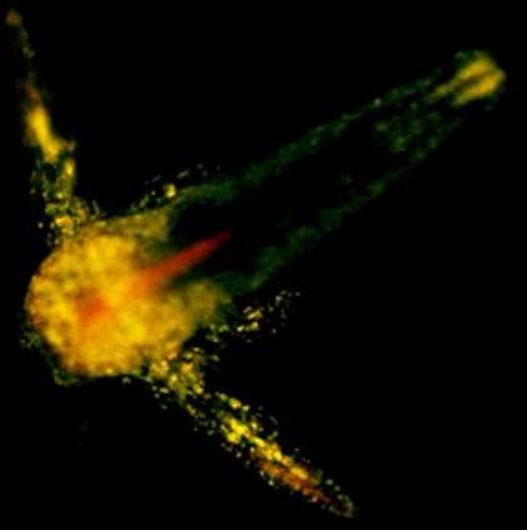
VNIVERSITAT DE VALÈNCIA Facultad de Ciencias Biológicas

Instituto de Acuicultura de Torre de la Sal (CSIC)



Optimización del enriquecimiento de nauplios de *Artemia* mediante el uso de emulsiones lipídicas formuladas a partir de aceites sintéticos ricos en DHA

Elena Viciano Delibano ~ *Tesis Doctoral*

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CSIC IATS INSTITUTO DE ACUICULTURA DE TORRE DE LA SAL

Optimización del enriquecimiento de nauplios de *Artemia* mediante el uso de emulsiones lipídicas formuladas a partir de aceites sintéticos ricos en DHA

Memoria presentada por Elena Viciano Delibano para optar al grado de Doctora en Ciencias Biológicas

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A mis padres

A Manu

"Todo parece imposible hasta que se hace"

Nelson Mandela

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Introducción general

1.1. Estado actual de la acuicultura

La Organización Mundial para la Agricultura y la Alimentación (*Food and Agriculture Organization of the United Nations*, FAO) y la Comisión Europea definen la acuicultura como "el cultivo de organismos acuáticos, incluyendo peces, moluscos, crustáceos y plantas acuáticas, en aguas continentales y costeras, que implica la intervención del hombre en el proceso de cría para aumentar la producción en operaciones como la siembra, la alimentación, la protección contra depredadores, etc.".

La acuicultura a pequeña escala ha existido desde tiempos antiguos en varios países. Estos orígenes están documentados desde el 2500 A.C., pero no es hasta mediados del siglo XX cuando se inicia una evolución hacia la producción industrial a gran escala. En el transcurso de medio siglo la acuicultura ha pasado de ser prácticamente insignificante a equipararse con la producción de pesca de captura. Este sector ha tenido que evolucionar, incluir innovaciones técnicas y adaptarse para satisfacer las necesidades de la población. Debido al crecimiento demográfico de estos últimos siglos, se ha observado una sobreexplotación de los recursos pesqueros, con lo que el desarrollo de la acuicultura a gran escala juega un papel vital en la sociedad actual. La FAO (2014) estima que más del 89% de las especies capturadas con valor comercial están en situación de

sobreexplotación, lo que ha provocado el estancamiento del sector pesquero. Estas poblaciones sobreexplotadas necesitan planes de ordenación rigurosos para restablecer la abundancia y recuperar una productividad plena y biológicamente sostenible. La FAO (2014) estima que en el 2030, el 65% de los animales acuáticos procederán de la acuicultura.

Hoy en día, la acuicultura es una importante actividad económica de producción de alimentos y de materias primas de uso industrial y farmacéutico, que da empleo a más de 12 millones de personas en el mundo. La acuicultura actual utiliza procesos cada vez más innovadores como parques flotantes o fijos en el fondo, balsas de cultivo, esteros o balsas naturales que aprovechan el agua de las mareas, estanques en tierra, etc. para el cultivo de moluscos, crustáceos, peces o algas.

En 2012 la pesca de captura y la acuicultura suministraron aproximadamente 158 millones de toneladas de pescado, crustáceos y moluscos (Figura 1.1). De esta cantidad, más del 86% fue destinada al consumo humano (alrededor de 136 millones de toneladas), proporcionando el 17% de la ingesta *per capita* de proteína de origen animal a nivel mundial (FAO, 2014).

La FAO (2014) estimó la producción global en acuicultura en el 2012 en 90,4 millones de toneladas, de las cuales 66,6 millones de toneladas fueron pescado comestible (incluido

peces, crustáceos y moluscos), 23,8 millones de toneladas fueron algas (mayoritariamente marinas) y 22.400 toneladas fueron de productos no alimentarios (conchas, perlas, etc.).

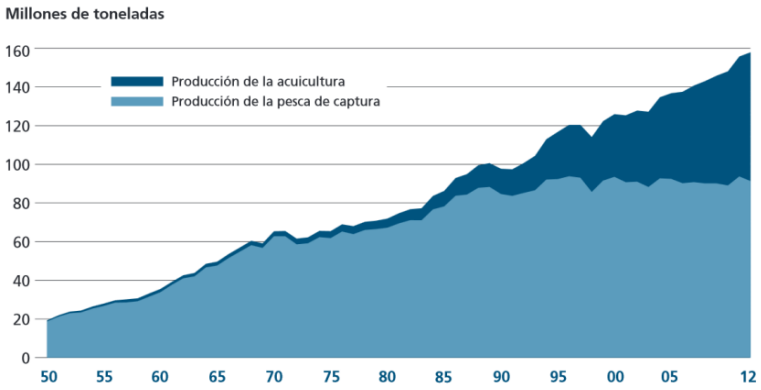

Figura 1.1. Evolución de la producción pesquera en el mundo en el periodo 1950-2010 (FAO, 2014).

El crecimiento de la producción acuícola mundial sigue siendo elevado. La contribución de la acuicultura a la producción mundial total de pescado en 2012 fue del 42,2%, frente al 25,7% que hubo en 2000 (FAO, 2014), esta contribución se ha duplicado de los 32,4 millones de toneladas obtenidos en el año 2000 a los 66,6 millones de toneladas del año 2012. Sin embargo, la tasa de crecimiento de la acuicultura ha comenzado a descender, siendo más llamativa en países como Malasia o EE.UU. Europa también ha mostrado un estancamiento de la producción, siendo la tasa media de

crecimiento anual para los países miembro de la Unión Europea (UE) del 0,7 en el periodo 2000-2012 (APROMAR, 2013).

En 2011, la UE produjo 1,27 millones de toneladas de productos de la acuicultura, este dato supone una reducción del 1,3% respecto a lo puesto en el mercado en 2010, y un descenso acumulado del 12,7 % desde el pico de producción europea que tuvo lugar en 1999 (APROMAR, 2014). Por el contrario, el consumo de pescado se ha disparado en la UE, ya que se ha visto que los productos acuáticos tienen un papel esencial en la dieta debido principalmente a que son fuentes ricas en aceites Omega 3, sobre todo de ácido docosahexaenoico (DHA, 22:6n-3) y ácido eicosapentaenoico (EPA, 20:5n3), importantes tanto para la salud cardiovascular y el desarrollo neurológico y cerebral del feto (Tocher, 2015). Sin embargo este aumento del consumo no se ha traducido en un incremento de la producción acuícola, sino en un aumento de la importación de pescado. Se espera que estas importaciones aumenten en los próximos años.

A pesar de que en la UE se cultivan bastantes especies, los principales productos de la acuicultura se reducen a unas pocas especies de pescados y moluscos. La principal especie producida en la UE es el mejillón, de la que se cultivan dos especies, el mediterráneo (*Mytilus galloprovincialis*) y el común (*Mytilus edulis*), seguida de la trucha arco iris (*Oncorhynchus mykiss*) y el salmón atlántico (*Salmo salar*).

Una parte de los productos acuáticos son procesados en forma de harinas y aceites de pescado para su uso como ingredientes alternativos al aceite y harinas de pescado derivado de pesquerías (APROMAR, 2013). En torno al 35% de la producción mundial de harina de pescado se obtuvo de residuos de pescado en el 2010 (FAO, 2014). A pesar de que esta práctica supone un avance hacia una mayor sostenibilidad de la acuicultura, lo cierto es que la acuicultura ha sido considerada como una actividad con consecuencias negativas para el medio ambiente entre las que se incluyen el deterioro de los ecosistemas litorales, la fuga de especies cultivadas que pueden convertirse en invasoras del medio ambiente local, la contaminación por desechos líquidos de los recursos hídricos locales, la transmisión de enfermedades de las especies cautivas a las poblaciones nativas de la región, etc. Pero también tiene beneficios, ya que la acuicultura puede reducir la presión sobre las poblaciones naturales sobreexplotadas, puede permitir la recuperación de poblaciones diezmadas, impulsar la producción natural de peces en libertad así como la diversidad de especies, etc.

Por tanto, el ritmo de crecimiento actual de la acuicultura y su capacidad para satisfacer la demanda mundial de alimentos de una población creciente dependen del logro de un modelo sostenible de desarrollo que minimice el impacto ambiental (Duarte y cols., 2007). El éxito de la acuicultura moderna se

basa en la adecuada gestión de la biología de las especies cultivadas, en la introducción de innovaciones tecnológicas y en el desarrollo de alimentos específicos, con el fin último de minimizar los efectos negativos señalados anteriormente.

1.1.1. La acuicultura en España

España es el estado miembro de la UE con un mayor volumen de producción en acuicultura con 264.162 toneladas en 2012 (21% del total de la UE) (Figura 1.2), seguido por Francia (205.210 toneladas, 16,3% del total de la UE) y Reino Unido (203.036 toneladas, 16,1% del total de la UE). Al considerar el valor económico de la producción, Reino Unido es el principal estado miembro productor, seguido de Francia y Grecia. España ocupa la 5ª posición con 395 millones (APROMAR, 2014).

El consumo de productos acuáticos en España es de 44,8 kg por habitante al año.

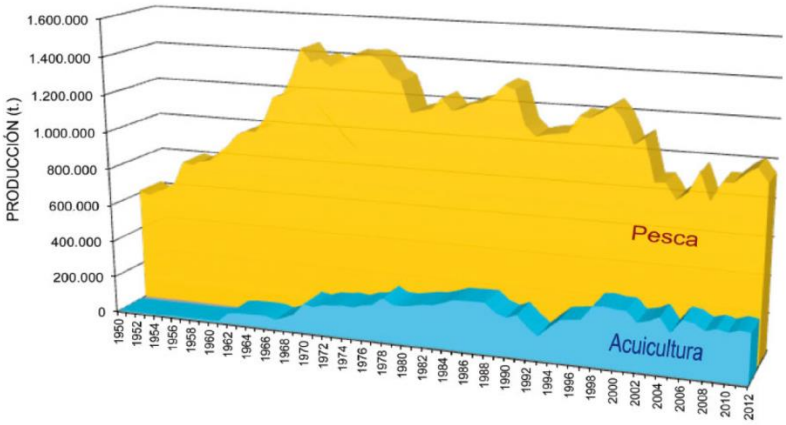

Figura 1.2. Evolución de la producción acuática total (acuicultura y pesca) en España en el periodo 1950-2012 (FAO, 2014)

La principal especie producida es el mejillón mediterráneo (*Mytilus galloprovincialis*), y en cuanto a peces son la dorada (*Sparus aurata*), la trucha arco iris (*Oncorhynchus mykiss*) y la lubina (*Dicentrarchus labrax*) (APROMAR, 2013), seguidas a menor escala de la corvina (*Argyrosomus regius*), la anguila (*Anguilla anguilla*) o el besugo (*Pagellus bogaraveo*) (Instituto Valenciano de Competitividad Empresarial, IVACE, 2014). La actual crisis económica ha provocado un estancamiento de la producción acuícola de toda España y un descenso notable en las inversiones en investigación para lograr el cultivo de nuevas especies.

En cuanto a la Comunidad Valenciana, las capturas pesqueras desembarcadas en la Comunidad Valenciana en 2011 fueron de 31.646 toneladas. La Comunidad Valenciana es líder

en producción de peces de crianza de mar con cerca del 35% del total de la producción española. Es la primera región productora de anguila de acuicultura (76% del total nacional) y de doradas (50%) del total de España, la segunda de corvina (37%) y la cuarta de lubina (18%) (IVACE, 2014).

1.2 Larvicultura de peces marinos

La larvicultura es la obtención de formas larvarias de peces, crustáceos y moluscos mediante el cultivo de estas especies con el objetivo de obtener individuos de talla comercial. Cuando esta obtención se lleva a cabo de forma directa en el medio natural mediante la pesca, conlleva problemas como la sobreexplotación y alteración de las cadenas tróficas marinas, entre otros. Actualmente, el mantenimiento en cautividad de *stocks* de reproductores de las especies de interés, es una práctica habitual de la acuicultura intensiva y ciertamente en la cría de especies marinas de interés comercial en España y en la UE como la dorada, lubina, etc. A partir de estos reproductores se obtienen las formas larvarias, que serán cultivadas atendiendo a sus requerimientos alimenticios, para lograr un correcto crecimiento de los individuos y asegurar niveles de supervivencia que garanticen la viabilidad económica de la explotación.

El alimento natural de las larvas de especies marinas son el fitoplancton y el zooplancton, y concretamente los copépodos.

La captura de estos organismos del medio natural conlleva los mismos problemas económicos, materiales y ecológicos que la captura de las propias larvas. En la práctica, la acuicultura ha solucionado este problema mediante el cultivo de algunas especies de fitoplancton y de zooplancton como los rotíferos (diferentes especies del género *Brachionus*) y la utilización masiva del crustáceo *Artemia* (branquiópodo anostráceo) en sus diferentes estadios de desarrollo (Medina, 2012).

El primer eslabón para la alimentación de las larvas de especies marinas consiste en la utilización de microalgas del fitoplancton. Las microalgas son una fuente de alimentación directa para animales filtradores como los moluscos y son vitales en los primeros estadios de desarrollo de algunos crustáceos. Además, también se utilizan microalgas como base para la producción de zooplancton (rotíferos y *Artemia*) y para la metodología de cría larvaria denominada "en agua verde" (Barnabé y René, 1972).

El siguiente eslabón en la cadena alimentaria de larvas marinas son los rotíferos. Los rotíferos son metazoos de 80-250 µm usados como presa viva en las primeras fases larvarias de la mayoría de las especies comerciales de peces y crustáceos (Barnabé y René, 1972). La especie más comúnmente cultivada en acuicultura es *Brachionus plicatillis*.

El último eslabón de la cadena trófica simplificada desarrollada en larvicultura marina la constituyen los estados larvarios (nauplios) del crustáceo *Artemia*. *Artemia* es capaz de

formar huevos de resistencia (quistes) que son fácilmente utilizables y almacenables y que permiten la obtención de nauplios vivos a demanda en un periodo de tiempo muy corto (aproximadamente 24 h) y sin necesidad de instalaciones de cultivo sofisticadas. Todo esto ha hecho de *Artemia* uno de los recursos de alimentación en acuicultura más importantes y de los que depende en gran medida la producción global de juveniles de muchas especies marinas de gran valor comercial. A pesar de las enormes ventajas, el uso de *Artemia* ofrece ciertas desventajas con respecto a presas naturales como los copépodos, y estas se relacionan principalmente con su escaso valor nutricional para larvas de peces y crustáceos marinos.

