, diatom consumption appears significantly higher during summer. This should be expected because there is a spring bloom of diatoms in this area (Leblanc et al., 2003). As shown by the low overall values of the above mentioned indices and by other works (e.g. Conway et al., 1999; Tudela et al., 2002; Islam and Tanaka, 2009; Morote et al., 2010) anchovy larvae normally do not feed on phytoplankton. Thus, it is likely that any trace of phytoplankton in the larvae comes either indirectly from the consumed zooplankton or from accidentally ingested phytoplankton. On the contrary, our find of moderately high levels of 18:1(ω-9) and long-chain monounsaturated fatty acids points out that calanoid copepods are important prey for anchovy late larvae (Werner and Auel, 2005), as shown in other studies with the carnivory indices 18:1(ω-9)/18−1(ω-7) and PUFA/SFA (Cripps and Atkinson, 2000; Garrido et al., 2008).

To date, the knowledge of European anchovy reproductive behavior in the western Mediterranean indicates that the spawning period begins in the spring, when the water starts to heat up and reaches 14 to 15 °C, and finishes in late-September, when water temperature starts to decrease (Palomera, 1992). However, the growth rates estimated in this work, together with otoliths analysis and age determination, indicate that anchovy larvae gathered in December 2007 were hatched approximately between the end of October and mid- November, which is well after the end of the spawning period previously known for this species (Palomera, 1992; Palomera et al., 2007). Thus, a prolongation of the spawning period took place that year, likely brought about by a process of sea surface heating. During the last decades, mean temperatures in the Western Mediterranean have been rising (Salat and Pascual, 2002; Reynolds et al., 2002). If the autumn–winter anchovy larvae population is becoming a norm in the biology of the species, we may assume that the spawning period of *Engraulis encrasicolus* is being extended, favoring a wider spawning period and perhaps the survival of these late larvae of European anchovy in colder months.

Our analysis regarding the growth of both August and December cohorts shows that growth rates were significantly different among cohorts, being higher in August. This difference could be due to water temperature or to food availability (Takahashi and Watanabe, 2005; Aldanondo et al., 2008). Mean temperature during August 2007 was 19 °C, while during December cruise it was 12 °C, a difference that could cause statistically different growth rates, yet still in agreement with the similar nutritional condition recorded in this study, as explained above in terms of different energetic expenditure.

Feeding activities have a positive correlation to water temperature (Houde, 1989), so lower growth rates in December may be due to a reduction in food intake and metabolic rates, rather than to food shortage (Takahashi and Watanabe, 2005), as the good nutritional condition observed in this period seems to confirm. Moreover, the Gulf of Lions is a rich environment in terms of food availability, favoring an adequate nutritional condition in anchovy larvae, as exposed by García et al. (1998).

The noted lack of significant differences both in the TAG/CHOL index and in the polar lipids content between seasons in the anchovy larvae would imply a major relevance of temperature in the differences found in growth rates. These differences are also confirmed by Takasuka and Aoki (2006), who found a direct relationship between temperature and growth rate in Japanese anchovy larvae.

The present study does not indicate that either of the populations of larvae has a greater probability of survival than the other. Further research concerning the recruitment success of these late larvae populations is needed. Some studies suggest that mortality of anchovy larvae of the Mediterranean is inversely related to growth rates (Allain et al., 2003; Palomera et al., 2007; La Mesa et al., 2009), supported by the idea that larvae with slower growth rate remain as larvae longer ("stage-duration" mechanism, see Chambers and Leggett (1987)) and extends the exposure to predation. In this sense, we think that the "bigger is better" hypothesis, as described by Leggett and DeBlois (1994), is a paradigm that fits our results.

Takasuka et al. (2003) also found that larvae of Japanese anchovy with lower growth rates were proportionally more abundant within predators' stomachs than in the sea, inferring that they were more vulnerable to predation. If this holds true, anchovy larvae found in December 2007 would be more vulnerable to predation and have less probability to reach the adult stage. However, further studies regarding the biology of predators that feed on anchovy larvae should be carried out, in order to estimate how their feeding activity could affect the probability of mortality of anchovy larvae.

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# Ontogenetic and seasonal changes in the feeding habits and trophic levels of two small pelagic fish species

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ABSTRACT: We investigated ontogenetic and seasonal changes in the feeding ecology of 2 small pelagic fish species, the European anchovy *Engraulis encrasicolus* and the European pilchard *Sardina pilchardus* in the Gulf of Lions (NW Mediterranean). By analysing the stable isotopes $\delta^{13}C$ and $\delta^{15}N$, we determined the seasonal variation in the food sources and in the trophic level of these species, and we examined dietary shifts during development. The results of these investigations provided estimates of the diets of both species. We compared the values observed during different seasons (summer, autumn and winter) and at different developmental stages (late-larvae, juveniles and adults) for both species, together with the values of potential groups of prey (microplankton, cladocerans, copepods and appendicularians). Late-larvae preferred to feed on microplankton, although differences in the diet appeared after metamorphosis. Cladocerans were usually the preferred prey when available (summer), and appendicularians were the preferred prey in autumn. During the winter, the diets seemed to be more heterogeneous. Different feeding behaviours between the late-larvae of the 2 species were the most likely reason for the slightly different trophic levels found in the present study. This research demonstrates that studies with stable isotopes can furnish an alternative and/or complementary method for determining the diet of small pelagic fishes over extended periods and provides comprehensive knowledge of the functioning of the pelagic ecosystem.

KEY WORDS: *Engraulis encrasicolus* · *Sardina pilchardus* · Stable isotopes · NW Mediterranean · Trophic ecology · Microplankton · Copepods · Appendicularians · Cladocerans

## INTRODUCTION

An understanding of the primary ecological processes in marine ecosystems, such as variation in food sources, trophic transfer through the food web and nutrient cycling, is fundamental for relating ecosystem functioning to management. In this context, the description of the trophic ecology of marine organisms is vital to determine the intrinsic factors that control their distribution, abundance, and, ultimately, their function within the ecosystem. Fishes have developed a wide variety of feeding-related structures (e.g. gill rakers, pyloric caeca, or teeth) and behaviours (e.g. filter feeding vs. particulate feeding) that may undergo modifications through ontogeny or season (Gerking 1994), with diverse ecological implications. Many studies have addressed the trophic roles of both top and bottom species of the food web, i.e. marine predators and plankton communities, respectively (Hunter & Price 1992, Rice 1995, Pace et al. 1999), and, among these studies, some have focused on a group that is particularly important ecologically: the small pelagic fishes (Bakun 1996, Cury et al. 2000, Palomera et al. 2007).