1.2.1. Importancia de los ácidos grasos en la larvicultura de peces marinos

Los ácidos grasos son ácidos orgánicos de cadena larga formados por una cadena hidrocarbonada hidrófoba de longitud variable con un grupo metilo (-CH_3) y carboxílico (-COOH) en ambos extremos de la molécula. Normalmente tienen un número par de átomos de carbono y se clasifican atendiendo al número de insaturaciones o dobles enlaces en: saturados, todos los átomos de carbono están unidos por un enlace simple, o insaturados, los cuales tienen uno (monoinsaturados) o más (poliinsaturados) dobles enlaces (insaturaciones) en su estructura. Dentro de los ácidos grasos poliinsaturados (PUFA,

Polyunsaturated Fatty Acid) se diferencian aquellos que tienen 20 átomos de carbono o más y la presencia de tres o más dobles enlaces, a estos se les denomina comúnmente como ácidos grasos altamente insaturados (HUFA, *Highly Unsaturated Fatty acid*). El sistema de nomenclatura más común de los PUFA es la denominada nomenclatura en "n" o "ω", que se caracteriza por especificar la posición del primer doble enlace desde el extremo metilo terminal, además de indicar también el tamaño de la cadena de carbono y el número de insaturaciones. Por ejemplo, el ácido graso 22:6n-3 (DHA), consta de una cadena de 22 átomos de carbono, con 6 dobles enlaces, el primero de ellos situado en el tercer carbono desde el extremo metilo (Rodríguez, 2014).

Las larvas de organismos marinos necesitan HUFA como EPA, DHA y ácido araquidónico (ARA, 20:4n-6) para satisfacer sus necesidades fisiológicas que garanticen su normal crecimiento y desarrollo (Tocher, 2010). Sin embargo, la maquinaría enzimática de elongación (elongasas) y desaturación (desaturasas) necesaria para su biosíntesis (producción endógena) es limitada en la mayoría de especies de organismos marinos y la demanda debe ser cubierta por tanto a través de la dieta (Watanabe y cols., 1982; Lavens y cols., 1989; Rainuzzo y cols., 1994; Sargent y cols., 1997, 2002; Bell y cols., 2003; Tocher y cols., 2003). En el caso de peces marinos, EPA, ARA y DHA se consideran ácidos grasos esenciales (EFA, *Essential*

Fatty Acid) (Tocher, 2010). Hay estudios que reflejan que la insuficiencia de estos nutrientes esenciales en la dieta de peces marinos se traduce en una disminución del desarrollo visual y neural de las larvas con consecuencias fisiológicas y comportamentales (Sargent y cols., 1999; Bell y cols., 2001, 2003).

Se considera una dieta óptima para los reproductores aquella que permite obtener huevos con un contenido lipídico igual al perfil que tienen los huevos de ejemplares salvajes (Sargent y cols., 2002; Cahu y cols., 2009). Los lípidos, en concreto los HUFA, juegan un papel muy importante en el proceso reproductivo, la ontogenia embrionaria y, en general, los primeros estadios de desarrollo larvario (Tocher, 2010). Muchos estudios sobre la calidad de las dietas centran su atención sobre su fracción lipídica, en concreto, sobre la composición en EFA presentes en el alimento (Rodríguez y cols., 1998; Ibarra-Zatarain y cols., 2015; Mozanzadeh y cols., 2015). La alimentación de los reproductores es un elemento determinante para poder obtener puestas de calidad. La composición bioquímica del alimento vivo es importante para los peces, ya que contiene la mayoría de los elementos nutritivos que garantizan la supervivencia y el óptimo desarrollo de las larvas.

1.3. Importancia del uso de *Artemia* en acuicultura

Artemia es la especie de presa viva más utilizada en el cultivo de larvas de peces y crustáceos (Aragão y cols., 2004). El género *Artemia* se subdivide normalmente en seis especies bisexuales y un gran número de poblaciones partenogenéticas (Amat y cols., 2005, 2007; Nunes y cols., 2006). En ambos tipos de reproducción las hembras pueden dar lugar a dos clases de huevos: a) los que finalizan su desarrollo embrionario en el interior del útero y que dan lugar a nauplios completamente formados (proceso ovovivíparo); b) los que detienen su desarrollo en el estado de blástula avanzada, cubriéndose de un corion resistente y quedando en forma de quiste o huevo de resistencia (oviparismo). La reproducción ovípara (formación de quistes) está asociada a la presencia de condiciones adversas en el medio natural y los quistes producidos de ésta, pueden permanecer en estado de latencia (diapausa) hasta que vuelvan a establecerse las condiciones óptimas para su eclosión.

La explotación de quistes de *Artemia* tiene un elevado interés económico, ya que de ella depende la producción mundial de larvas de peces (sobre todo marinas) y de crustáceos. Uno de los principales productores de quistes de *Artemia* es el *Great Salt Lake* (GSL) de Utah (EE.UU.). En la década de 1980 los quistes procedentes de este lago monopolizaron

aproximadamente el 90% de las existencias en el mercado mundial (Medina, 2012). El incremento de la demanda, las cosechas irregulares y la explotación no sostenible de un recurso natural, provocaron una crisis que llevó a cuestionarse si la disponibilidad de *Artemia* podría constituir un factor limitante para el desarrollo de la acuicultura. Las consecuencias de esta crisis han sido un incremento desmesurado de los precios, baja calidad del producto e incertidumbre sobre biodiversidad y origen. Esto se tradujo en una ausencia de garantía sobre la viabilidad de los quistes tras un periodo largo de almacenaje, falta de uniformidad del tamaño naupliar y carencia de estándares nutricionales en cuanto a su composición lipídica, necesarios para una adecuada nutrición de las larvas de especies marinas. Continua la búsqueda de fuentes alternativas al GSL y algunos estudios valoran la capacidad productora de quistes de *Artemia* de algunos lagos salados de América (Cohen y cols., 1999) y Asia (Van Stappen, 2005).

El uso de nauplios de *Artemia* como presa viva tiene algunas ventajas: rápida y sencilla disponibilidad (como se ha comentado anteriormente), adaptabilidad a un amplio rango de salinidades (5 – 250 g L^{-1}) y temperaturas (6 – 35 ºC), ciclo de vida corto, elevada adaptabilidad a condiciones ambientales adversas, elevada fecundidad, versatilidad reproductiva (bisexual/partenogenética, ovoviparismo/oviparismo), pequeño tamaño, movimiento constante, ausencia de respuesta de escape

y adaptación a una variada fuente de alimento al tratarse de un filtrador no selectivo (Nunes y cols., 2006).

1.4. Carencias nutricionales de *Artemia*

La calidad nutricional de los quistes de *Artemia* varía en función de la cepa y el lote (Moraiti-Ioannidou y cols., 2009), situación que puede ocasionar problemas asociados a su uso como alimento para larvas de peces y crustáceos (Léger, 1986; 1987). A mediados de los años sesenta y principios de los setenta, comenzaron a aparecer en la literatura numerosos trabajos relacionados con mortalidades masivas de larvas de diferentes especies de peces y crustáceos, cuando se empleaban nauplios de *Artemia* de determinadas razas o variedades como único alimento (Shelbourne, 1968; Reeve, 1969; Provenzano y Goy, 1976). Esta mortalidad no se observaba exclusivamente al utilizar una única población de *Artemia* sino en varias de diverso origen geográfico (Watanabe y cols., 1978; Watanabe, 1979). Estos hechos unidos a la enorme demanda de quistes necesarios para el desarrollo de la acuicultura, impulsaron el interés por conocer el valor nutritivo de los nauplios destinados a alimentar las formas larvarias en cultivo (Monroig, 2006). Léger y cols. (1986) pusieron de manifiesto la escasez de ciertos EFA para animales marinos en la composición lipídica de los nauplios y se observaron diferencias de composición entre las diferentes especies estudiadas e incluso entre una misma población

muestreada en diferentes épocas del año. Todo esto desembocó en la aceptación de la clasificación propuesta por Watanabe y cols. (1978), en la que se diferencian dos grandes tipos de *Artemia* según el contenido en determinados ácidos grasos: el tipo dulceacuícola, con elevados niveles de ácido linolénico (LNA, 18:3n-3) y de ácido linoleico (LA, 18:2n-6) y ausencia de EPA; y el tipo marino, con menores niveles de LNA y presencia de LA. En ninguno de los dos tipos aparecen cantidades apreciables de DHA.

Los lípidos de la dieta son particularmente importantes en los estadios tempranos de desarrollo de las larvas de peces marinos (Sargent y cols., 2002), debido a que representan una fuente de energía importante para las larvas y una fuente de EFA necesarios en la formación de estructuras celulares y un crecimiento larval normal (Izquierdo y cols., 2000, Lall y Lewis-McCrea, 2007, Cahu y cols., 2009). Uno de los mayores cuellos de botella en la cría de peces en cautividad es la natural deficiencia en EFA de las presas vivas, como rotíferos y nauplios de *Artemia*, usadas de manera rutinaria en los criaderos (*hatcheries*) para alimentar a las larvas de peces marinos (Conceição y cols, 2010). Los nauplios de *Artemia* carecen de DHA y tienen contenidos generalmente bajos de EPA, y por tanto, deben ser enriquecidos con productos ricos en estos ácidos grasos, para garantizar la supervivencia, el crecimiento y

la adecuada metamorfosis de las larvas de peces marinos (Sargent y cols., 1999; Hamre y cols., 2013).

1.5. Uso de emulsiones lipídicas como sistema de suministro de nutrientes

Como se ha introducido anteriormente, el escaso valor nutricional de los nauplios de *Artemia* para larvas de organismos marinos relacionado con su bajo contenido en EFA como DHA y EPA, hace necesario someter a los nauplios a un proceso de enriquecimiento previo a su uso como presa viva. El enriquecimiento consiste en la incorporación de productos ricos en EFA en los nauplios de *Artemia* aprovechando su condición de filtradores pasivos (Léger y cols., 1986). Así, los nauplios actúan como un vehículo vivo incorporando en su tracto digestivo partículas con un rango de tamaños determinado y que se encuentran en suspensión en el medio.

Existen dos métodos de enriquecimiento: el indirecto y el directo. El método indirecto consiste en la vehiculación de otros organismos, tales como levaduras o microalgas (Watanabe y cols., 1982; Aragão y cols., 2004), que contienen los nutrientes esenciales para las larvas y que se hallan en niveles subóptimos en los nauplios. En el método directo, los nauplios se ponen en contacto con las sustancias enriquecedoras en forma de micropartículas (Southgate y Lou, 1995), liposomas (Hontoria y cols., 1994; McEvoy y cols., 1996; Monroig y cols., 2003,

2006a, b, c) y mucho más generalmente, emulsiones lipídicas (Léger y cols., 1986; Evjemo y cols., 1997, Han y cols., 2000, 2001, 2005). De hecho, uno de los métodos de enriquecimiento directo más extendido en la actualidad es la utilización de emulsiones ricas en EFA. En contacto con el agua de mar, estas emulsiones lipídicas se dispersaran formando pequeñas gotas o partículas que el nauplio puede filtrar e ingerir hasta incorporarlo en el tracto digestivo. Las emulsiones se formulan a base de aceites de pescado ricos en HUFA como el DHA y el EPA, así como otros componentes como ésteres etílicos de DHA, vitaminas, emulgentes, etc.

Existen problemas asociados al proceso de enriquecimiento tales como la elevada mortalidad naupliar (Figueiredo y cols., 2009), la autoxidación de los HUFA (McEvoy y cols., 1995, Sargent y cols., 1997) y el elevado coste económico de las emulsiones lipídicas (Southgate y Lou, 1995). Además, el proceso de enriquecimiento es ineficiente debido a la imposibilidad de obtener valores de PUFA similares a los presentes en el plancton marino, y a la retroconversión selectiva que realizan los nauplios de *Artemia*, que catabolizan DHA en EPA durante el enriquecimiento (Evjemo y cols., 1997, Navarro y cols., 1999, Han y cols., 2001). Estas dificultades para el enriquecimiento de *Artemia* en DHA, son la principal limitación para su utilización como primera presa viva para las larvas de peces (Bell y cols., 2003; Haché y Plante, 2011). También hay

que tener en cuenta que la composición final en términos de ácidos grasos de la presa viva enriquecida está en gran medida condicionada por su propia composición basal deficiente en HUFA, y de mayor peso específico respecto al del enriquecedor.

Así, el enriquecimiento específico y preferencial en DHA parece un paso clave para aumentar los niveles de este EFA en los nauplios de *Artemia* de forma que, a pesar de la ineludible retroconversión a EPA, los niveles de DHA sigan siendo suficientemente elevados como para satisfacer los requerimientos de las larvas de organismos marinos. A pesar de los avances obtenidos en los últimos años y a la variedad de productos comerciales disponibles en la actualidad, lo cierto es que el proceso de enriquecimiento sigue siendo ineficiente con resultados de contenido en EFA por debajo del alimento natural. Así, parece obvio que deben desarrollarse nuevas estrategias de enriquecimiento que permitan garantizar niveles más elevados de DHA en presas vivas y, por tanto, más similares a los de las presas naturales. Para lograr este cometido, el presente estudio aborda la caracterización de una nueva emulsión lipídica rica en DHA formulada a partir de un aceite sintético (DHA-Algatrium) con un contenido en DHA del 70% del total de ácidos grasos. La emulsión lipídica se desarrolló en el Instituto de Agroquímica y Tecnología de los Alimentos (IATA-CSIC, Valencia, España) en colaboración con Archivel Technologies S.L. (Barcelona, España) y se denominó M70. Mediante la utilización de la

emulsión M70 se pretende aumentar de forma notable el nivel de DHA en los nauplios de *Artemia* y así lograr revertir la relación DHA/EPA considerada como un indicador bioquímico para evaluar la idoneidad nutricional de las dietas en la larvicultura de peces marinos (Reitan y cols., 1994; Evjemo y cols. 1997).

El desarrollo de la acuicultura, unido al agotamiento de los recursos pesqueros, justifica la inversión en investigación y tecnología depositada en este sector. En este contexto se encuadra el presente trabajo de investigación, el cual se centra en cómo mejorar la alimentación larvaria de los organismos marinos obtenidos en explotaciones acuícolas.

1.6. Presentación de los trabajos y justificación de la unidad temática

Teniendo en cuenta los numerosos trabajos que destacan la importancia de los ácidos grasos en la alimentación larvaria de organismos marinos, nos proponemos probar y obtener información acerca de la utilización de una nueva emulsión lipídica rica en PUFA, concretamente en DHA (contiene un 70% de este ácido graso), para alimentar nauplios de *Artemia*, empleados como presa viva en la cría de larvas de organismos marinos. Esto significaría realizar un enriquecimiento selectivo en DHA, para paliar la catabolización que tiene lugar en los nauplios y revertir la relación DHA/EPA, alcanzando así los

niveles de DHA conseguidos al alimentar los nauplios con su alimento natural (fito y zooplancton).