Small pelagic fish are essential elements of marine ecosystems owing to their significant biomass at intermediate levels of the food web (Cury et al. 2000, Palomera et al. 2007). Pelagic fish play a significant

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role in connecting the lower and upper trophic levels (Cury et al. 2000). Their massive populations, which exert a huge pressure on zooplankton and, at the same time, are the main food for many species, may vary greatly in size under intensive exploitation or following changes in productivity. Therefore, fluctuations in small pelagic populations owing to fishing or natural factors modify the structure and functioning of the marine ecosystem (e.g. Cury et al. 2000, Shannon et al. 2000, Daskalov 2002). The significant abundance and success of pelagic fish in upwelling areas have been attributed to the flexibility of their feeding behaviour (van der Lingen et al. 2009). Pelagic fish have also been identified as important groups in many ecosystems (Libralato et al. 2006), e.g. in upwelling areas, where they exhibit a 'wasp-waist' flow control (Cury et al. 2000). Therefore, the interactions among different populations of small pelagic fishes can also be strongly influenced by regime climatic shifts and can have strong impacts on fisheries (e.g. Klyashtorin 1998, Rodríguez-Sanchez et al. 2002).

Low trophic-level species are directly influenced by the remarkable environmental differences between seasons (Calbet et al. 2001). The populations of small pelagic fish may be affected by any environmental change that influences the plankton community, which is the basis of the diet of these fishes. In fact, the seasonal unevenness of oceanographic parameters (i.e. salinity, fluorescence and, most importantly, temperature) and of river runoff has been shown to have important effects on the biology and viability of these fish populations (Lloret et al. 2001, 2004). The early life stages (i.e. larvae and juveniles) are especially sensitive to such effects (Govoni 2005, Ruiz et al. 2006, Costalago et al. 2011). Several species of small pelagic fish co-occur in the Mediterranean Sea. Unquestionably, the European anchovy *Engraulis encrasicolus* and the European sardine *Sardina pilchardus* are the most relevant of these species in terms of both biomass and fishery catches (Palomera et al. 2007). These 2 small pelagic fish are also key species at mid-trophic levels in the Mediterranean Sea (Coll & Libralato 2012). *E. encrasicolus* was found to be a key species in the North Adriatic (Coll et al. 2007, Barausse et al. 2009) and in the Aegean Sea (Tsagarakis et al. 2010). On the contrary, in the NW Mediterranean, *S. pilchardus* is among the leading keystone species and is also considered to exhibit 'wasp-waist' trophic control (Coll et al. 2006, Palomera et al. 2007, Navarro et al. 2011). Small pelagic fish are the most vulnerable to any environmental shift among fish species (Coll et al. 2008). At the same time, they are the main constituents of the diet of several pelagic, demersal and apical species (e.g. Coll et al. 2006, Palomera et al. 2007, Navarro et al. 2009).

A significant number of studies on the ecology of anchovy and sardine in the Mediterranean Sea have been conducted (see reviews by Palomera et al. 2007, Morello & Arneri 2009), but relatively few of these studies address dietary composition. Previous papers that included dietary information focussed on larval stages of *Engraulis encrasicolus* and *Sardina pilchardus* (Conway et al. 1998, Tudela et al. 2002, Catalán et al. 2010, Morote et al. 2010), the adults of *E. encrasicolus* (Tudela & Palomera 1997, Plounevez & Champalbert 2000), or the juveniles and adults of *E. encrasicolus* (Borme et al. 2009). However, none of the previous works, that, in addition, have usually been limited in temporal resolution, included data about sardines, except that of Morote et al. (2010), and they studied only larvae <1.6 cm in total length. A more comprehensive understanding of the trophic dynamics of assemblages of small pelagic fish from a seasonal and ontogenetic perspective is therefore essential to highlight the important role of these populations within the marine ecosystem. The present study intends to provide this necessary knowledge more comprehensively by gathering and analyzing data from different seasons and life stages of these 2 pelagic species for the first time in the Mediterranean.

All previous studies on the trophic ecology of these small pelagic fish species have been based on the direct analysis of stomach contents (Tudela & Palomera 1997, Conway et al. 1998, Plounevez & Champalbert 2000, Borme et al. 2009, Morote et al. 2010). This approach involves implicit methodological errors because it cannot accurately quantify the importance of prey items that are readily digested and because it does not identify the prey items that are actually assimilated following ingestion. The stable isotope approach can augment conventional means of dietary analysis because stable isotopes reflect time-integrated dietary records and present a perspective on trophic dynamics that involves a more substantial time period than the analysis of stomach contents can provide (Polunin & Pinnegar 2008). Therefore, stable isotope analysis yields information that cannot always be obtained from direct observation and can support hypotheses about the developmental changes in the feeding strategies of a species and about species interactions because these hypotheses are based on data about assimilated food rather than ingested food. In fish, the stable isotope values of muscular tissue integrate dietary information

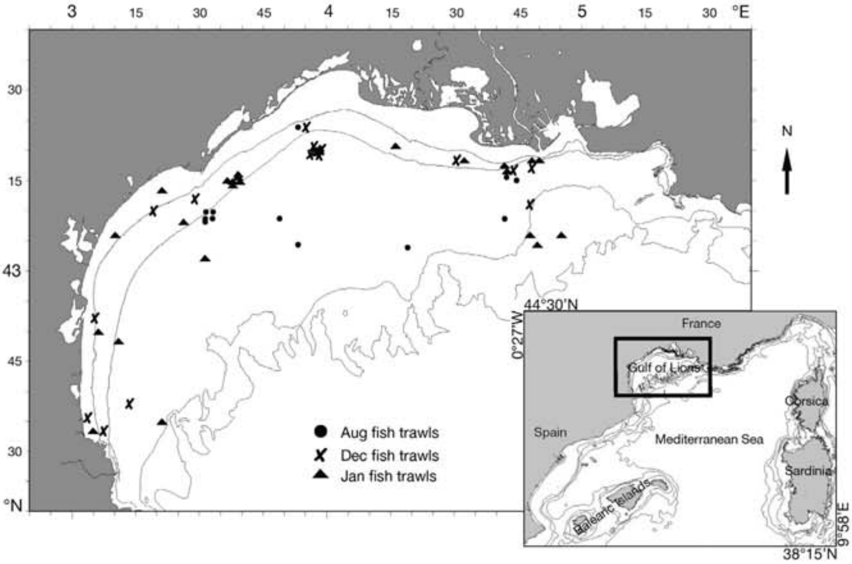

Fig. 1. Study area (Gulf of Lions, NW Mediterranean), indicating fish and plankton sampling locations. The bathymetric lines correspond to depths of 20, 50, 100 and 200 m