Dentro de este planteamiento se justifica la unidad temática de los tres trabajos de investigación que componen esta tesis doctoral, presentados a continuación.

Artículos del compendio:

Artículo 1:

Viciano, E., Monroig, Ó., Salvador, A., Amat, J., Fiszman, S., Navarro, J.C., 2015. **Enriching *Artemia* nauplii with a high DHA- containing lipid emulsion: search for an optimal protocol.** Aquaculture Research 46, 1066-1077.

Este estudio tuvo como objetivo investigar estrategias prácticas para optimizar el uso de una nueva emulsión lipídica (M70) muy rica en ácido docosahexaenoico (DHA, 22:6n-3). Teniendo en cuenta su contenido particularmente alto en DHA (70%), se evaluó la utilización de M70 para enriquecimientos de nauplios de *Artemia* en una serie de seis experimentos. Concretamente se estudió la eficiencia de la bioencapsulación de M70 en nauplios bajo diferentes condiciones experimentales: fuente de oxígeno, flujo de aireación, temperatura, concentración y dosificación de la emulsión y densidad naupliar.

Los resultados mostraron que una utilización óptima de M70 se consigue con temperaturas de incubación de 28 ºC, aireación moderada y densidades naupliares de 300 individuos por mL. La emulsión se puede dispensar en el medio de enriquecimiento en una única dosis de 0,8 g L^{-1} sin efectos perjudiciales aparentes sobre su estabilidad a la oxidación y la supervivencia de nauplios de *Artemia* durante el enriquecimiento.

Artículo 2:

Viciano, E., Monroig, Ó., Barata, C., Navarro, J.C., 2015. **Antioxidant activity and lipid peroxidation in *Artemia* nauplii enriched with a new DHA-rich emulsion and the effect of adding an external antioxidant based on hydroxytyrosol.**

Aquaculture Research, *In press*.

Durante el enriquecimiento de los nauplios de *Artemia* con emulsiones lipídicas, particularmente en aquellas con elevados contenidos en DHA, como la emulsión experimental M70, los nauplios y la emulsión están sometidos a unas condiciones prooxidantes que pueden causar estrés a los nauplios y oxidar los ácidos grasos presentes en la emulsión. Para probar esto se

midieron diferentes enzimas antioxidantes y la peroxidación lipídica en los nauplios enriquecidos bajo varios tratamientos: cultivo con levaduras de panificación (*Saccharomyces cerevisiae*), microalgas (*Tetraselmis suecica*), y las emulsiones lipídicas M70 y la comercial DC Super Selco (Inve, Dendermonde, Bélgica). Además se estudió el efecto de una sustancia antioxidante basada en Hidroxitirosol, antioxidante natural extraído del aceite de oliva, sobre M70 y su eficacia enriquecedora. Los resultados muestran diferencias significativas al utilizar el antioxidante externo para controlar y evitar que aumente la peroxidación lipídica.

Artículo 3:

Viciano, E., Iglesias, J., Lago, M.J., Sánchez, F.J., Otero, J.J., Navarro, J.C., 2011. **Fatty acid composition of polar and neutral lipid fractions of *Octopus vulgar*is Cuvier, 1797 paralarvae reared with enriched on-growth *Artemia***. Aquaculture Research 42, 704-709.

El cultivo del pulpo (*Octopus vulgaris*) está limitado por las complicaciones durante la fase de paralarva lo que produce unas elevada mortalidad. En este estudio se compara el uso de *Artemia* enriquecida con *Nannochloropsis* sp. y con una emulsión muy rica en PUFA (M70) para alimentar a las paralarvas de pulpo. Se llevaron a cabo análisis de ácidos grasos

y de composición en clases lipídicas de las paralarvas de pulpo alimentadas con los dos tratamientos y se observaron diferencias en la composición de lípidos polares y neutros de las paralarvas. Tras 28 días de experimento, la supervivencia de las paralarvas se estimó en un 3% para M70 y 22,5% para *Nannochloropsis* sp. El análisis de la composición de ácidos grasos de las presas enriquecidas con los dos tratamientos, de las paralarvas de pulpo y el fraccionamiento y análisis de ácidos grasos de los lípidos totales, clases lipídicas polares y neutras, seguido de un Análisis de Componentes Principales, reveló que, independientemente de la dieta, ambas fracciones de lípidos mostraros patrones de ácidos grasos distintos. La composición de los lípidos polares se mantuvo invariable, mientras que la de los lípidos neutros estuvo más influenciada por la dieta, lo que refleja la variabilidad entre los tratamientos dietéticos.

1.7. Bibliografía

Amat, F., Hontoria,, F., Ruiz, O., Green, A.J., Sánchez, M.I., Figuerola, J., Hortas, F., 2005. The American brine shrimp as an exotic invasive species in the western Mediterranean. Biological Invasions 7, 37-47.

Amat, F., Hontoria, F., Navarro, J.C., Vieira, N., Mura, G., 2007. Biodiversity loss in the genus *Artemia* in the Western Mediterranean Region. Limnetica 26, 387-404.

Aragão, C., Conceiçao, L.E.C., Dinis, M.T., Fyhn, H., 2004. Amino acid pools of rotifers and *Artemia* under different conditions: nutritional implications for fish larvae. Aquaculture 234, 429-445.

APROMAR, Asociación Empresarial de Productores de Cultivos Marinos de España, 2013. La acuicultura en España. 97 pp.

APROMAR, Asociación Empresarial de Productores de Cultivos Marinos de España, 2014. La acuicultura en España. 91 pp.

Barnabé, G. y René, F. 1973. Reproduction contrôlée et production chez la dorade *Sparus auratus* Linné 1978. Comptes Rendus de l'Acadèmie des Sciences de Paris 276: 1621-1623.

Bell, M.V., Dick, J.R., Porter, E.A., 2001. Biosynthesis and tissue deposition of docosahexaenoic acid (22:6n-3) in rainbow trout (*Oncorhynchus mykiss*). Lipids 36, 1153-1159.

Bell, J.G., McEvoy, L.A., Estévez, A., Shields, R.J., Sargent, J.R., 2003. Optimising lipid nutrition in first-feeding flatfish larvae. Aquaculture 227, 211-220.

Cahu, C.L., Gisbert, E., Villeneuve, L.A.N., Morais, S., Hamza, N., Wold, P., Zambonino-Infante, J.L., 2009. Influence of dietary phospholipids on early ontogenesis of fish. Aquaculture Research 40, 989-999.

Cohen, R.G., Amat, F., Hontoria, F y Navarro, J.C., 1999. Preliminary characterization of some Argentinean *Artemia* populations from La Pampa and Buenos Aires provinces. International Journal of Salt Lake Research 8, 329-340.

Conceição, L.E.C., Yúfera, M., Makridis, P., Morais, S., Dinis, M.T., 2010. Live feeds for early stages of fish rearing. Aquaculture Research 41, 613-640.

Duarte, C, Marbá, N. y Holmer, M., 2007. Rapid domestication of marine species. Science 316: 382-383.

Evjemo, J.O., Coutteau, P., Olsen, Y., Sorgeloos, P., 1997. The stability of docosahexaenoic acid in two *Artemia* species following enrichment and subsequent starvation. Aquaculture 155, 135-148.

FAO, Food and Agriculture Organization or the United Nations, 2014. El estado actual de la pesca y acuicultura. FAO Fisheries department, Roma, 253 pp.

Figueiredo, J., Van Woesik, R., Lin, J., Narciso, L., 2009. *Artemia franciscana* enrichment model – How to keep them small, rich and alive? Aquaculture 294, 212-220.

Haché, R. y Plante, S., 2011. The relatioship between enrichment, fatty acid profiles and bacterial load in cultured rotifers (*Brachionus plicatillis* L-Strain) and *Artemia* (*Artemia salina* strain *Franciscana*). Aquaculture 311, 201-208.

Hamre, K., Yúfera, M., Rønnestad, I., Boglione, C., Concenição, L.E.C., Izquierdo, M., 2013. Fish larval nutrition and feed formulation: knowledge gaps and bottlenecks for advances in larval rearing. Reviews in Aquaculture 5, S26-S58.

Han, K., Geurden, I., Sorgeloos, P., 2000. Enrichment strategies for *Artemia* using emulsions providing differents levels of n-3 highly unsaturated fatty acids. Aquaculture 183, 335-347.

Han, K., Geurden, I., Sorgeloos, P., 2001. Fatty acid changes in enriched and subsequently starved *Artemia franciscana* nauplii enriched with different essential fatty acids. Aquaculture 199, 93-105.

Han, K., Geurden, I., Van der Meeren, P., Bai, S.C., Sorgeloos, P., 2005. Particle size distribution in two lipid emulsion used for the enrichment of *Artemia* nauplii as a function of their preparation method and storage time. Journal of the World Aquaculture Society 36, 196-202.

Hontoria, F., Crowe, J.H., Crowe, L.M., Amat, F., 1994. Potential use of liposomes in larviculture as a delivery system through *Artemia* nauplii. Aquaculture 127, 255-264.

Ibarra-Zatarain, Z., Morais, S., Bonacic, K., Campoverde, C., Duncan, N., 2015. Dietary fatty acid composition significantly influenced the proactive-reactive behaviour of Senegalense sole (*Solea senegalensis*) post-larvae. Applied Animal Behaviour Science 171, 233-240.

IVACE, Instituto Valenciano de competitividad empresarial, 2014. Acuicultura y pesca de la Comunidad Valenciana. Generalitat Valenciana. 12 pp.

Izquierdo, M.S., Socorro, J., Arantzamendi, L., Hernández-Cruz, C.M., 2000. Recent advances in lipid nutrition in fish larvae. Fish Physiology and Biochemistry 22, 97-107.

Lall, S.P., Lewis-McCrea, L.M., 2007. Role of nutrients in skeletal metabolism and pathology in fish – An overview. Aquaculture 267, 3-19.

Lavens, P., Léger, P., Sorgeloos, P., 1989. Manipulation of the fatty acid profile in *Artemia* offspring produced in intensive culture system. En: Aquaculture – A biotechnology in progress. De Pauw, N., Jaspers, E., Ackefors, H., Wilkins, N. (Eds). European Aquaculture Society, Bredene, Belgium, pp 731-739.

Léger, P., Bengtson, D.A., Simpson, K.L., Sorgeloos, P., 1986. The use and nutritional value of *Artemia* as a food source. Oceanography and Marine Biology - Annual Review 24, 521-623.

Léger, P., Bengtson, D.A., Sorgeloos, P., Simpson, K.L., Beck, A.D., 1987. The nutritional value of *Artemia*: a review. En: *Artemia* research and its applications. Sorgeloos, P., Bengtson, D.A., Decleir, W., Jaspers, E. (Eds.), Universia Press, Wetteren, Belgium, pp. 357-372.

McEvoy, L.A., Navarro, J.C., Bell, J.G., Sargent, J.R., 1995. Autoxidation of oil emulsions during the *Artemia* enrichment process, Aquaculture 134, 101-112.

McEvoy, L.A., Navarro, J.C., Hontoria, F. Amat, F., Sargent, J.R., 1996. Two novel *Artemia* enrichment diets containing polar lipid. Aquaculture 144, 339-352.

Medina, G.R., 2012. El recurso de *Artemia* de Argentina: Biodiversidad y uso en acuicultura. Tesis doctoral. Universidad de Valencia. 259 pp.

Monroig, O., Navarro, J.C., Amat, I., González, P., Amat, F., Hontoria, F., 2003. Enrichement of *Artemia* nauplii in PUFA, phospholipids, and wáter-soluble nutrients using liposomes. Aquaculture International 11, 151-161.

Monroig, O., Navarro, J.C., Amat, F., González, P., Bermejo, A., Hontoria, F., 2006a. Enrichment of *Artemia* nauplii in essential fatty acids with different types of liposomes and their use in the rearing of gilthead sea bream (*Sparus aurata*) larvae. Aquaculture 251, 491-508.

Monroig, O., Navarro, J.C., Amat, F., González, P., Hontoria, F., 2006b. Effect of aeration on the efficiency of *Artemia* enrichment with EFA-rich emulsion and liposomes. Aquaculture 257, 382-392.

Monroig, O., Navarro, J.C., Amat, F., González, P., Hontoria, F., 2006c. Effects of nauplial density, product concentration and product dosage on the survival of the nauplii and EFA incorporation during *Artemia* enrichment with liposomes. Aquaculture 261, 659-669.

Monroig, O., 2006. Diseño y optimización de liposomas para su uso como sistema de suministro de nutritientes a larvas de peces marinos. Tesis doctoral. Universidad de Valencia. 239 pp.

Moraiti-Ioannidou, M., Castritsi-Catharios, J., Miliou, H., Sorgeloos, P., 2009. Biochemical composition and digestive enzyme activity during naupliar development of *Artemia* spp from three solar saltworks in Greece. Aquaculture 286, 259-265.

Mozanzadeh, M.T., Marammazi, J.G., Yavari, V., Agh, N., Mohammadian, T., Gisbert, E., 2015. Dietary n-3 LC-PUFA requirements in silvery-black porgy juveniles (*Sparidentex hasta*). Aquaculture 448, 151-161.

Navarro, J.C., Henderson, R.J., McEvoy, L.A., Bell, M.V., Amat, F., 1999. Lipid conversion during enrichment of *Artemia*. Aquaculture 174, 155-166.

Nunes, B., Carvalho, F., Guilhermino, L., Van Stappen, G., 2006. Use of the genus *Artemia* in ecothoxicity testing. Environmental Pollution 144, 453-462.

Provenzano, A.J., Goy, J.W., 1976. Evaluation of a sulphate lake strain of *Artemia* as food for larvae of the grass shrimp, *Palaemonetes pugio*. Aquaculture 9, 343-350.

Rainuzzo, J.C., Reitan, K.I., Jorgensen, L., Olsen, Y., 1994. Lipid composition in turbot larvae feed cultured by emulsions of different lipid classes. Comparative Biochemistry Physiology 107A, 699-710.

Reitan, K.I., Rainuzzo, J.R., Olsen, Y., 1994. Influence of lipid composition of live feed on growth, survival and pigmentation of turbot larvae. Aquaculture International 2, 22-48.

Reeve, M.R., 1969. The laboratory culture of the prawn *Palaemon serratus*. Fisheries Investigation 26, 1-38.

Rodríguez, C., Pérez, J.A., Badía, P., Izquierdo, M.S., Fernández-Palacios, H., Lorenzo, A., 1998. The *n*-3 highly unsaturated fatty acids requirements of gilthead seabream (*Sparus aurata* L.) larvae when using an appropriate DHA/EPA ratio in the diet. Aquaculture 169, 9-23.

Rodríguez, D., 2014. Reconstrucción de Pedigrí y valoración de los requerimientos en ácidos grasos en reproductores de *Seriola dumerili*. Tesis doctoral. Universidad de La Laguna. 160 pp.

Sargent, J.R., McEvoy, L.A., Bell. J.G., 1997. Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. Aquaculture 155, 177-127.

Sargent, J.R., McEvoy, L.A., Estévez, A., Bell, G., Bell, M., Henderson, J., Tocher, D., 1999. Lipid nutrition of marine fish during early development: current status and future directions. Aquaculture 179, 217-229.