between 40 and 80 d prior to sampling (Bode et al. 2007, Buchheister & Latour 2010). Stable isotopes of nitrogen ($\delta^{15}N$) are indicators of trophic positions because consumers are predictably enriched in $\delta^{15}N$ relative to their food (Post 2002, Vanderklift & Ponsard 2003). Stable carbon isotope values ($\delta^{13}C$) give information on primary production and are useful for tracing the origin of the prey consumed (Vander Zanden et al. 1999, Pinnegar & Polunin 2000). Furthermore, by combining stable isotope values for consumers and their prey, powerful isotopic mixing models can be applied to obtain estimates of the relative contribution of each potential prey item to the diet of the consumer (e.g. stable isotope analysis in R [SIAR] isotopic mixing model; Parnell et al. 2010). These models add useful information to investigations of food selectivity and can be used to complement data from stomach content analysis (Peterson 1999, Lin et al. 2007, Tripp-Valdez & Arreguín-Sánchez 2009).

Flaherty & Ben-David (2010) pointed out that there are limitations in the use of isotopic mixing models because of the important differences in isotope values that can be found if there is spatial heterogeneity, so that habitat-derived variation in consumers' isotopes would be mistaken as diet variation in resource isotope values. Since populations of *Engraulis encrasicolus* and *Sardina pilchardus* in the Gulf of Lions are widespread and homogenously distributed over the whole of the continental shelf and therefore feeding on the same available resources, we could assume that this limitation would not be of great influence in our case.

The present study uses $\delta^{13}C$ and $\delta^{15}N$ values as indicators of the trophic relationships between *Engraulis encrasicolus* and *Sardina pilchardus* for different age groups (late-larvae, juveniles and adults) during different seasons (summer, autumn and winter) and therefore under different environmental conditions. The study investigates seasonal and ontogenetic changes in the food sources and trophic levels of these species, and it examines possible dietary shifts during development. These analyses yield estimates of the diet composition of both species. All of these findings are important because they can supply the knowledge needed to fill current information gaps existing in both experimental and direct observational studies and allow improved management of fish stocks.

## MATERIALS AND METHODS

### Study area and sample collection

The present study was conducted in the Gulf of Lions (Fig. 1), one of the most productive areas of the NW Mediterranean (Salat 1996). In terms of biomass, it is also the most important area of the Mediterranean for small pelagic fish species (Barangé et al. 2009). During 3 different oceanographic cruises on board the RV 'L'Europe' (IFREMER, France), we collected late-larvae, juveniles and adults of *Engraulis encrasicolus* and *Sardina pilchardus*. The standard length ranges considered to classify the individuals within these groups were: for late-larvae 2.0–3.5 cm for *E. encrasicolus* and 2.5–4.0 cm for *S. pilchardus*, for juveniles 3.6–8.5 cm for *E. encrasicolus* and 4.1–10.5 cm for *S. pilchardus* and for adults 8.6–12 cm for *E. encrasicolus* and 10.6–14 cm for *S. pilchardus*. All individuals were considered as adults when they reached the minimum length at first maturity observed during the cruises. The first cruise was conducted during autumn (12 to 21 December 2007), the second cruise was conducted during summer (21 to 29 July 2008) and the third was conducted during winter (11 to 27 January 2009). All specimens were caught with a pelagic trawling net equipped with a small-mesh codend (mesh length: 5 mm; ISO 1107) and towed at an average speed of 3.6 knots over a 30 to 40 min period. The samples were immediately frozen (−20°C) after sorting on board.

During each season, plankton samples were collected at the same sites where the pelagic trawls were made. Sampling was done at 16 plankton stations in summer, 15 in autumn and 13 in winter using a standard WP2 net with a mesh size of 200 µm and a scaled-down WP2 net with a mesh size of 53 µm. The WP2 net sample was sieved through a 3000 µm plankton mesh to obtain the 200 to 3000 µm mesozooplankton fraction and the scaled-down WP2 net was sieved through a 200 µm plankton mesh to obtain the 53 to 200 µm microplankton fraction. All plankton samples were split with a Motoda plankton splitter (Motoda 1959). One-half of each sample was preserved in buffered 4% formaldehyde–seawater solution for subsequent qualitative analyses of plankton community composition, whereas the other half was frozen (−20°C) on board for biomass measurements and stable isotopic determination. Qualitative analysis of plankton was performed in the laboratory, and individuals were identified to the lowest taxonomical level possible under a stereomicroscope (Leica MZ12) with a magnification of up to 100×. The mesozooplankton samples were analysed in aliquots representing about 10% of the sample and repeated until counting at least 400 copepods in each; additional subsamples were also taken for any other abundant organism (i.e. cladocerans during summer). Microplankton samples were subsampled differently: 1 to 2% of the original volume was analysed to estimate the presence of nauplii, dinoflagellates, ciliates and diatoms; small copepods (mainly copepodites) were analysed in volumes sufficient to count at least 400 individuals. The number of individuals of each identified taxon and abundances (ind. m$^{-3}$) were calculated.

### Stable isotope analysis

A portion of dorsal muscle (without skin) was extracted from each individual (late-larvae, juveniles and adults). Muscle has been defined as the most appropriate tissue to analyse stable isotope in fish (Sweeting et al. 2005). Plankton samples from each season were defrosted in the laboratory, pooled together and sorted into different potential prey groups (microplankton, copepods and appendicularians in all the seasons, and also cladocerans in summer) which were selected according to previous studies of stomach contents (Morote et al. 2010 for larvae, D. Costalago unpubl. data for juveniles, Plounevez & Champalbert 2000 for adults).

All fish and plankton samples were freeze-dried, powdered, and 0.9 to 1.0 mg of each sample was packed into tin capsules. The samples were then oxidised with CuO and $CO_3O_4$/Ag at approximately 900°C in a Flash EA 1112 Elemental Analyser coupled with a pyrolyser TC-EA and a gas bench through a Conflo III Finnigan MAT interface. $NO_x$ was reduced with Cu at 680°C. The combustion products $N_2$ and $CO_2$ were introduced into a Delta C Finnigan MAT mass spectrometer through an $MgClO_4$ drying column. The isotope ratio mass spectrometry facility at the Serveis Científico-Tècnics of the University of Barcelona (Spain) applies international standards, generally run for every 12 samples: IAEA CH7 (87% of C), IAEA CH6 (42% of C) and USGS 24 (100% of C) for $\delta^{13}C$ and IAEA N1 and IAEA N2 (with 21% of N) and IAEA $NO_3$ (13.8% of N) for $\delta^{15}N$.