Sargent, J.R., Tocher, D.R., Bell, J.G., 2002. The lipids. En: Halver, J.E., Hardy, R.W. (Eds.), Fish Nutrition. Academic Press, San Diego, pp. 181-257.

Shelbourne, J.E., 1968. The culture of marine fish larvae with special references to the plaice *Pleuronectes platessa* and the sole *Solea solea*. Tesis doctoral. London University, Londres, Reino Unido.

Southgate, P.C., Lou, D.C., 1995. Improving the n-3 HUFA composition of *Artemia* using microcapsules containing marine oils. Aquaculture 134, 91-99.

Tocher, D.R., 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Reviews in Fisheries Science 11, 107-184.

Tocher, D.R., 2010. Fatty acid requirements in ontogeny of marine and freshwater fish. Aquaculture Research 41, 717-732.

Tocher, D.R., 2015. Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. Aquaculture, *In press*

Van Stappen, G., 2005. *Artemia* zoogeography and taxonomy: an up-to-date overview. *Artemia* Seminar, September 2005, Ghent, Belgium

Watanabe, T., Oowa, F.,Kitajima, C., Fujita. S., 1978. Nutritional quality of brine shrimp, *Artemia salina*, as a living feed from the viewpoint of essential fatty acids for fish. Bulletin of the Japanese Society for the Science of Fish 44, 1115-1121.

Watanabe, T., 1979. Nutritional quality of living feeds used in seed production of fish. En: G. Yamamoto (Ed.), Proc. 7th Japan-Soviet Joint Symposium on Aquaculture. Tokai University, Tokio (Japón), 49-57.

Watanabe, T., Ohta, M., Kitajima, C., Fujita, S., 1982. Improvement of dietary value of brine shrimp *Artemia salina* for fish larvae by feeding them on w3 highly unsaturated fatty acids. Bulletin of the Japanese Society for the Science of Fish 48, 1775-1782.

CAPÍTULO 2

Objetivos

2.1. Objetivo general

La investigación desarrollada en la presente Tesis Doctoral se enmarcó dentro del proyecto PETRI de investigación "Desarrollo de productos enriquecedores de alto contenido en ácidos grasos poliinsaturados de cadena larga para complementar el valor nutritivo de presas vivas como alimento larvario" (Ref. PTR1995-0714-OP-02-01).

El objetivo principal de la presente tesis doctoral es emplear aceites de gran pureza y ricos en DHA para formular una emulsión lipídica que permita enriquecer más eficazmente nauplios de *Artemia* en DHA a pesar de las complicaciones del proceso señaladas anteriormente. Para ello se formularon emulsiones lipídicas enriquecedoras en cuyo perfil de ácidos grasos predomina el DHA respecto al resto.

2.2. Objetivos específicos

Este objetivo general, se subdivide en varios objetivos específicos:

1. Estudiar la estabilidad física de la emulsión elaborada a partir de aceite rico en DHA, denominada "M70", durante su utilización como producto enriquecedor de nauplios de *Artemia*.

2. Estudiar los procesos oxidativos en nauplios de Artemia enriquecidos con la emulsión M70.
3. Definir los protocolos óptimos de enriquecimiento con M70 y su repetitividad.
4. Establecer la eficacia enriquecedora de la emulsión M70 mediante pruebas de alimentación larvaria.

Tras los dos capítulos preliminares (Introducción y Material y Métodos), los resultados obtenidos se presentan en los Capítulos 4-6[1], que corresponden con las tres publicaciones SCI que han constituido la base de la presente tesis doctoral:

- Capítulo 4: **Viciano, E.**, Monroig, Ó., Salvador, A., Amat, J., Fiszman, S., Navarro, J.C., 2015. Enriching *Artemia* nauplii with a high DHA-containing lipid emulsion: search for an optimal protocol. Aquaculture Research 46, 1066-1077.
- Capítulo 5: **Viciano, E.**, Monroig, O., Barata, C., Navarro, J.C., 2014. Antioxidant activity and lipid peroxidation in *Artemia* nauplii enriched with a new DHA-rich emulsion and the effect of adding an external antioxidant based on hydroxytyrosol. Aquaculture Research, *Aceptado*.

[1] *Los Capítulos 4-6 mantienen los requisitos de uniformidad de las revistas en las que se publicaron, aunque se han editado para facilitar su lectura y adaptarlos al formato de la presente Tesis Doctoral.*

- Capítulo 6: **Viciano, E.**, Iglesias, J., Lago, M.J., Sánchez, F.J., Otero, J.J., Navarro, J.C., 2011. Fatty acid composition of polar and neutral lipid fractions of *Octopus vulga*ris Cuvier, 1797 paralarvae reared with enriched on-growth *Artemia*. Aquaculture Research 42, 704-709.

Finalmente, el Capítulo 7 incluye una discusión general abordando globalmente las cuestiones planteadas a lo largo del desarrollo de la presente Tesis Doctoral.

CAPÍTULO 3

Material y Métodos comunes

3.1. Elaboración de emulsiones lipídicas a partir de aceites ricos en DHA

Las emulsiones son sistemas coloidales formados por dos o más líquidos inmiscibles, estando uno de ellos disperso en el otro en forma de pequeñas gotas. En la mayoría de emulsiones alimentarias los líquidos inmiscibles son aceite y agua, por lo que es necesaria una agitación mecánica para su dispersión.

Es necesario que la emulsión sea estable durante un periodo razonable de tiempo, para ello se utilizan agentes emulsionantes y estabilizantes, que dan consistencia, coherencia y una textura óptima a la emulsión. El término *emulsionante* incluye aquellos compuestos que facilitan la formación de la emulsión al disminuir la tensión interfacial aceite-agua, proporcionando una estabilidad a corto plazo por la formación de una película protectora alrededor de las gotas de la fase dispersa. El término *estabilizante*, por su parte, se refiere a aquellos compuestos que no poseen un carácter tensoactivo pero que proporcionan mayor estabilidad a las emulsiones a través de la restricción de las interacciones entre las partículas dispersas debido al aumento de viscosidad de la fase continua que producen (Dickinson, 1993).

Cuando el sistema se desestabiliza se produce la separación de ambas fases. Esta desestabilización, la cual puede

ser un proceso reversible o irreversible, se puede ocasionar por medio de varios procesos:

- Cremado: Es debido a la diferencia de densidad de las dos fases que forman la emulsión y a la gravedad terrestre. Es un proceso reversible porque no supone una diferencia en el tamaño de las partículas. El sistema se puede recuperar redispersando la emulsión.
- Floculación: Implica la agregación de pequeñas gotas bajo la influencia de fuerzas atractivas formando grupos. Es reversible porque no implica una diferencia en el tamaño individual de las gotas. Se puede redispersar por agitación.
- Coalescencia: Es la unión irreversible de las gotas para dar lugar a otras mayores. Puede conducir a la separación completa de las dos fases.

En acuicultura, para el enriquecimiento de presas vivas, se suelen emplear aceites de pescado por su riqueza en HUFA de la serie n-3 como el DHA y el EPA. El método más habitual de enriquecimiento es mediante la utilización de emulsiones lipídicas formuladas a partir de estos aceites de pescado. Para la presente tesis doctoral se elaboró una nueva emulsión lipídica a partir de DHA-Algatrium (Brudy Technology, Barcelona, España), aceite con la particularidad de ser especialmente rico en DHA (70% del total de ácidos grasos). La emulsión

resultante se denominó M70. La fabricación de las emulsiones se realizó en el Laboratorio de Propiedades Físicas y Sensoriales de Alimentos del Instituto de Agroquímica y Tecnología de los Alimentos (IATA-CSIC, Valencia, España) en colaboración con la empresa Archivel Technologies S.L. (Mataró, Barcelona, España). Además, se efectuaron estudios de estabilidad y pruebas para determinar la textura óptima (Giner, 2005).

Las emulsiones se realizaron mezclando 30 mL de agua y aceite, junto con el estabilizante y el emulsionante. La mezcla fue emulsionada mediante un homogeneizador Polytron PT 45/80 (Kinematica GmbH, Littau/Lucerna, Suiza) a 3000 rpm durante 3 min (Giner, 2005). Los estabilizantes probados fueron: goma Xantana (derivado del maíz, E-415), goma de Garrofín (obtenido de las semillas de algarrobo *Ceratonia siliqua*, E-410) y goma de Guar (obtenido a partir de *Cyamopsis tetragonolobus*, E-412). Los emulsionantes probados fueron: Tween 80 (monooleato de sorbitan polioxietilenado), montanox 80 VG PHA (monooleato de sorbitan polietoxilado), lecitina de soja y datem (ésteres de ácido diacetil tartárico de mono y diglicéridos).

La composición de ácidos grasos de las emulsiones resultantes se analizaron en el Instituto de Acuicultura de Torre de la Sal (IATS–CSIC, Castellón, España) mediante cromatografía gaseosa como se describe en la sección 3.3. Tras un periodo de almacenaje de aproximadamente 60 d, se

comprobó la estabilidad de las preparaciones emulsionantes y se probaron en enriquecimientos con nauplios de *Artemia* según se describe con más detalle en la sección 3.2.

Estos ensayos preliminares permitieron escoger aquella combinación de aceite, estabilizante y emulsionante que dio mejores resultados en cuanto a estabilidad de la emulsión y a la eficacia de enriquecimiento en términos de contenido de DHA en los nauplios.

La emulsión lipídica utilizada en los experimentos del presente estudio se formuló a partir de aceite DHA-Algatrium rico en DHA (70%) (Tabla 3.1) suministrado por Brudy Technology (Barcelona, España) y Archivel Technology (Barcelona, España). Su composición consistió en: 50/50 aceite:agua, 0,36% de goma Xantana (System Bio-Industries-BSI, Getafe, Madrid) como estabilizante y 0,1% de Tween 80 (Fluka, Sigma-Aldrich, St. Louis, Missouri) como emulsionante (Giner, 2005).

Tabla 3.1. Composición de DHA-Algatrium, aceite sintético utilizado en la preparación de la nueva emulsión M70. Análisis según ficha técnica.

Propiedades	**Límites**	**Resultados**
Índice de acidez	6 máx	5,50%
DHA	70 mín	73,90%
EPA	10 máx	19,70%
DPA	20 máx	1,90%
Ácidos grasos ω3 totales	85 mín	95,50%
Índice de peróxidos	5 máx	0,9 Meq/Kg
Metales pesados	2,0 máx	Nivel permitido
Cadmio	0,1 máx	Nivel permitido
Cobre	0,1 máx	Nivel permitido
Hierro	1,0 máx	Nivel permitido
Mercurio	0,1 máx	Nivel permitido
Plomo	0,1 máx	Nivel permitido
Estaño	2,0 máx	Nivel permitido
Arsénico	0,1 máx	Nivel permitido

Un aspecto importante a la hora de desarrollar las nuevas emulsiones es el tamaño de partícula que resulta al dispersar la emulsión en agua de mar. Un tamaño de micela (o gotícula) adecuado es esencial para ser eficientemente filtrado por las estructuras filtradoras de los nauplios de *Artemia* antes de ser incorporado en el tracto digestivo. Jones y Gabbott (1976) establecieron que el tamaño óptimo de partícula para filtradores pasivos está comprendido entre 5 y 100 µm. Además del tamaño, la eficiencia en la ingesta de partículas de emulsión

depende de su concentración en el medio de enriquecimiento (Han y cols., 2005).

En el IATA-CSIC, en colaboración con el Instituto de Ciencias de los Materiales de la Universidad de Valencia (ICMUV), se midió el tamaño de partícula de las emulsiones, tanto de las comerciales como de M70. El análisis de la distribución del tamaño de partícula se realizó con un analizador Malvern Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, Reino Unido), basado en la tecnología de difracción de láser. En el blanco óptico, la muestra es atravesada por un láser produciéndose la dispersión de la luz sobre las partículas iluminadas. Unos detectores miden la intensidad de la luz dispersada en función del ángulo de dispersión (Malvern, 2014). Mediante ésta técnica se consigue medir tamaños de partícula comprendidos entre 0,06 y 2000 µm (ICMUV, 2009). Los cálculos del tamaño de partícula se basan en la teoría de difusión de Mie y se realizaron con el software provisto con el equipo (Mastersizer 2000 v5.40). Las mediciones se realizaron por triplicado para cada muestra.

La emulsión comercial DC Super Selco (DCSS, Inve, Dendermonde, Bélgica) tuvo un diámetro de partícula de 0,5 µm, mientras que en el resto de emulsiones tales como Easy DHA Selco, Easy Super Selco (Inve, Dendermonde, Bélgica) y M70, se estimó un diámetro de partícula de 3 µm. Considerando los resultados del perfil de ácidos grasos en nauplios y la

distribución de tamaños, se concluyó que las diferencias encontradas en el tamaño de partículas fueron demasiado pequeñas para afectar a la eficiencia de la filtración de los nauplios de *Artemia*. Por lo tanto, las posibles diferencias encontradas en los perfiles de ácidos grasos de los nauplios se relacionan con la propia composición lipídica de las emulsiones.

Otro aspecto a tener en cuenta para estudiar la estabilidad de emulsiones enriquecedoras es la tendencia de algunas de éstas a formar grumos durante el proceso de enriquecimiento de presas vivas. Esto ensucia el medio de cultivo debido a que al filtrar los nauplios tras el enriquecimiento, los grumos se quedan retenidos junto a ellos y puede favorecer contaminaciones o infecciones de las larvas de peces. Se realizaron pruebas preliminares con y sin nauplios. M70 no formó grumos durante el proceso de enriquecimiento, pero DCSS formó pequeños grumos al enriquecer nauplios de *Artemia*.

3.2. Enriquecimiento de nauplios de *Artemia*

3.2.1. Eclosión de quistes de *Artemia*

Los quistes utilizados en todos los experimentos procedieron de lotes comerciales grado EG (Inve, Dendermonde, Bélgica), con bajo contenido en HUFA. Para la eclosión de quistes, se utilizó un recipiente de 60 L junto con un

calentador termostatizado a 28ºC en el que se sumergieron los tubos de eclosión. Éstos se llenaron con agua de mar y se aclimataron a la temperatura de 28 ± 1 ºC. La densidad de quistes fue inferior a 4 g L^{-1}, tal y como recomienda Van Stappen (1996). Los quistes fueron sometidos a agitación vigorosa por medio de aeración continua e iluminación constante mediante un tubo fluorescente que aportó aproximadamente 1500 lux al sistema.

Tras un periodo de 23 h el sistema de aireación se detuvo facilitando así la separación de los nauplios recién eclosionados, que se desplazaron al fondo del tubo de eclosión, de las cáscaras que flotaron en la parte superior del mismo. Las cáscaras fueron eliminadas por decantación y los nauplios recién eclosionados se filtraron por una malla de 100 µm. Los nauplios retenidos se enjuagaron con abundante agua corriente para eliminar los metabolitos de eclosión. A continuación, se vertieron en probetas de 1 o 2 L para hacer dos lavados más, uno con agua corriente y el último con agua de mar, con el fin de eliminar la mayor cantidad de quistes no eclosionados (Monroig, 2006). Tras los lavados, los nauplios se mantuvieron en agua de mar con aireación, logrando así una dispersión homogénea para llevar a cabo los contajes mediante el muestreo de seis alícuotas de 150 µL. Tras la estimación de la densidad naupliar obtenida en cada eclosión, se procedió al cálculo del volumen a transferir

a cada tubo de enriquecimiento para que la densidad final en los tubos de enriquecimiento fuera de 300 nauplios mL^{-1}.

3.2.2. Enriquecimiento de los nauplios de *Artemia*

El proceso de enriquecimiento supone incubar los nauplios recién eclosionados junto con el producto enriquecedor. La emulsión comercial DCSS (Inve) se utilizó como control en todos los experimentos. La concentración de producto enriquecedor en el medio de enriquecimiento se fijó en 420 mg lípido L^{-1} (cantidad que equivale a una dosis final de 0,6 g L^{-1} de producto comercial según recomienda el fabricante), expresada en términos de la masa de la fracción lipídica por unidad de volumen del medio de enriquecimiento (Monroig, 2006). Las emulsiones creadas para esta tesis doctoral, al contener en su formulación más cantidad de agua, necesitaron mayor cantidad de producto para conseguir la cantidad total de lípidos recomendada.

Tras pesar la cantidad necesaria de los enriquecedores correspondientes a la dosis requerida (420 g lípido L^{-1}), éstos se dispersaron en agua de mar con ayuda de una batidora y se añadieron a los tubos con los nauplios. Las condiciones de enriquecimiento fueron las mismas que las de eclosión, es decir temperatura de 28 ± 1 ºC, nivel de iluminación de 1500 lux y

aireación suficiente para que el oxígeno disuelto no descendiera de 4 mg L^{-1} (Van Stappen, 1996).

Tras un periodo de 21 h, la aireación se detuvo y el contenido de los tubos de enriquecimiento se filtró a través de una malla de 100 µm en la que quedaron retenidos los nauplios de *Artemia*. Éstos se lavaron con abundante agua corriente para eliminar los posibles restos de producto enriquecedor que hubieran quedado adheridos al nauplio. Finalmente se lavaron con agua destilada para eliminar restos de sal y se liofilizaron y congelaron a -20ºC hasta su análisis.

3.3. Análisis químico

3.3.1. Análisis de lípidos totales

La extracción de lípidos totales en muestras de nauplios de *Artemia* enriquecidos y emulsiones se realizó mediante una modificación del método propuesto por Folch y cols. (1957). Las muestras fueron liofilizadas se pesaron utilizando una balanza de precisión XS105 dual Ray (Mettler Toledo, L'Hospitalet de Llobregat, España) y homogeneizadas mecánicamente mediante disgregadores tipo Potter, con una mezcla de cloroformo:metanol (2:1, v/v) que contenía un 0,01% (p/v) de antioxidante hidroxitolueno butilado (BHT, Sigma, Alcobendas, España). Al volumen total de homogeneizado se le

añadió una cuarta parte de una disolución acuosa 0,88% de KCl. Tras agitar la muestra y centrifugarla a 2000 rpm durante 2 min, la fase orgánica (infranadante) se filtró a través de un filtro de papel (Whatman, Maidsteno, Reino unido). Se repitió el mismo proceso con el *pellet*, con el fin de maximizar la extracción de lípido. La fase orgánica obtenida tras el filtrado se secó bajo flujo de N_2. El extracto de lípido se trasvasó a un vial de vidrio pesado previamente y se secó completamente mediante flujo de N_2. La determinación de lípidos totales se realizó gravimétricamente tras 24 h en desecador al vacío. Los extractos de lípidos se mantuvieron a una concentración conocida en una mezcla de cloroformo:metanol (2:1, v/v) con un 0,01% de BHT (p/v), bajo atmósfera de N_2 desplazando el aire presente en los viales para minimizar la oxidación durante su almacenamiento a -20ºC.

3.3.2. Análisis de las clases lipídicas

Las clases lipídicas fueron analizadas por cromatografía de capa fina de alta resolución (*High Performance Thin Layer Chromatography*, HPTLC), según el método descrito por Olsen y Henderson (1989). Brevemente, 15 µg de los extractos lipídicos se aplicaron en placas de gel de sílice G 20 de 10x10 cm (Merck, Darmstadt, Alemania) mediante jeringas tipo Hamilton. A menos que se indique lo contrario, las placas se sometieron a una doble elución. En los primeros 5 cm de la

placa desde el punto de aplicación, los lípidos polares se separaron con un primer sistema de solventes compuesto por acetato de metilo, isopropanol, cloroformo, metanol y una solución acuosa de KCl 0,25% (p/v) en la proporción 25:25:25:10:9 (v/v/v/v/v). Tras el secado de la placa, se utilizó un segundo sistema de solventes compuesto por hexano, éter dietílico y ácido acético glacial, en una proporción 80:20:2 (v/v/v) que separó las clases lipídicas de tipo neutro. Este segundo sistema de solventes migró hasta el borde superior. A continuación se procedió a la evaporación de los restos de solvente mediante secado al vacío. Las placas fueron rociadas con una disolución acuosa 30% de $(CH_3CO)_2Cu$ (p/v) que contenía un 8% H_3PO_4 (v/v) (Fewster y cols., 1969) y se introdujeron en una estufa a 160ºC donde se carbonizaron las distintas fracciones de las muestras. La densidad óptica de las distintas clases de lípidos se cuantificó mediante un densitómetro de imágenes calibrado (GS-710 Bio-Rad, San Francisco, California, USA). Los resultados se expresaron en términos de porcentajes de cada clase lipídica en relación al total de lípidos.

La Figura 3.1 es un típico ejemplo de doble desarrollo con las diferentes clases de lípidos separadas e identificadas. La cuantificación se realizó tras la sustracción del ruido de fondo (en forma de mancha) que acompaña ineludiblemente a los frentes de elución.

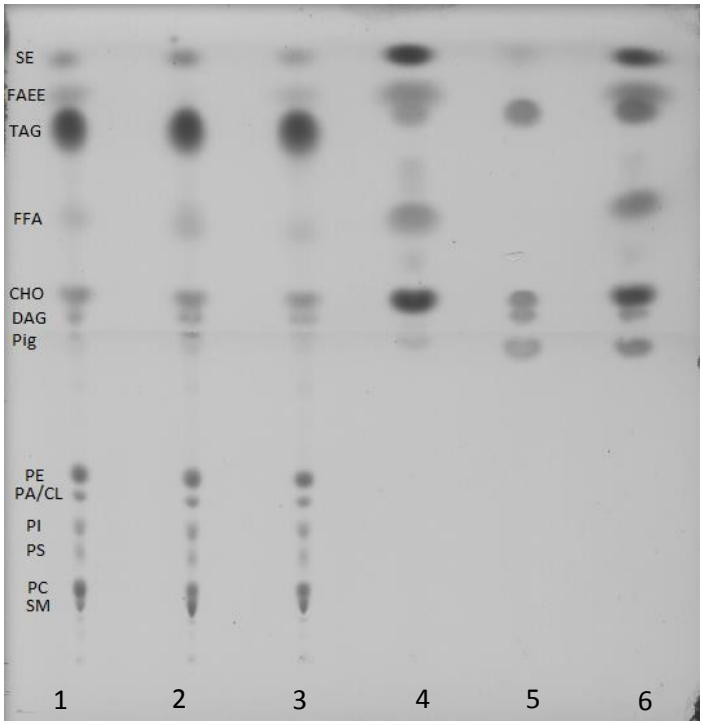

Figura 3.1. Placa de HPTLC (Merck, Darmstadt, Alemania) de doble desarrollo. Separación de clases lipídicas de lípidos polares y neutros de muestras de nauplios enriquecidos con: 1) emulsión M70, 2) emulsión con un 50% de DHA, 3) DCSS. Las carreras 4, 5 y 6 corresponden a estándares consistentes en una mezcla de lípidos neutros. Sm, esfingomielina; PC, fosfatidilcolina; PS, fosfatidilserina; PI, fosfatidilinositol; PA/CL, ácido fosfatídico/cardiolipina; PE, fosfatidiletanolamina; Pig, pigmentos; DAG, diacilglicéridos; CHO, colesterol; FFA, ácidos grasos libres; TAG, triacilglicéridos; FAEE, ésteres etílicos de ácidos grasos, SE, ésteres de esterol.

3.3.3. Análisis de ácidos grasos por cromatografía de gases

3.3.3.1. Derivatización de ácidos grasos

El análisis de ácidos grasos se realizó mediante cromatografía de gases a partir de los ésteres metílicos de los ácidos grasos (FAME, *Fatty Acids Methyl Esters*) obtenidos por derivatización de los extractos lipídicos de las muestras. La derivatización consistió en la transmetilación catalizada por ácido (Christie, 2003). Este proceso consiste en incubar una cantidad conocida del extracto lipídico en 1 mL de tolueno y 2 mL de una disolución 1% de H_2SO_4 en metanol a 50ºC durante un periodo aproximado de 16 h. A la muestra se le añadió el ácido graso 19:0 y que actuó como estándar y que sirvió para cuantificar los ácidos grasos presentes en la muestra.

Tras la transmetilación catalizada por ácido, los FAME se recuperaron mediante dos extracciones líquido:líquido consecutivas. La primera se realizó añadiendo a la muestra incubada 5 mL de agua destilada y 5 mL de hexano:éter dietílico (1:1, v/v) con un 1% de BHT. Tras su agitación y centrifugación durante 2 min a 2000 rpm, se recogió el sobrenadante. Sobre el infranadante se realizó un segundo lavado con 5 mL de hexano:éter dietílico (1:1, v/v) para maximizar la recuperación de los FAME. Tras una nueva agitación y centrifugación, el sobrenadante se añadió al anterior y, sobre éste, se realizó una

segunda extracción añadiendo 3 mL de una disolución al 2% de $KHCO_3$ (p/v) a la fase sobrenadante recogida. Tras agitar la muestra, se centrifugó durante 2 min a 2000 rpm y la fase sobrenadante se trasvasó a otro tubo donde se secó con N_2 seco. La muestra seca fue transferida a un vial de vidrio, donde se conservó disuelta en 140 µL de hexano:éter dietílico (1:1, v/v) con una 1% de BHT disuelto, hasta la realización de la purificación de los FAME.

3.3.3.2. Purificación de FAME

La purificación de los FAME recogidos se llevó a cabo mediante cromatografía de capa fina en placas TLC (*Thin Layer Chromatography*) de gel de sílice G 60 de 20x20 cm (Merck, Darmastadt, Alemania). El solvente utilizado fue una mezcla de hexano:éter dietílico:ácido acético glacial (85:15:1,5, v/v/v). La adición de un estándar en la misma placa, permitió estimar la posición de la fracción de los FAME de las muestras tras su elución en el sistema de solventes indicado. La fracción correspondiente a cada muestra se recuperó mediante el rascado del gel de sílice en el área donde se hallaba la fracción de FAME. Los FAME presentes en el gel de sílice se eluyeron con hexano:éter diétilico (1:1, v/v) mediante agitación suave y posterior centrifugación de 2 min a 2000 rpm. La fracción líquida se trasvasó a unos nuevos tubos donde se llevó a cabo el secado con N_2 seco. Los FAME purificados se trasvasaron a un

vial de vidrio mediante en 230 µL de hexano con un 0,01% de BHT hasta el posterior análisis cromatográfico.

3.3.3.3. Condiciones cromatográficas

Los ácidos grasos fueron analizados en un cromatógrafo Fisons Instruments GC 8000 Series (Fisons Instruments, Rodano, Italia) equipado con una columna tubular abierta de 30 m x 0,25 mm (Tracer, TRWAX, espesor de capa: 0,25 µm, Tecknokroma, Barcelona, España) y detección por ionización de llama. Las muestras se eluyeron con helio como único componente de la fase móvil, bajo un gradiente térmico de 50 a 220ºC. Las señales procedentes de un detector de llama (Fisons Instruments, Rodano, Italia) se recogieron mediante un sistema informático equipado con la aplicación Azur software package (versión 4.0.2.0. Datalys, France). La identificación de los diferentes ácidos grasos se realizó por comparación con estándares bien caracterizados como el que aparece en la Figura 3.2.

3.3.4. Análisis de la peroxidación lipídica

La reacción de peroxidación lipídica consta de los siguientes pasos: a) formación de radicales libres, iniciando el proceso de oxidación; b) formación de hidroperóxidos como

productos primarios de reacción; c) formación de productos secundarios de oxidación; y d) formación de productos terciarios de oxidación (Kamal-Eldin yPokorny, 2005).

La determinación de la peroxidación lipídica presente en las muestras de nauplios de *Artemia* enriquecidos, en las emulsiones lipídicas y en el medio de enriquecimiento de los nauplios, se realizó mediante una variación del método del ácido tiobarbitúrico (*Thiobarbituric Acid Reactive Substances*, TBARS) propuesto por Ohkawa y cols. (1979) o por la medición de los niveles de peroxidación lipídica (LPO) (Esterbauer y cols., 1991).

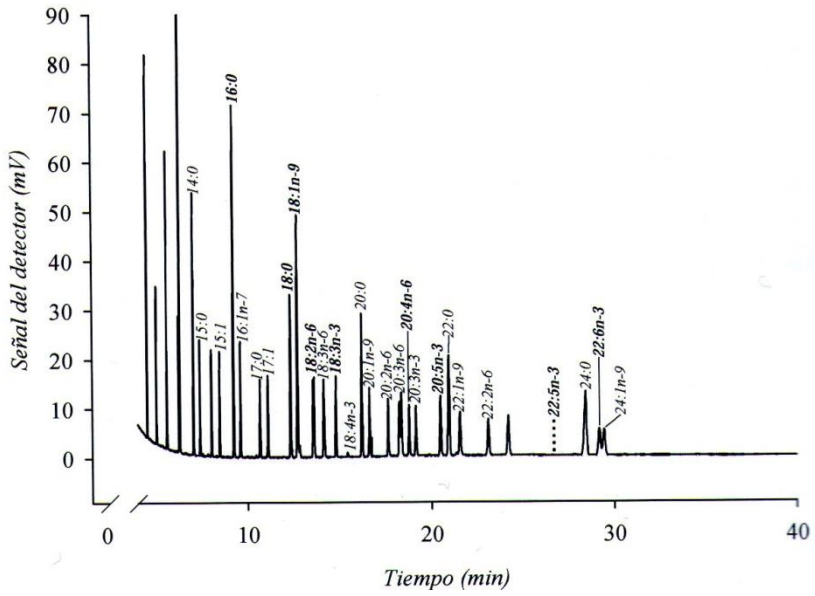

Figura 3.2. Separación de ésteres metílicos de ácidos grasos mediante una técnica de GC con una columna tubular abierta y detección por ionización de llama de un patrón comercial (37 Component FAME Mix de Supelco (Sigma-Aldrich Co.).