The $\delta^{13}C$ values were corrected for the effect of lipids both in fish and prey samples following Logan et al. (2008). This procedure reduces the time and uncertainty associated with lipid extraction procedures, and it improves the estimates of dietary proportions derived from stable-isotope mixing models (Phillips & Gregg 2001, Logan et al. 2008).

### Isotopic mixing model

To estimate the diet composition at each age (late-larvae, juveniles and adults) during each season (summer, autumn and winter) we applied a Bayesian model in SIAR 4.1.1 (Stable Isotope Analysis in R 2.12.2). This model runs under the free software R (R Development Core Team 2009). The model allows the inclusion of sources of uncertainty. In particular, the variability in the isotope signatures (mean and standard deviation) of prey species can be incorporated into the model (Parnell et al. 2010). SIAR uses Markov-chain Monte Carlo modelling, takes data on animal stable isotopes and fits a Bayesian model of the diet habits based on a Gaussian likelihood function with a Dirichlet prior mixture distribution for the mean.

The model also assumes that each target value (i.e. the stable isotope data for each individual) comes from a Gaussian distribution with an unknown mean and standard deviation. The structure of the mean is a weighted combination of the food sources' isotopic values. The standard deviation depends on the uncertainty in the fractionation corrections and the natural variability among target individuals within a defined group. We used the isotopic discrimination of 1.01 ± 0.17‰ for C and 3.56 ± 0.17‰ for N as the average of discrimination factors estimated for muscle analyses of different marine fish species provided in Caut et al. (2009).

### Trophic level

To estimate the trophic levels (TL) of the different individuals we used the equation:

$$TL_{consumer} = TL_{basal} + (\delta^{15}N_{consumer} - \delta^{15}N_{prey})/\Delta\delta^{15}N \quad (1)$$

$TL_{consumer}$ refers to the mean TL of each individual fish. $\delta^{15}N_{prey}$ and $\delta^{15}N_{consumer}$ were, respectively, the isotopic values of microplankton and individual fish obtained in the present study (in each season). We applied a basal trophic level ($TL_{basal}$) of 1.5, assuming that the microplankton (mostly composed by phytoplankton) showed a trophic level between 1 of primary producers and 2 of micro- and mesozooplankton (Coll et al. 2006). The values of the isotopic discrimination factor for N ($\Delta\delta^{15}N$) were the same used for the isotopic mixing model (estimated from Caut et al. 2009).

### Selectivity index

The output data of the SIAR model, together with the relative composition of the functional groups of plankton in the environment, were used to estimate the Ivlev diet selectivity index (Ivlev 1961, Krebs 1989) for each case analyzed. The value of the index was calculated with the equation:

$$(r_i - p_i) / (r_i + p_i) \quad (2)$$

where $r_i$ is the proportion of prey item $i$ calculated from the SIAR model and $p_i$ is the proportion of prey item $i$ available from the marine environment.

### Statistical analysis

ANOVA tests were used to examine the differences in both $\delta^{13}C$ and $\delta^{15}N$ values among species and ages in each season (summer, autumn and winter). Post hoc comparisons for observed means were performed with a Tukey test. The assumptions of ANOVA were checked with a Kolmogorov-Smirnov test for normality and a Levene test for homogeneity of variances. All analyses were performed with SPSS v.19 statistical software. A significance level of $p < 0.05$ was used for all tests unless otherwise stated.

## RESULTS

### Plankton composition

The microplankton samples were composed primarily of phytoplankton (mainly diatoms; to a lesser extent, dinoflagellates, such as *Ceratium* spp. and *Peridinium* spp.; and tintinnids), together with high numbers of copepod nauplii during the summer and winter and small copepods, especially *Oncaea* spp., throughout the year and, during the winter, *Paracalanus parvus*. Mesozooplankton was dominated by copepods (mainly calanoids). The plankton community also included a relatively less important number of appendicularians during the autumn and winter (Fig. 2). Cladocerans occurred during summer but not during other seasons (Fig. 2).

### Isotopic differences

*Sardina pilchardus* showed lower $\delta^{15}N$ values in summer and the highest $\delta^{15}N$ values in autumn, except for late-larvae (winter), which had significantly lower $\delta^{15}N$ values than in any other age group and season (Table 1, Fig. 3). Regarding *S. pilchardus* $\delta^{13}C$ values (juveniles and adults), there were no significant differences ($p = 0.25$) between autumn and winter seasons (Table 2), while summer $\delta^{13}C$ val-

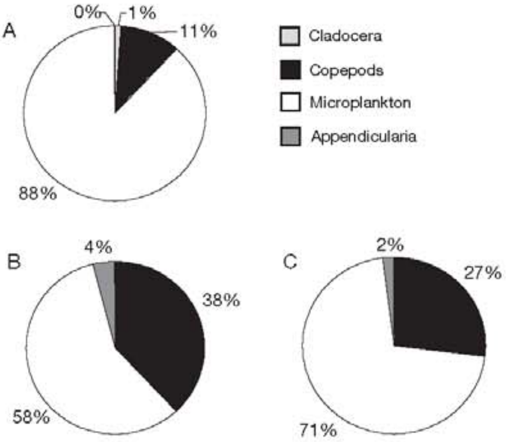

Fig. 2. Composition of plankton community in the Gulf of Lions during (A) summer, (B) autumn and (C) winter showing proportions of the 4 dietary functional groups defined in the present study

ues were statistically different from the other 2 seasons (Tables 1 & 2, Fig. 3). *S. pilchardus* late-larvae (winter) also showed significantly lower values of $\delta^{13}C$ compared to adults and juveniles (Table 2). *S. pilchardus* values of $\delta^{13}C$ and $\delta^{15}N$ did not differ in autumn between adults and juveniles ($p = 0.870$ for $\delta^{15}N$, $p = 0.213$ for $\delta^{13}C$; Table 1). For both *S. pilchardus* age groups (juveniles and adults) analysed together, $\delta^{13}C$ appeared to differ between the summer and other seasons, and $\delta^{15}N$ differed between winter and the other seasons (Table 1, Fig. 3).

*Engraulis encrasicolus* $\delta^{15}N$ isotopic values in both autumn and winter displayed marked differences between adults and juveniles (Table 1, Fig. 3). *E. encrasicolus* late-larvae (summer) had significantly higher values of $\delta^{15}N$ than juveniles (Table 1, Fig. 3). No differences in $\delta^{15}N$ values were observed among seasons for adult *E. encrasicolus*, whereas $\delta^{15}N$ values were statistically different for juveniles during autumn than in other seasons. When adults and juveniles were considered together, significant differences in $\delta^{15}N$ values were found among the 3 periods (Table 1, Fig. 3). *E. encrasicolus* $\delta^{13}C$ values exhibited substantial differences during the summer for both juveniles and adults. *E. encrasicolus* latelarvae (summer) had higher $\delta^{13}C$ values than adults.