El método TBARS se basa en la reacción, bajo condiciones controladas de temperatura y pH, del malondialdehido (MDA) con el ácido tiobarbitúrico (TBA), que forman un complejo coloreado que se puede medir espectrofluométricamente (máximo de absorbancia a 532 nm; λex = 532 / λem = 553). En tubos de polipropileno de 2 mL se mezclaron 36 µL de una disolución acuosa 8,1% (p/v) de dodecil sulfato de sodio (SDS) con un 0,05% de BHT, 270 µL de una solución pH 3,5 al 20% (v/v) de ácido acético glacial, 270 µL de una disolución 0,8% (p/v) de TBA en agua destilada y 144 µL de muestra de nauplios homogeneizados o del medio de enriquecimiento (Monroig y cols., 2007). Los tubos se cerraron herméticamente y se incubaron durante 60 min en un baño a 95ºC. Pasado este periodo, la reacción se detuvo enfriando los tubos sumergiéndolos en agua con hielo durante aproximadamente 3 min, y se les añadió 180 µL de agua destilada y 900 µL de la disolución n-butanol:piridina (15:1, v/v). Tras su agitación para favorecer la mezcla de ambas fases, se centrifugaron durante 3 minutos a 10000 rpm a 4 ºC. La fluorescencia de la fase orgánica (sobrenadante) se midió en un espectrofluorímetro F-2500 Hitachi (Tokio, Japón) a longitudes de onda de excitación y emisión de 515 y 530 nm respectivamente. La concentración de peróxidos se calculó a través de una curva patrón obtenida con disoluciones de concentraciones crecientes del compuesto tetratoxipropano

(TEP), que fueron sometidas a las mismas condiciones que la muestra.

El método de LPO también se basa en la reacción del MDA pero en esta ocasión con el N-metil-2-fenilindol (NMPI) para formar un complejo coloreado con una absorbancia conocida (586 nm) (Esterbauer y cols., 1991). Mediante éste método la detección de otros productos de la peroxidación lipídica que interfieren en los resultados, como el 4-hidroxialkenal, se minimiza.

3.4. Análisis estadístico

Todos los análisis estadísticos se llevaron a cabo usando el programa estadístico SPSS versión 15.0 (SPSS Inc., Chicago, Illinois, EE.UU). La condición de normalidad de las variables fue analizada mediante la prueba de Kolmogorov-Smirnov (Sokal y Rohlf, 1981) y se realizaron las transformaciones más adecuadas para aquellas variables que no presentaban una distribución normal. La homogeneidad de las varianzas se analizó mediante la prueba de Levene (SPSS).

Las diferencias entre medias de variables evaluadas en dos tratamientos experimentales (dos niveles de factor de variación) se comprobaron mediante la prueba T de Student para muestras independientes. Las diferencias entre medias de variables

evaluadas en más de dos tratamientos experimentales (más de dos niveles de variación del factor), se determinaron mediante el análisis de la varianza (*Analysis of Variance*, ANOVA), seguido de la prueba de Tukey para la comparación múltiple de medias (Sokal y Rohlf, 1981).

En el caso de existir heterogeneidad de varianzas se usaron las pruebas robustas de Welch, para determinar diferencias entre medias y de Games-Howell para el establecimiento de diferencias entre grupos (SPSS Inc., Chicago, Illinois, EE.UU). Estas dos pruebas estadísticas no requieren homogeneidad de las varianzas, al contrario de las pruebas ANOVA y Tukey. El nivel de significación en todas las pruebas estadísticas se fijó en 0,05.

Los resultados de los ácidos grasos fueron analizados mediante un análisis multivariante de componentes principales (*Principal Component Analysis*, PCA). El PCA es una técnica multivariante encuadrada dentro del grupo de técnicas de simplificación o reducción de la dimensión. Con esta metodología, el conjunto de variables (los ácidos grasos) es reducido a una serie menor de componentes. El primer componente principal (PC1) recoge la mayor parte de la variación que hay en el conjunto de datos, y los siguientes componentes contienen tanta variabilidad como sea posible dentro de la restante. Hay varios criterios para decidir el número de factores que permita definir la estructura correcta de los datos

y que posibilite su posterior interpretación. Los criterios más importantes son el porcentaje de la varianza explicada y el contraste de caída o gráfico de sedimentación de Catell (1966).

Los factores (componentes) pueden ser reflejados en gráficas junto con las variables medibles para dar una idea de su correlación con el factor correspondiente. Por tanto, el gráfico de puntuaciones, que es una representación gráfica de los valores individuales en los PC, ilustra la relación entre los casos individuales y las variables.

Como paso previo al PCA hay que analizar la idoneidad estadística utilizando para ello la prueba de esfericidad de Bartlett y la medida de Kaiser-Meyer-Oklin (KMO). La prueba de esferidad de Barlett permite contrastar la hipótesis nula de que la matriz de correlaciones es una matriz de identidad, cuya aceptación implicaría el replanteamiento de la utilización del PCA. La medida de adecuación de KMO es útil para comparar los valores de los coeficientes de correlación observados con los coeficientes de correlación parcial, de modo que valores pequeños indican que el PCA no es aconsejable. Kaiser (1970) recomienda como límite de aceptación de este índice KMO valores superiores a 0,6 (Ruiz, 2008).

3.5. Bibliografia

Catell, R.B., 1966. The screen test for the number of factors. Multivariate Behavioral Research 1, 245-276.

Christie, W.W., 2003. Lipid analyses, third ed. Oily Press, Bridgwater.

Dickinson, E., 1993. Towards more natural emulsifiers. Trends in Food & Science Technology 4, 330-334.

Esterbauer, H., Schaur, R.J., Zollner, H., 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radical Biology & Medicine 11, 81-128.

Fewster, M.E., Burns, B.J., Mead, J.F., 1969. Quantitative densitometric thin-layer chromatography of lipids using copper acetate reagent. Journal of Chromatography 43A, 120-126.

Folch, J., Lees, M., Sloane-Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry 226, 497-509.

Giner, E., 2005. Desarrollo de emulsiones de alto contenido en ácidos grasos poliinsaturados para complementar el valor nutritivo de presas vivas en acuicultura. Tesis licenciatura. Universidad politécnica de Valencia, España. pp.75.

Han, K., Geurden, I., Van der Meeren, P., Bai, S.C., Sorgeloos, P., 2005. Particle size distribution in two lipid emulsion used for the enrichment of *Artemia* nauplii as a function of their preparation method and storage time. Journal of the World Aquaculture Society 36, 196-202.

ICMUV (Instituto Universitario de Ciencia de los Materiales): Guía Malvern Mastersizer 2000. Accesible en la web: Accesible en la web: http://www.uv.es/icmuv/c/inve/tec24.htm.

Jones, D.A. y Gabbott, P.A., 1976 Prospects for the use of microcapsules as food particles for marine particulate feeders. In J.R. Nixon (Editor), Microencapsulation, New York, Marcel Dekker, Inc. pp. 77–91.

Kaiser, H.F., 1970. A second generation little jiffy. Psychometrika 35, 401-415.

Kamal-Eldin, A. y Pokorny, J., 2005. Lipid oxidation products and methods used for their analysis. In: Kamal-Eldin, A., Pokorny, J. (Editors), Analysis of lipid oxidation. AOCS press. Champain, Ill. USA. 1-7 pp.

Malvern, 2014. Mastersizer 2000: brochure. Accesible en la web: http://www.malvern.com/en/products/product-range/mastersizer-range/mastersizer-2000.

Monroig, O., 2006. Diseño y optimización de liposomas para su uso como sistema de suministro de nutrientes a larvas de peces marinos. Tesis doctoral. Universidad de Valencia, España. pp.239.

Monroig, O., Navarro, J.C., Amat, F., Hontoria, F., 2007. Enrichment of *Artemia* nauplii in vitamin A, vitamin C and methionine using liposomes. Aquaculture 269, 504-513.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry 95, 351-358.

Olsen, R.E. y Henderson, R.J., 1989. The rapid analysis of neutral and polar marine lipids using double-development HPTLC and scanning densitometry. Journal of Experimental Marine Biology and Ecology 129, 189-197.

Ruiz, O., 2008. Caracterización de diversas poblaciones de *Artemia* desde el punto de vista de su composición en ácidos grasos y de sus patrones moleculares. Tesis doctoral. Universitat de València, España. pp. 232.

Sokal, R.R., Rohlf, F.L., 1981. Biometry. The principles and practice of statistics in biological research. 2nd ed. W.H. Freeman &Co., New York. pp. 859.

Van Stappen, G., 1996. Introduction, biology and ecology of *Artemia*. En: Lavens, P., Sorgeloos, P., (Eds.), Manual on the production and use of

live food for aquaculture (FAO). Fisheries Technical Paper Nº 361. Roma, Italia, pp. 101-121.

CAPÍTULO 4

Enriching *Artemia* nauplii with a high DHA-containing lipid emulsion: search for an optimal protocol

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Abstract

The present study aimed to investigate practical strategies to optimise the use of a high-DHA lipid emulsion (M70), a product with great potential in live prey enrichments for marine larviculture. Considering its particularly high content in docosahexaenoic acid (22:6n-3, DHA), the adequate utilisation of the emulsion for *Artemia* enrichments was evaluated in a series of six experiments. More specifically, the bioencapsulation efficiency of M70 into *Artemia* nauplii was tested under different experimental conditions of oxygen source, aeration flow, incubation temperature, concentration and dosage, as well as nauplial densities. Our results showed that an optimal utilisation of M70 is achieved with incubation temperatures of 28 ºC, moderate aeration flows and nauplial densities of 300 ind mL^{-1}. In addition, the emulsion can be dispensed in the enrichment medium in one single dose of 0.8 g L^{-1}, with no apparent detrimental effects on its oxidative stability and *Artemia* nauplii survival during enrichment.

Keywords: Artemia*; docosahexaenoic acid; essential fatty acid; lipid emulsion; live prey enrichment*

Resumen

El presente estudio tiene como objetivo investigar las estrategias prácticas para optimizar el uso de una emulsión con un elevado contenido en DHA (M70), un producto con un gran potencial en el enriquecimiento de presas vivas destinadas a la larvicultura marina. Teniendo en cuenta su particularmente alto contenido en ácido docosahexaenoico (22:6n-3, DHA), se evaluó la adecuada utilización de la emulsión para enriquecimientos de Artemia *en una serie de seis experimentos. Más concretamente, se analizó la eficiencia de bioencapsulación de M70 en los nauplios de* Artemia *bajo diferentes condiciones experimentales de fuente de oxígeno, flujo de aireación, temperatura de incubación, concentración y dosis del enriquecedor y densidad naupliar. Nuestros resultados mostraron que una utilización óptima de M70 se consigue mediante la incubación a 28 ºC, flujos de aireación moderados y densidades de 300 ind* mL^{-1}*. Además, la emulsión se puede dispensar en el medio de enriquecimiento en una única dosis de 0,8 g L-1, sin ningún perjuicio en su estabilidad oxidativa y supervivencia de los nauplios de* Artemia *durante el enriquecimiento.*

4.1. Introduction

Marine fish, and particularly their larval stages, have high requirements for highly unsaturated fatty acids (HUFA), including the physiologically active arachidonic (20:4n-6, ARA), eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic (22:6n-3, DHA) acids. Whereas the essential fatty acids (EFA) requirements of freshwater and salmonid species can be met by including the C18 polyunsaturated fatty acids (PUFA) α-linolenic acid (18:3n-3, LNA) and linoleic acid (18:2n-6, LA) (Buzzy *et al*. 1996; Bell *et al*. 2004), it is generally accepted that marine species have limited capability for the biosynthesis of HUFA from the C18 PUFA, although, exceptionally, some species have been proved to express genes encoding fatty acyl desaturases and elongases involved in these biochemical pathways (Yamamoto *et al*. 2010; Monroig *et al*. 2011; Morais *et al*. 2011). In consequence, adequate levels of pre-formed ARA, EPA and DHA need to be supplied through the diet to ensure normal growth and development (Bell *et al*. 1995, 2003; Estévez *et al*. 1998, 1999; Sargent *et al*. 2002; Tocher *et al*. 2003; Benitez- Santana *et al*. 2007).

Artemia nauplii are widely used as live prey in marine fish hatcheries worldwide, due to their availability and digestibility (Léger *et al*. 1986). The nutritional value of *Artemia* nauplii, however, is not adequate for marine fish larvae since they are largely deficient in essential HUFA, particularly DHA (Koven *et*

al. 1990). In order to overcome the problem, *Artemia* nauplii are enriched through incubations in seawater containing HUFA-rich products that are passively incorporate by the nauplii (Léger *et al*. 1986). This procedure is called bioencapsulation (Navarro *et al*. 1999). Many different enrichment diets have been used to enhance *Artemia* EFA contents: microalgae (Watanabe *et al*. 1982; Aragão *et al*. 2004), microcapsules (Southgate & Lou 1995), liposomes (Hontoria *et al*. 1994; McEvoy *et al*. 1996; Monroig *et al*. 2003; 2006a, b, c), and lipid emulsions (Léger *et al*. 1986; Evjemo *et al*. 1997; Han *et al*. 2000; 2001; 2005).

Lipid emulsions are arguably the most extended enrichment diets and a big variety of products are now commercially available. Composition of commercial lipid emulsions basically consists of fish oils, with other minor components including emulsifying agents, vitamins and fatty acid (FA) derivatives such as fatty acid ethyl esters (FAEE) added to compensate suboptimal natural profiles of fish oil (Monroig *et al*. 2007). Whereas the efficiency of enrichment protocols with emulsified diets is normally higher than that of other enrichment products (Coutteau & Sorgeloos 1997; Sorgeloos *et al*. 2001), their use has been related with detrimental side effects. Among them, the autoxidation of HUFA (McEvoy *et al*. 1995; Sargent *et al*. 1997) and the consequent bioaccumulation of potentially toxic lipid peroxides into larvae fed emulsion-enriched *Artemia* (Monroig *et al*. 2006)

have been described. Furthermore, autooxidation of HUFA-rich products might deplete dissolved oxygen (DO) levels in the enrichment medium and thus can further compromise the overall performance of the enrichment process as well as favour *Artemia* mortalities (Léger *et al*. 1987; Figueiredo *et al*. 2009). Importantly, the most hindering and yet unresolved drawback from the use of emulsion-based enrichers involves specific difficulties in increasing the DHA content of nauplii.

The low efficiency of DHA enrichment in *Artemia* nauplii has been acknowledged as a major obstacle for their use as live prey for first-feeding larvae of marine fish (Bell *et al*. 2003; Haché *et al*. 2011). While the bioencapsulation of other HUFA including EPA and ARA has been readily achieved (Villalta *et al*. 2005a, b), specific factors determining the limited DHA incorporation into *Artemia* appear to exist. On one hand, the autooxidation mechanisms mentioned above might be particularly severe for DHA compared to other HUFA, due to its longer chain length and higher unsaturation degree (Cosgrove *et al*., 1987). On the other hand, biological factors related to *Artemia* physiology also need to be considered. Naturally occurring trace levels of DHA found in *Artemia* lipids (Evjemo *et al*. 1997) suggest that *Artemia* DHA requirements are low, and excessive DHA input occurring during enrichment might be compensated through DHA retroconversion to EPA (Watanabe *et al*. 1983; Furuita *et al*. 1996; Barclay & Zeller 1996; Evjemo

et al. 1997; Navarro *et al*. 1999). In order to overcome such adverse effects, novel enrichment strategies to enhance the DHA contents of *Artemia* nauplii need to be developed.

In the present study, we investigated the efficiency of a DHA-rich oil emulsion (M70) as enrichment diet for *Artemia* nauplii. Considering the particular aspects derived from its DHA-rich nature, a series of experiments were carried out to establish an optimal use of M70 under different oxygenation regimes, incubation temperatures, enrichment product concentrations and dosage modes, as well as different nauplial densities. All the investigated parameters were previously shown as relevant aspects during live prey enrichment procedures (Van Stappen 1996; Han *et al*. 2000, 2001; Monroig *et al*. 2006a, b; Sui *et al*. 2007; Hamre & Harboe 2008; Figueiredo *et al*. 2009).

4.2. Materials and methods

4.2.1. Emulsion formulation

The experimental lipid emulsion M70 consisted of a 1:1 (v/v) oil/water suspension, of which the synthetic oil DHA Algatrium (Brudy, Barcelona, Spain) contained approximately 70% DHA of total FA in the form of ethyl esters. Xanthan gum (0.36 %) and Tween 80 (0.10 %) were used as stabiliser and emulsifier, respectively (Giner 2005). M70 formulation was

developed by Archivel Technologies S.L. (Barcelona, Spain) and the The Physical and Sensory Properties Laboratory at the Instituto de Agroquímica y Tecnología de los Alimentos (IATA-CSIC, Valencia, Spain). The FA profiles of the M70 emulsion are reported in Table 4.1.

4.2.2. *Artemia* nauplii enrichments: general conditions

Low HUFA containing *Artemia* nauplii were obtained from the hatching of EG grade cysts (Inve, Ghent, Belgium). After an incubation period of 23 h at 28 ºC, nauplii were collected and rinsed with tap water to remove the hatching metabolites and debris. Newly hatched nauplii were thereafter placed in 1 L cylinder-conical glass vessels containing seawater for further enrichment with the experimental emulsion M70. Unless otherwise stated, *Artemia* enrichments were carried out at 28 ºC, aeration of 1 L min^{-1}, diffusion system consisting of a 25-cm long and 0.5-cm-diameter section glass tube applied from the bottom of the vessel, nauplial density of 300 nauplii mL^{-1}, and product concentrations of 0.8 g L^{-1} for M70 dispensed in a single dose at the beginning of the enrichment process. All the enrichment treatments were run in triplicates (n=3). After an enrichment period of 21 h, samples of *Artemia* nauplii were collected by filtering the enrichment medium through a 100 µm mesh carefully washed with tap water in order to eliminate remains of emulsion adhered to the nauplial shells, and

subsequently rinsed with distilled water. *Artemia* samples were frozen at -20 ºC and freeze-dried previous to FA analysis.

Table 4.1. Fatty acid composition (percent of total fatty acids) of the enrichment products M70 and DC Super Selco (DCSS, Inve, Ghent, Belgium) utilised in the present study

Fatty acid	M70	DCSS
14:0	0.1	1.3
16:0	0.9	5.2
16:1n-7	0.4	1.9
18:0	0.4	2.7
18:1n-9	3.7	9.2
18:1n-7	0.5	1.6
18:2n-6	0.7	4.8
18:3n-3	0.6	1.0
18:4n-3	0.4	1.8
20:0	N.D.	0.4
20:1n-9	0.3	2.3
20:3n-6	0.1	0.1
20:4n-6	2.4	1.6
20:3n-3	0.1	0.2
20:4n-3	0.5	1.1
20:5n-3	7.7	31.6
22:0	0.1	0.4
22:1n-11	0.1	0.2
22:5n-3	2.7	2.2
22:6n-3	70.3	20.6
Saturated	1.8	10.3
Monounsaturated	5.2	18.5
Polyunsaturated	91.1	66.2
HUFA n-3	81.3	55.6
HUFA n-6	8.0	2.6
DHA/EPA ratio	9.1	0.6

N.D.: not detected; HUFA n-3: ≥20:3n-3; HUFA n-6: ≥20:2n-6; DHA/EPA: docosahexaenoic and eicosapentanoic fatty acid ratio.

4.2.3. *Artemia* nauplii enrichment with M70: protocol optimisation

Optimal conditions for the use of M70 in *Artemia* enrichments were assessed by analysing the FA composition of the nauplii obtained from a series of experiments (1-6) varying the type and source of aeration, temperature, dosage and concentration of enrichment product and nauplial density. Conditions for each subsequent experiment were established based on the results from preceding experiments (Table 4.2).

4.2.3.1. Experiment 1: Effects of oxygen source

The effects of air quality on the enrichment efficiency of the emulsion M70 were tested. Three different treatments, all providing a fairly constant dissolved oxygen content above the recommended 4 mg L^{-1} (Van Stapen, 1996), were tested: "oxygen", with pure oxygen; "air", with compressed air; and "mixture", with both pure oxygen and aeration.

4.2.3.2. Experiment 2: Effects of aeration

In order to assess the effects of the aeration level in the *Artemia* enrichment with M70, three different air flows were tested: "1 lpm", "2 lpm" and "3 lpm" with air flows of 1.0, 2.0 and 3.0 L min^{-1}, respectively, being used. Air flows were individually controlled in each enrichment vessel by means of a rotameter (Key Instruments, Trevose, PA, USA).

Table 4.2. Experimental conditions set up in the experiments carried out in the present study. DC Super Selco (DCSS, Inve, Ghent, Belgium) was used as control treatment in Experiment 6 as indicated in Materials and Methods

	Treatment	Oxygen source	Air flow (L min^{-1})	Temp. (ºC)	Product concentration (g L^{-1})	Doses	Nauplial density (nauplii mL^{-1})
Exp 1	oxygen	Oxygen	1	28	0.8	1	300
	air	Air	1	28	0.8	1	300
	mixture	Ox.+Air	1	28	0.8	1	300
Exp 2	1 lpm	Air	1	28	0.8	1	300
	2 lpm	Air	2	28	0.8	1	300
	3 lpm	Air	3	28	0.8	1	300
Exp 3	24C	Air	1	24	0.8	1	300
	28C	Air	1	28	0.8	1	300
Exp 4	0.6	Air	1	28	0.6	1	300
	0.8	Air	1	28	0.8	1	300
Exp 5	2 doses	Air	1	28	0.8	2	300
	1 dose	Air	1	28	0.8	1	300
Exp 6	low dens	Air	1	28	0.8	1	150
	high dens	Air	1	28	0.8	1	300
	DCSS	Air	1	28	0.6	1	300

4.2.3.3 Experiment 3: Effects of temperature

The effects of temperature in the enrichment process were assessed by incubating the nauplii in the presence of M70 at 24 ºC (treatment "24C") or 28 ºC (treatment "28C"). The temperature was kept (± 1 ºC) constant by placing the enrichment vessels in a thermostatic bath.

4.2.3.4 Experiment 4: Effects of enrichment product concentration

The effects of the M70 concentration on the enrichment efficiency were evaluated by enriching *Artemia* at two different concentrations: 0.8 g M70 L^{-1} (treatment "0.8"), this concentration being equivalent to 0.6 g L^{-1} recommended for commercial emulsions after correction for the water content difference; and 0.6 g M70 L^{-1} (treatment "0.6").

4.2.3.5. Experiment 5: Effects of product dosage

In order to assess the effects of the enrichment product dosage on *Artemia* bioencapsulation, two experimental treatments were established: "2 doses", with M70 emulsion being splitted into two doses supplying 0.4 g L^{-1} at the beginning of the enrichment process and 0.4 g L^{-1} after 7 h; "1 dose", with the enrichment diet M70 being supplied in a single dose of 0.8 g L^{-1} at the beginning of the enrichment process.

4.2.3.6 Experiment 6: Effects of nauplial density

The effect of nauplial density in the *Artemia* enrichment with M70 was evaluated by incubating the nauplii at two different densities, 150 (treatment "low dens") and 300 nauplii mL^{-1} (treatment "high dens"). As the last of our experiments aiming at optimisation of M70 as enrichment diet, Experiment 6

was carried out under those experimental conditions (oxygen source, aeration flow, temperature, product concentration and product dosage) that had produced the optimal enrichment results in the preceding Experiments 1-5. Moreover, we compared the enrichment performance of M70 with that of DC Super Selco ("DCSS", Inve, Ghent, Belgium), a commercial product basically consisting of a fish oil emulsion. DCSS enrichment was carried out under the same conditions of M70, except for the product concentration, 0.8 g L^{-1} for M70, and 0.6 g L^{-}1for DCSS, to compensate for the different water content of both products (50 % and 30 % for M70 and DCSS, respectively), as mentioned before.

4.2.4. Total lipids and FA analyses

Total lipids were extracted (Folch *et al*. 1957) from nauplii freeze-dried samples, measured gravimetrically (XS105 Mettler Toledo, Switzerland), and stored in chloroform: methanol (2:1; v/v) containing 0.01% butylated hydroxytoluene (BHT) at -20 ºC until further use. Total lipids were subjected to acid catalysed transmethylation for 16 h at 50 ºC using 1 mL toluene and 2 mL of 1% (v/v) sulphuric acid in methanol (Christie, 2003). Fatty acid methyl esters (FAME) were extracted with hexane:diethyl ether (1:1; v/v) containing 0.01% BHT and purified by thin-layer chromatography (Silica gel G60, 20 x 20 cm glass plates, Merck, Darmstadt, Germany) using hexane:diethyl ether:acetic

acid (85:15:1.5; v/v/v) as a solvent system. FAME were then analysed with a Fisons Instruments GC 8000 Series (Rodano, Italy) gas chromatograph. Peaks were recorded using the Azur software package (version 4.0.2.0. Datalys, France). Individual FAME were identified by comparison with known standards. The relative amount of each FA was expressed as a percentage of the total amount of FA.

4.2.5. Statistical analysis

Analytical data were expressed as means ± standard deviations (n=3). Differences between treatments were analysed by one-way analysis of variance (ANOVA), followed by either Bonferroni's multiple comparison test or a Student's *t*-test when only two groups were compared (Sokal & Rohlf, 1981). If heterogeneity of variances existed, Welch test was used to detect differences, followed by Games-Howell test to assess the differences between groups. When significance was $P \leq 0.05$, means were considered statistically different. The FA analytical data from Experiment 6, for which a comparison with the commercial product DCSS was carried out, were further analysed by multivariate Principal Components Analysis (PCA), in order to highlight the effects of factors enrichment diet and nauplial density. Statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, Illinois, USA).

4.3. Results

4.3.1 Experiment 1: Effect of oxygen source

Whereas the DHA bioencapsulation into *Artemia* nauplii did not show statistical differences among the treatments in Experiment 1, the levels of EPA and ARA in nauplii from "air" treatment were significantly higher than those of treatments "oxygen" and "mixture" (Table 4.3). Thus, the DHA/EPA ratio also showed statistically significant differences and *Artemia* from "oxygen" treatment had higher DHA/EPA values (1.8 ± 0.0) in comparison with nauplii from treatments "air" and "mixture" (1.6 ± 0.1). As a preliminary conclusion from Experiment 1, DHA levels were similar in nauplii from all treatments and thus use of pure oxygen did not improve the enrichment efficiency of a more readily available source like compressed air.

4.3.2 Experiment 2: Effect of aeration

The FA profiles of the *Artemia* nauplii enriched under different air flows ("1 lpm", "2 lpm" and "3 lpm") are shown in Table 4.3. Our results suggested that M70 enrichments are more efficient under relatively low air flows. Thus, the DHA contents of *Artemia* from treatment "1 lpm" were significantly higher than those of *Artemia* from treatment "3 lpm". This was also reflected in the DHA/EPA ratio of both treatments, being 1.4

and 1.3 for "1 lpm" and "3 lpm" treatments, respectively. Experiment 2 results allowed us to conclude that enrichment procedures at 1 L min^{-1} improved M70 bioencapsulation efficiency.

4.3.3 Experiment 3: Effect of temperature

Table 4.3 shows the FA profiles of *Artemia* nauplii enriched with M70 at 24 and 28 ºC. Our results indicated that the contents of ARA, EPA and DHA, as well the ratio DHA/EPA, were significantly higher in nauplii incubated at 28 ºC than at 24 ºC. Overall, 28 ºC was confirmed to be a more adequate incubation temperature for M70 enrichments compared to 24 ºC.

4.3.4 Experiment 4: Effect of product concentration

Experiment 4 compared the HUFA bioencapsulation performance of M70 dispensed to *Artemia* nauplii at product concentrations of 0.6 or 0.8 g L^{-1}. Generally, no differences among any of each individual FA analysed were detected between both treatments ($P>0.05$), possibly due to a remarkably high variability in the FA contents of "0.6" nauplii. Despite no significantly different, average DHA contents in nauplii from treatment "0.8" (8.3 ± 0.2) were still higher than those of "0.6" nauplii (6.6 ± 2.0) (Table 4.3). Moreover, statistically significant increases of the DHA/EPA ratio were observed for M70 concentrations of 0.8 g L^{-1}. We could therefore conclude that an

M70 concentration of 0.8 g L^{-1} produced better bioencapsulation results than 0.6 g L^{-1}.

4.3.5 Experiment 5: Effect of product dosage

The results from Experiment 5 indicated that dispensation of the enrichment product M70 in one or two doses did not produce differences in the FA profiles from *Artemia* lipids (Table 4.3). It was concluded that dispensing the product M70 in one single dose at the beginning of the enrichment process was a more practical and simpler strategy.

4.3.6 Effects of nauplial density

No effect of the nauplial density (150 and 300 nauplii mL^{-1}) on the enrichment performance with M70 was observed. Thus, no significant differences among any of the FA analysed could be established. Interestingly, a notable impact on *Artemia* FA profiles was observed when comparing the enrichment products M70 and the commercial emulsion DCSS, the control treatment in Experiment 6. The enrichment comparisons with DCSS showed that differences were mainly attributable to the effect of enrichment product (Table 4.3). This was further corroborated by the PCA, with the first component (PC1) explaining 92.5 % and the second 6.5 % (Fig. 4.1a), and subsequent score plot (Fig. 4.1b) that revealed two groups separated in the first component on the basis of the enrichment

Table 4.3. Fatty acids (percentage of total fatty acids) from enriched *Artemia* nauplii collected from Experiment 1-6 (see Materials and Methods for treatment details). Data represent means ± standard deviations (n=3).Treatments in each Experiment with different letter are significantly different ($P \leq 0.05$). If no superscript appears, values are not different.