The comparisons between the 2 species showed that, during the summer, the $\delta^{15}N$ values of *Sardina pilchardus* and *Engraulis encrasicolus* adults differed significantly. During the autumn, only differences between adults' $\delta^{15}N$ values were significant between species. During the winter, however, both the adults and juveniles exhibited significant species differences in $\delta^{15}N$ values. The $\delta^{13}C$ values of the juveniles also differed between the species in winter and summer (Tables 1 & 2, Fig. 3). A comparison of the late-larvae (*E. encrasicolus* in summer and *S. pilchardus* in winter) found statistically significant differences in the values of both $\delta^{13}C$ and $\delta^{15}N$ (Table 2).

Concerning types of prey, appendicularians showed higher $\delta^{15}N$ values than other types in all seasons (Table 3, Fig. 3). Excluding summer, microplankton presented the lowest $\delta^{15}N$ values. The $\delta^{13}C$ values are generally variable between seasons (Fig. 3).

Table 1. *Engraulis encrasicolus*, *Sardina pilchardus*. Sample size, mean ± SD of standard length (SL), stable isotope values ($\delta^{15}N$ and $\delta^{13}C$) and trophic level (TL) of different age groups of *E. encrasicolus* and *S. pilchardus* (adult, juvenile and late-larva) during summer, autumn and winter in the Gulf of Lions

| | n | SL (cm) | $\delta^{13}C$ (‰) | $\delta^{15}N$ (‰) | TL |
|---|---|---|---|---|---|
| ***E. encrasicolus*** | | | | | |
| **Summer** | | | | | |
| Adult | 15 | 11.57 ± 0.44 | −19.56 ± 0.16 | 8.23 ± 0.34 | 2.42 ± 0.09 |
| Juvenile | 15 | 6.37 ± 0.85 | −18.26 ± 0.41 | 7.78 ± 0.41 | 2.27 ± 0.12 |
| Late-larva | 15 | 2.15 ± 0.16 | −18.38 ± 0.11 | 8.14 ± 0.32 | 2.39 ± 0.09 |
| **Autumn** | | | | | |
| Adult | 14 | 10.58 ± 0.18 | −17.83 ± 0.32 | 8.16 ± 0.48 | 2.90 ± 0.13 |
| Juvenile | 15 | 8.11 ± 0.49 | −17.67 ± 0.29 | 8.53 ± 0.45 | 3.05 ± 0.12 |
| **Winter** | | | | | |
| Adult | 7 | 8.80 ± 0.23 | −17.42 ± 0.22 | 8.07 ± 0 33 | 2.63 ± 0.09 |
| Juvenile | 15 | 7.57 ± 0.17 | −17.38 ± 0.24 | 7.54 ± 0.53 | 2.45 ± 0.15 |
| ***S. pilchardus*** | | | | | |
| **Summer** | | | | | |
| Adult | 17 | 13.62 ± 0.43 | −19.32 ± 1.16 | 8.52 ± 0.43 | 2.53 ± 0.12 |
| Juvenile | 15 | 7.02 ± 0.20 | −17.51 ± 0.21 | 7.97 ± 0.29 | 2.33 ± 0.08 |
| **Autumn** | | | | | |
| Adult | 15 | 12.91 ± 0.87 | −17.68 ± 0.71 | 8.91 ± 0.48 | 3.22 ± 0.13 |
| Juvenile | 15 | 9.11 ± 0.93 | −17.97 ± 0.52 | 8.95 ± 0.84 | 3.24 ± 0.23 |
| **Winter** | | | | | |
| Adult | 15 | 11.35 ± 0.91 | −17.64 ± 0.37 | 8.69 ± 0.41 | 2.87 ± 0.12 |
| Juvenile | 14 | 9.74 ± 0.52 | −17.77 ± 0.41 | 8.88 ± 0.53 | 2.95 ± 0.14 |
| Late-larva | 15 | 3.12 ± 0.11 | −18.10 ± 0.21 | 6.74 ± 0.42 | 2.19 ± 0.12 |

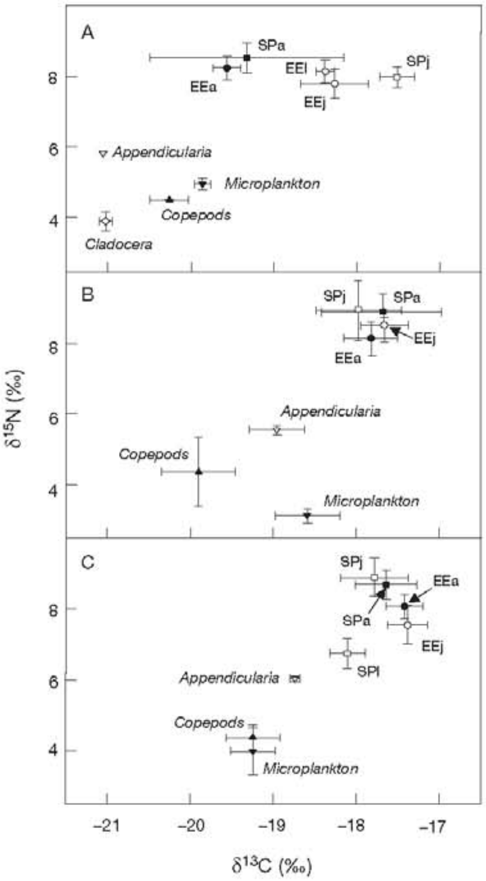

Fig. 3. *Engraulis encrasicolus, Sardina pilchardus.* $\delta^{15}N$ and $\delta^{13}C$ values (mean ± SD) of *E. encrasicolus* late-larvae (EEl), juveniles (EEj) and adults (EEa), and *S. pilchardus* late-larvae (SPl), juveniles (SPj) and adults (SPa) during (A) summer, (B) autumn and (C) winter. Reference values for the main prey groups (see Fig. 2) in each season are also given

### Dietary differences

The diets estimated with the SIAR model (isotopic values in Tables 1 & 3) indicated that during the summer, *Engraulis encrasicolus* adults fed on cladocerans (37.8%) and appendicularians (24.7%), whereas juveniles fed primarily on cladocerans (33.8%) and copepods (35.5%). The juveniles also fed on microplankton (27.1%). The late-larvae fed primarily on microplankton (50.2%) and also on copepods (35%) (Fig. 4). During the summer, *Sardina pilchardus* adults had a heterogeneous diet, with appendicularians (29.2%) as the main prey. The diet of *S. pilchardus* juveniles was based on cladocerans

Table 2. Summary of the ANOVA results for inter-seasonal (summer, autumn and winter) variation in stable isotopes over species and age of *Engraulis encrasicolus* and *Sardina pilchardus* in the Gulf of Lions. Abbreviations for age combinations in Tukey post hoc test summaries are—L: late-larvae; J: juvenile; A: adults; pairs of means differing significantly ($p = 0.05$) by Tukey test are linked with an 'x'

| | Effect | *F* | df | p | Post hoc |
|---|---|---|---|---|---|
| **Summer** | | | | | |
| $\delta^{15}N$ | Species | 6.55 | 1, 75 | <0.001 | |
| | Age | 14.83 | 1, 75 | <0.001 | AxL |
| | Species × Age | 0.25 | 2, 75 | 0.62 | |
| $\delta^{13}C$ | Species | 10.87 | 1, 75 | 0.002 | |
| | Age | 57.54 | 2, 75 | <0.001 | AxL, JxL, A |
| | Species × Age | 2.89 | 1, 75 | 0.11 | |
| **Autumn** | | | | | |
| $\delta^{15}N$ | Species | 14.71 | 1, 58 | <0.001 | |
| | Age | 1.85 | 1, 58 | 0.18 | |
| | Species × Age | 1.19 | 1, 58 | 0.28 | |
| $\delta^{13}C$ | Species | 0.38 | 1, 58 | 0.54 | |
| | Age | 0.27 | 1, 58 | 0.61 | |
| | Species × Age | 2.98 | 1, 58 | 0.89 | |
| **Winter** | | | | | |
| $\delta^{15}N$ | Species | 51.58 | 1, 65 | <0.001 | |
| | Age | 96.81 | 2, 65 | <0.001 | LxJ, A |
| | Species × Age | 7.03 | 1, 65 | 0.07 | |
| $\delta^{13}C$ | Species | 11.45 | 1, 65 | <0.001 | |
| | Age | 8.29 | 2, 65 | <0.001 | LxJ, A |
| | Species × Age | 0.93 | 1, 65 | 0.34 | |

Table 3. Sample size (n), mean ± SD of stable isotope values ($\delta^{15}N$ and $\delta^{13}C$) of prey groups during summer, autumn and winter in the Gulf of Lions

| | n | $\delta^{13}C$ (‰) | $\delta^{15}N$ (‰) |
|---|---|---|---|
| **Summer** | | | |
| Cladocera | 3 | −21.03 ± 0.08 | 3.90 ± 0.27 |
| Copepods | 3 | −20.26 ± 0.23 | 4.50 ± 0.01 |
| Microplankton | 3 | −19.85 ± 0.09 | 4.95 ± 0.17 |
| Appendicularia | 1 | −21.06 | 5.82 |
| **Autumn** | | | |
| Copepods | 3 | −19.90 ± 0.44 | 4.38 ± 0.97 |
| Microplankton | 4 | −18.59 ± 0.38 | 3.13 ± 0.20 |
| Appendicularia | 2 | −18.96 ± 0.33 | 5.55 ± 0.12 |
| **Winter** | | | |
| Copepods | 3 | −19.24 ± 0.32 | 4.37 ± 0.36 |
| Microplankton | 5 | −19.24 ± 0.27 | 3.98 ± 0.67 |
| Appendicularia | 3 | −18.74 ± 0.06 | 6.03 ± 0.08 |

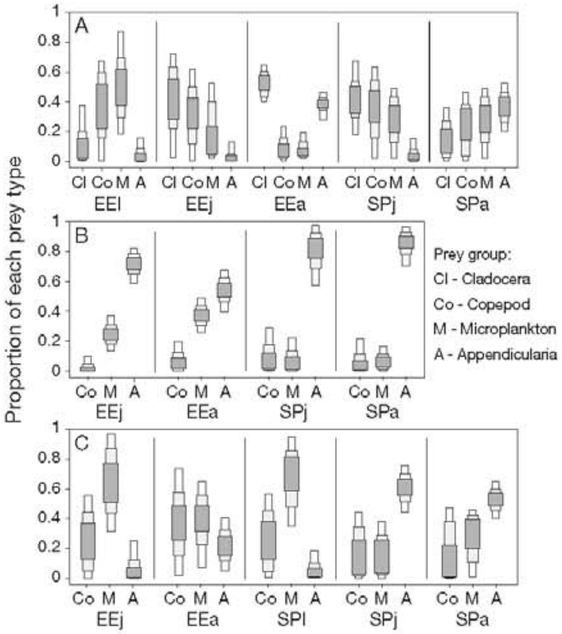

Fig. 4. *Engraulis encrasicolus*, *Sardina pilchardus*. Results of stable isotope analysis in R (95, 75 and 50% credibility intervals) showing estimated prey contributions to the diet of *E. encrasicolus* late-larvae (EEl), juveniles (EEj) and adults (EEa), and *S. pilchardus* late-larvae (SPl), juveniles (SPj) and adults (SPa) from the Gulf of Lions (northwestern Mediterranean) during (A) summer, (B) autumn and (C) winter

(36.8 %), copepods (33.5 %) and microplankton (25.1 %) (Fig. 4).

During the autumn, the diets of adults were similar to the diets of juveniles for both *Sardina pilchardus* and *Engraulis encrasicolus*. The main prey types for *S. pilchardus* were appendicularians (88 % in adults and 82.8 % in juveniles). Appendicularians were also the primary prey item for *E. encrasicolus* (59.4 % in adults and 75.4 % in juveniles) (Fig. 4).

*Sardina pilchardus* adults and juveniles in winter also showed a diet based on appendicularians (49 % in adults and 58.1 % in juveniles), but the primary prey item of late-larvae was microplankton (56.1 %). In the same season *Engraulis encrasicolus* juveniles fed primarily on microplankton (69 %), whereas adult *E. encrasicolus* fed primarily on copepods and appendicularians in similar amounts (40.7 and 40.8 %, respectively) (Fig. 4).

### Prey selectivity

Ivlev's selectivity index showed that during the summer, both cladocerans and appendicularians were the most heavily selected prey (Fig. 5). Positive selection was also exhibited for copepods, while microplankton was negatively selected in all cases (Fig. 5). During autumn, appendicularians were generally preferred. An apparent neutral selection was shown for copepods, and negative selection was shown for microplankton (Fig. 5). During the winter, copepods, followed by appendicularians, were highly selected in nearly all cases. The only exception was *Sardina pilchardus* late-larvae, which did not select appendicularians. Microplankton was not positively selected in any case (Fig. 5).

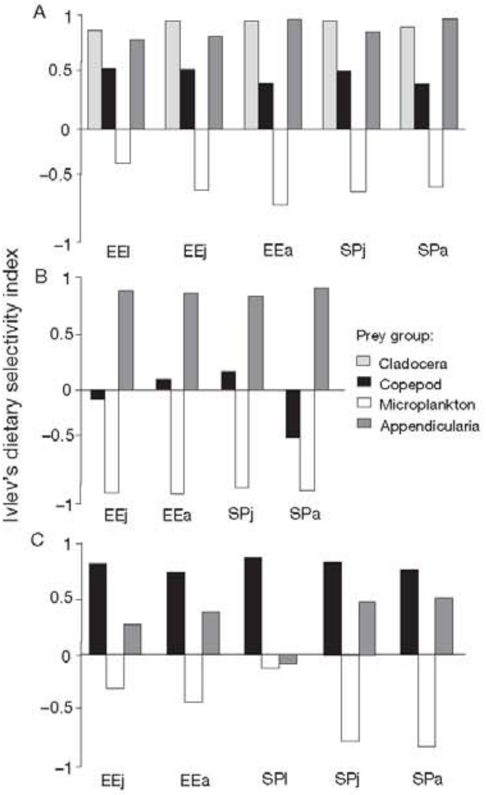

Fig. 5. *Engraulis encrasicolus*, *Sardina pilchardus*. Ivlev's dietary selectivity index for *E. encrasicolus* late-larvae (EEl), juveniles (EEj) and adults (EEa), and *S. pilchardus* late-larvae (SPl), juveniles (SPj) and adults (SPa) during (A) summer, (B) autumn and (C) winter

## DISCUSSION

Our study determined the trophic dynamics of anchovy *Engraulis encrasicolus* and sardine *Sardina pilchardus* during different seasons and at different life stages. The results of the study emphasised the feeding plasticity of these species in the Gulf of Lions, as already observed in the Adriatic Sea (Borme et al. 2009). The results showed that the late-larvae of both species feed more abundantly on microplankton than on any other prey and that a remarkable difference in the diet occurred after metamorphosis. If cladocerans were available (i.e. during the summer), they were usually the preferred prey. The high selectivity for appendicularians in autumn seemed to explain the high trophic levels found for this season because appendicularians are the prey group with the highest $\delta^{15}N$ values. These high $\delta^{15}N$ values of appendicularians could be due to the retention of zooplankton organisms of high $\delta^{15}N$ values, such as carnivorous copepods or even small larvae, on their houses (Deibel & Lee 1992); this is in accordance with the data of Hobson et al. (2002), who obtained a higher value of stable-nitrogen isotope and a higher trophic level in appendicularians than in mixed zooplankton samples.

The difference in trophic levels between *Engraulis encrasicolus* and *Sardina pilchardus* late-larvae resulted from their distinct feeding behaviour. *E. encrasicolus* late-larvae, which showed values of $\delta^{15}N$ similar to those in the other stages, generally fed on copepods and microplankton, a diet similar to that of *S. pilchardus* late-larvae. However, despite the high proportion of these types of prey in the sea during summer, *E. encrasicolus* late-larvae have been found to feed preferentially on appendicularians and cladocerans if these food items are available. Morote et al. (2010) found that *E. encrasicolus* larvae <15 mm standard length (SL) fed primarily on copepods and cladocerans (see also Tudela et al. 2002), whereas *S. pilchardus* ate a relatively high number of protists along with copepod nauplii. Other studies of *S. pilchardus* larvae >13 mm SL reported a diet based on phytoplankton (Rasoanarivo et al. 1991). Given the mean SL of the larvae examined in the current study, it is probable that they did not retain the isotopic signal from parental feeding activity (Pepin & Dower 2007). In view of these considerations and the results cited, it seems reasonable to suppose that *S. pilchardus* late-larvae, whose diet is more herbivorous, would exhibit a lower trophic level than that of *E. encrasicolus* late-larvae. This difference is even clearer from the selectivity indices, which indicate that *E. encrasicolus* late-larvae tend to feed on prey with higher $\delta^{15}N$ values rather than on the microplankton that represent a primary constituent of the diet of *S. pilchardus* late-larvae.

The observed differences between ages during the winter and summer for *Engraulis encrasicolus* and *Sardina pilchardus* reflected a change in the diet across the ontogenetic development of the fishes. We did not find differences during autumn, because no late-larvae of any species were collected during that period. These findings suggested the hypothesis that the diet shift occurred primarily at the time of metamorphosis (Lindsay et al. 1998), whereas juveniles and adults maintained similar diets. However, *E. encrasicolus* during summer exhibited clear differences between juveniles and the other 2 stages. Lindsay et al. (1998) also found a drastic change in $\delta^{15}N$ in Japanese anchovy *E. japonicus* as individuals grew from 15 to 30 mm SL and another change between 30 and 70 mm SL. These size ranges are almost coincident with the sizes of the juveniles in our study (6.37 ± 0.85 cm of SL). Therefore, dietary changes may also occur after metamorphosis.

The most frequently cited explanation for the ontogenetic dietary shift refers to the development of the feeding apparatus of the fishes (June & Carlson 1971, King & Macleod 1976, MacNeill & Brandt 1990, Gerking 1994). According to these authors, larvae become able to filter-feed when the development of their gill rakers is complete. Thus, differences in the diet are related to the minimum prey sizes that are efficiently retained by the feeding apparatus.

In contrast, Tanaka et al. (2006) analysed the stomach contents and gill-raker morphology of 3 species of planktivorous pelagic fishes. These authors found that the differences in the diets of these species were explained by differences in feeding behaviour (filter-feeding vs. particulate feeding) rather than by differences in morphology. This conclusion supports our results for juveniles and adults of *E. encrasicolus* during summer. These fish, caught at different locations and different times, are considered to have a fully developed filtering apparatus. Consequently, the differences in the diet indicated here by stable isotopes could depend on shifts in feeding habits mediated by food density. For example, the fish could shift between filter-feeding and particulate feeding, depending on the concentrations of different prey items (Bulgakova 1996).

Previous studies based on stomach contents analysis argued that *Engraulis encrasicolus* in the Gulf of Lion is mainly zooplanktivorous (Tudela & Palomera 1997, Plounevez & Champalbert 2000, Morote et al.

2010, Costalago et al. 2011), whereas *Sardina pilchardus* also feeds significantly on microheterotrophs (Rasoanarivo et al. 1991, Morote et al. 2010). These observations, together with our results, led us to hypothesise that both *E. encrasicolus* and *S. pilchardus* are omnivorous all through their life cycles. However, our results demonstrated a slightly higher trophic level for *S. pilchardus* in all seasons and stages, except late-larvae, a similar pattern to that described for the Atlantic coast of the Iberian Peninsula (Bode et al. 2007). This discrepancy was explained by van der Lingen (1998) and Bode et al. (2006), who demonstrated that sardines primarily obtain protein nitrogen from zooplankton rather than from phytoplankton. This argument is also supported by the observation that herbivores generally have higher $\delta^{15}N$ variability (Mill et al. 2007). Moreover, the differences between the present study and those by Coll et al. (2006) and Navarro et al. (2011), both focused on the Catalan Sea, could be considered a consequence of the diet data used by those authors. In particular, they based their results on anchovy diet data reported by Tudela & Palomera (1997) and on sardine diet data from the eastern Mediterranean (Demirhindi 1961), resulting in a higher trophic level in *E. encrasicolus* than in sardine (3.05 and 2.97, respectively). Alternatively, some differences in the diets of *E. encrasicolus* and *S. pilchardus* between areas may also have influenced these results.

A comparison of the trophic levels found in our study with values from upwelling areas shows that adults of both anchovy and sardine in the Gulf of Lions normally exhibited trophic levels similar to the trophic levels found for homologous species in regions with upwelling. Bode et al. (2007) found trophic levels of 3.5 for *Sardina pilchardus* and of 3.4 for *Engraulis encrasicolus* for the northern Iberian Atlantic coast. Miller et al. (2010) found trophic levels of 2.9 for *S. sagax* and of 3.1 for *E. mordax* for the California Current. Moreover, the trophic level of 2.9 derived for *S. sagax* in the southern Benguela Current is lower than the value for *S. pilchardus* in the Gulf of Lions (van der Lingen & Miller 2011). In this context, Miller et al. (2011) showed that fish from less productive areas exhibited relatively higher trophic levels than those from more productive areas. Therefore, the food web of areas with relatively low average primary production, like the Gulf of Lions, is more linear than the food web in zones with upwelling (e.g. the Galician coast, where the trophic structure is more intricate and ramified) (Agostini & Bakun 2002). A more linear food web implies that individuals, in this case small pelagic fish in the Mediterranean sea, are more dependent on the adjacent lower trophic level than those small pelagic fish from more highly productive areas (Miller et al. 2011), a pattern that could be interpreted as a bottom-up ecosystem structure in the Mediterranean. However, in view of the high biomass of the mid-trophic level small pelagic fish in the northwestern Mediterranean, this structure is actually closer to a 'wasp-waist' system, as found in the Adriatic Sea (Coll et al. 2007).

The trophic dynamics of zooplankton and small pelagic fish occupy the most significant position within marine pelagic food webs (Shannon et al. 2009). In the Gulf of Lions, a substantial amount of seasonal variability affecting the lower trophic levels has been widely reported (Molinero et al. 2005). These temporal fluctuations reflect an important feature of the area—the effect of the Rhone River. The Rhone is the primary source of the runoff entering the Mediterranean. The mean annual flow of the river is 1700 $m^3 s^{-1}$, and its catchment area is 98 000 $km^2$ (Darnaude et al. 2004). In fact, correlations between river discharge and marine pelagic fish abundance in the northwestern Mediterranean have been extensively studied (García & Palomera 1996, Lloret et al. 2001, 2004) and are generally explained by the enhancement of planktonic production produced by the input of nutrients from rivers. We found significant differences in the $\delta^{13}C$ signatures of fishes among seasons in all cases. These results demonstrate the importance of seasonal variability for the structure of the food web.

Lindsay et al. (1998) also found that, in central Japan, rivers may supply the coastal trophic web with different $\delta^{15}N$ signatures depending on the season. In agreement with this result, our study showed that this heavy nitrogen isotope was generally more abundant in both food sources and fish samples during autumn. The difference may reflect the higher amount of rainfall during autumn in this region. Moreover, Odum (1985) suggested that the augmented contributions of flows to detritus could serve as a marker of disruption in energy transport from lower to higher trophic levels, conferring higher signature to $\delta^{15}N$ than expected with a less direct transmission to predators. With this in mind, it can be argued that nutrients derived from the Rhone River in autumn tend to have heavier N. Correspondingly, we showed that $\delta^{15}N$ values in summer were generally lower for the 2 fish species than in the other 2 studied seasons, probably because summer is the driest season and the river discharges are lower.

In conclusion, the present study shows that adults of *Engraulis encrasicolus* and *Sardina pilchardus* generally prey over larger plankton than juveniles and late-larvae. We also illustrate the importance of appendicularians in the diet of both species, especially when cladocerans are not available, and prove that stable isotope analysis is an essential tool for complement dietary studies based on direct observations of stomach contents, because appendicularians are likely easily digested (Capitanio et al. 2005) and could have been underestimated in some cases (Capitanio et al. 1997, D. Costalago unpubl. data). Moreover, based on our output data from the SIAR model and the data we gathered from the plankton samples, we also showed an innovative manner of calculating dietary selectivity through Ivlev's selectivity index. To our knowledge, this approach has never previously been attempted, and we believe that, with limitations and future improvements, it can be useful for drawing a more comprehensive and accurate picture of trophic dynamics.

Assessing the isotopic values of different types of prey in different seasons is therefore essential in understanding whether isotopic seasonal variations of pelagic fish are caused by changes in diet or by variations of basal isotopic levels due to environmental oscillations. Anthropogenic alterations in the ecosystem, such as overfishing, eutrophication, or climate change often entail alterations in the trophic structure of the communities; therefore, the above-discussed findings may be useful in future management scenarios that would take into account an ecosystem approach to fisheries in the northwestern Mediterranean.

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Este libro no empezó en el año 2007. Este libro no trata sobre peces pelágicos, ni sobre ecología marina. Este libro no sirve para hacer que la pesca de anchoa y sardina sea sostenible. Este libro no me convertirá en Doctor.

Este libro, sin embargo, contiene todo aquello que quieran encontrar en él las personas que lo han hecho posible. Y han sido muchas, como muchos sus ánimos, sus esfuerzos y sus impuestos.

Este trabajo es vuestro trabajo, es por y para vosotras y vosotros.

Pero sobre todo, esta tesis es para mi padre, mi madre y mi hermana.