	Experiment 1			Experiment 2			Experiment 3		Experiment 4		Experiment 5		Experiment 6		
% Fatty acid	oxygen	air	mixture	1 lpm	2 lpm	3 lpm	24C	28C	0.6	0.8	2 doses	1 dose	low dens	high dens	DCSS
14:0	0.5±0.0	0.5±0.0	0.6±0.3	0.6±0.0	0.7±0.0	0.6±0.0	0.5±0.0	0.5±0.0	1.0±0.7	0.6±0.0	0.6±0.0	0.6±0.0	0.6±0.0^{a}	0.6±0.0^{a}	0.8±0.0^{b}
15:0	0.5±0.0	0.6±0.0	0.5±0.0	0.9±0.0	0.9±0.1	0.9±0.0	0.7±0.1	0.7±0.0	0.6±0.0	0.6±0.0	0.9±0.0	0.9±0.0	0.4±0.3	0.6±0.0	0.3±0.3
16:0	9.5±0.1	9.3±0.0	9.6±0.7	10.1±0.1	10.5±0.4	10.4±0.1	10.0±0.0	9.7±0.2	11.1±1.1	10.4±0.1	10.0±0.1	9.8±0.1	9.9±0.2	10.0±0.1	10.0±0.4
16:1n-9	0.5±0.0	0.5±0.0	0.5±0.0	0.4±0.0^{a}	0.6±0.2^{b}	0.7±0.0^{c}	0.6±0.0	0.6±0.0	0.5±0.1	0.6±0.0	0.6±0.2	0.6±0.2	0.6±0.0^{a}	0.5±0.0^{a}	0.5±0.0^{b}
16:1n-7	1.9±0.0	1.9±0.0	2.1±0.3	4.7±0.0^{a}	4.7±0.2^{ab}	4.5±0.0^{b}	2.9±0.1	2.9±0.1	2.7±0.9	2.1±0.0	4.4±0.2	4.4. ±0.1	2.1±0.1^{a}	2.1±0.0^{a}	2.3±0.0^{b}
16:2	0.0±0.0	0.0±0.0	0.0±0.0	0.6±0.0	0.7±0.0	0.7±0.1	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.6±0.0	0.7±0.0	0.5±0.0^{a}	0.5±0.0^{a}	0.4±0.0^{b}
16:3	0.5±0.0	0.5±0.0	0.5±0.0	0.7±0.2	0.9±0.1	0.8±0.1	0.6±0.0	0.6±0.0	0.2±0.3	0.0±0.0	0.9±0.1	0.7±0.2	0.0±0.0	0.0±0.0	0.1±0.0
18:0	4.7±0.2	4.6±0.1	4.5±0.1	4.1±0.0^{a}	4.3±0.2^{ab}	4.3±0.1^{b}	4.2±0.0	4.3±0.1	4.4±0.2	4.7±0.0	4.1±0.0	4.20±0.1	4.5±0.1^{a}	4.4±0.0^{a}	4.1±0.2^{b}
18:1n-9	16.1±0.2	16.0±0.2	16.0±0.1	22.4±0.2^{a}	23.2±1.1^{ab}	23.3±0.1^{b}	19.2±0.1^{a}	18.7±0.1^{b}	16.8±0.6	16.8±0.1	22.2±0.3	21.7±0.2	16.4±0.2^{a}	16.4±0.1^{a}	17.0±0.2^{b}
18:1n-7	6.0±0.1	6.1±0.1	6.1±0.1	8.2±0.1^{a}	8.6±0.5^{ab}	8.8±0.1^{b}	7.5±0.2	7.4±0.1	6.3±0.4	6.3±0.0	8.3±0.1	8.4±0.3	6.3±0.0^{a}	6.3±0.0^{a}	5.5±0.1^{b}
18:2n-6	4.9±0.1	4.7±0.0	4.7±0.1	4.3±0.0	4.4±0.2	4.4±0.0	4.8±0.0^{a}	4.6±0.0^{b}	4.9±0.5	5.0±0.0	4.3±0.0	4.3±0.0	4.8±0.1^{a}	4.9±0.0^{a}	6.3±0.0^{b}
18:3n-3	24.2±0.9	24.6±0.3	24.4±0.9	19.1±0.3	19.9±1.0	19.8±0.2	25.6±0.1^{a}	23.9±0.4^{b}	25.2±3.2	25.4±0.1	19.1±0.2	19.3±0.1	24.8±0.6^{a}	24.6±0.1^{a}	19.8±0.0^{b}
18:4n-3	3.0±0.1	2.9±0.1	3.0±0.1	1.8±0.0^{a}	1.9±0.1^{ab}	1.7±0.0^{b}	3.0±0.0	2.6±0.1	3.1±0.2	3.2±0.1	1.8±0.0	1.9±0.0	3.1±0.1^{a}	3.1±0.0^{a}	2.8±0.1^{b}
20:0	0.1±0.0	0.1±0.0	0.1±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.0	0.1±0.0	0.0±0.0	0.0±0.0	0.1±0.0	0.1±0.0	0.1±0.0
20:1n-9	0.6±0.1	0.6±0.0	0.6±0.1	0.5±0.0	0.5±0.0	0.5±0.0	0.4±0.0	0.5±0.0	0.5±0.1	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0^{a}	0.5±0.0^{a}	0.9±0.0^{b}
20:2n-6	0.2±0.0	0.2±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.0±0.0	0.1±0.0	0.1±0.1	0.2±0.0	0.2±0.0	0.0±0.0	0.1±0.1	0.2±0.0	0.2±0.0	0.2±0.0
20:3n-6	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.0±0.0	0.0±0.0	0.1±0.0	0.0±0.0	0.1±0.0	0.1±0.0	0.0±0.0	0.0±0.0	0.1±0.0	0.1±0.0	0.1±0.0
20:4n-6	1.3±0.0^{a}	1.5±0.0^{b}	1.4±0.0^{ab}	1.5±0.0	1.4±0.1	1.5±0.0	1.2±0.0^{a}	1.5±0.0^{b}	1.0±0.1	1.1±0.0	1.6±0.0	1.7±0.0	1.2±0.1^{a}	1.2±0.0^{a}	1.0±0.0^{b}
20:3n-3	0.7±0.0	0.7±0.0	0.7±0.0	0.2±0.0	0.2±0.1	0.1±0.1	0.5±0.0	0.5±0.0	0.6±0.1	0.6±0.0	0.1±0.1	0.1±0.1	0.6±0.0^{a}	0.6±0.0^{a}	0.5±0.0^{b}
20:4n-3	0.6±0.0	0.6±0.0	0.6±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.5±0.0	0.4±0.0	0.6±0.0	0.6±0.0	0.2±0.0	0.1±0.1	0.6±0.0^{a}	0.6±0.0^{a}	0.8±0.0^{b}
20:5n-3	5.7±0.2^{a}	6.2±0.1^{b}	6.4±0.2^{b}	6.2±0.1^{a}	5.6±0.6^{ab}	5.7±0.0^{b}	5.2±0.0^{a}	6.2±0.2^{b}	5.0±1.3	4.6±0.0	6.2±0.1	6.5±0.0	5.2±0.2^{a}	5.1±0.1^{a}	13.8±0.5^{b}
22:0	0.3±0.1	0.2±0.0	0.2±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.2	0.0±0.0	0.2±0.0	0.2±0.0	0.1±0.1	0.0±0.0	0.2±0.0^{a}	0.2±0.0^{a}	0.3±0.0^{b}
22:5n-3	0.7±0.0	0.6±0.0	0.6±0.0	0.3±0.0^{a}	0.2±0.1^{ab}	0.1±0.0^{b}	0.2±0.2	0.1±0.0	0.4±0.1	0.4±0.0	0.1±0.1	0.1±0.2	0.5±0.0^{a}	0.5±0.0^{a}	0.6±0.0^{b}
22:6n-3	10.4±0.4	10.2±0.1	10.3±0.5	8.2±0.2^{a}	6.2±2.8^{ab}	7.3±0.1^{b}	6.9±0.1^{a}	9.1±0.3^{b}	6.6±2.0	8.3±0.2	9.0±0.6	9.4±0.1	9.2±1.0^{a}	9.6±0.3^{a}	4.9±0.2^{b}
Saturated	15.7±0.4	15.5±0.1	15.8±1.1	15.8±0.2	16.6±0.7	16.2±0.0	15.6±0.3	15.3±0.3	17.5±1.7	16.7±0.1	15.7±0.2	15.4±0.2	15.8±0.5	16.0±0.1	15.2±0.6
Monounsat.	25.8±0.2	25.7±0.2	25.8±0.5	36.3±0.3^{a}	37.7±1.8^{ab}	37.8±0.2^{b}	30.6±0.2^{a}	30.0±0.2^{b}	27.0±0.6	26.5±0.2	35.9±0.4	35.5±0.6	26.0±0.2^{a}	26.0±0.1^{a}	27.1±0.3^{b}
Poliunsat.	53.3±0.5	53.7±0.1	53.6±1.3	43.4±0.4	41.7±2.4	42.4±0.2	49.2±0.2	50.1±0.6	49.3±1.8	50.6±0.4	44.1±0.6	44.9±0.4	51.0±0.7	51.7±0.4	51.8±0.9
HUFA n-3	18.1±0.6	18.3±0.2	18.5±0.4	15.2±0.3^{a}	12.4±3.6^{ab}	13.5±0.1^{b}	13.2±0.1^{a}	16.3±0.5^{b}	13.2±3.0	14.6±0.2	15.7±0.7	16.3±0.4	16.2±1.2^{a}	16.5±0.4^{a}	20.6±0.7^{b}
HUFA n-6	2.3±0.1	2.3±0.1	2.2±0.1	1.7±0.0^{a}	1.5±0.2^{ab}	1.5±0.0^{b}	1.4±1.1	1.6±0.1	1.7±0.2	1.9±0.0	1.7±0.1	1.8±0.1	2.0±0.1^{a}	2.1±0.0^{a}	1.6±0.0^{b}
DHA/EPA	1.8±0.0^{a}	1.6±0.1^{b}	1.6±0.1^{b}	1.4±0.0^{a}	1.1±0.4^{ab}	1.3±0.0^{b}	1.3±0.0^{a}	1.5±0.0^{b}	1.4±0.4^{a}	1.8±0.0^{b}	1.4±0.1	1.4±0.0	1.8±0.1^{a}	1.9±0.0^{a}	0.3±0.0^{b}
% lipids (DW)	31.4±0.8	27.6±0.5	27.5±1.0	25.7±1.6	24.0±2.0	26.4±0.6	22.7±0.2	22.9±0.4	25.2±0.4	26.6±1.8	31.6±0.7	31.3±0.8	25.7±1.0	22.4±1.2	28.8±0.6

HUFA n-3: ≥20:3n-3; HUFA n-6: ≥20:2n-6; DHA/EPA: docosahexaenoic and eicosapentaenoic acid ratio; FAME: Fatty acid methyl ester.

product used, whereas no separation based on the nauplial density was achieved. The association of the two groups to their respective variables allowed to identify 18:1n-9, LA and EPA with DCSS and 16:0, 18:0, 18:1n-7, LNA, ARA and DHA with M70 in the first component.

4.4. Discussion

It has been often reported in the literature that bioencapsulation of HUFA into *Artemia* nauplii is particularly difficult to achieve (Navarro *et al*.1999; Bell *et al*. 2003). Among the factors believed to account for such limited efficiency for HUFA delivery, some have a particular relevance when DHA-rich enrichment products are used. Here, we investigated diverse practical strategies to optimise the use of the emulsion M70, a product with great potential for its use in *Artemia* enrichments.

Dissolved oxygen (DO) availability in the culture medium is critical for *Artemia* survival during enrichment, and DO concentrations above 4 mg L^{-1} have been proposed (Van Stappen 1996). While standard enrichment procedures involve the use of compressed air to provide adequate levels DO, pure oxygen supply is particularly recommended when DO levels can be potentially compromised. That might be the case of live prey enrichments with high DHA contents, as oxygen depleting processes such as lipid peroxidation are especially favoured

(McEvoy *et al*. 1995; Ries 2009). We therefore compared the performance of pure oxygen *vs*. compressed air when using M70 as enrichment diet, and conclude that no difference in either the HUFA bioencapsulation into *Artemia* nor nauplial mortality were observed. Altogether, Experiment 1 allowed us to conclude that oxygen supplied with compressed air is an effective diffusion system to meet the DO demands in enrichment procedures with M70, and that other more expensive alternatives such as pure oxygen are not required.

Other factors like air flow and temperature during the enrichment procedure also need to be considered when a compromise between lipid peroxidation, oxygen supply and enrichment efficiency is pursued. In addition to oxygen solubility and its potential consequences in lipid peroxidation, the air flow during enrichment also determines the overall turbulence in the medium. It has been suggested that highly turbulent conditions during enrichment might impede the normal swimming behaviour of nauplii and damage the nauplial filtratory structures, thus ultimately hindering the incorporation of the enrichment product particles (Navarro *et al*. 1999; Monroig *et al*. 2006b). In agreement with those findings, the emulsion M70 exhibited a higher HUFA bioencapsulation efficiency when utilised at moderate air flows ("1 lpm") than that at highly turbulent conditions ("3 lpm"). In contrast with the results reported by Monroig *et al*. (2006b), who investigated the

enrichment efficiency of HUFA-rich liposomes under the same air flow conditions set for Experiment 2, the enrichment with M70 did not produce an excessive foam in the medium and thus the *Artemia* mortality caused by such an effect did not occur for any the air flow studied.

Temperature of the enrichment medium is a key factor for efficient bioencapsulation of HUFA into *Artemia*. Beyond controlling the oxygen solubility and lipid peroxidation mechanisms, temperature also determines the metabolic capability of *Artemia* nauplii and therefore important development landmarks such as the mouth opening and vitelum absortion, as well as the onset of filtering/natatory appendixes (Hochanchka & Somero 1984; Anger 2001). Our results indicated that enhanced HUFA enrichment was achieved at 28 ºC compared with 24 ºC, this being consistent with the vast majority of studies in which 28 ºC has been established as preferred enrichment temperature (Harel *et al*. 1999; Han *et al*. 2001; Sui *et al*. 2007; Boglino *et al*. 2012). In contrast, relatively low temperatures (21-22 ºC) were established in other studies (Garcia *et al*. 2008; Figueiredo *et al*. 2009), but unfortunately no specific reasons for the choice of these low temperatures, and the causes of their effects, were given. Since the enrichment efficiency of M70 did not appear to be compromised by potentially more pro-oxidant temperatures (“28C” *vs*. “24C"), we continued our investigations evaluating other experimental

parameters potentially affecting the oxidative stability of M70 and consequently its HUFA bioencapsulation efficacy.

Another strategy to preserve DHA-rich enrichment diets from oxidation involves an adequate dosage along the enrichment protocol. While one single dose at the beginning of the enrichment process appears to be the most extended dosage mode (Estevez *et al*. 1999; Villalta *et al*. 2005a, b), two doses, one at the beginning of the enrichment process and another one at mid-period (Evjemo *et al*. 1997, 2001; Estévez *et al*. 1998; Han *et al*. 2000, 2001; Sui *et al*. 2007; Hamre & Harboe, 2008) might contribute to reduce lipid peroxidation as the time of exposure to pro-oxidant conditions (continuous light, high temperature and limited dissolved oxygen) is minimised.

The results from the present study confirmed that no differences in HUFA bioencapsulation existed when M70 was dispensed in one or two doses, and thus the oxidative stability of M70 enables this enrichment diet to be dispensed in a unique dose without any evident detrimental effect in terms of HUFA incorporation over 21 h of incubation.

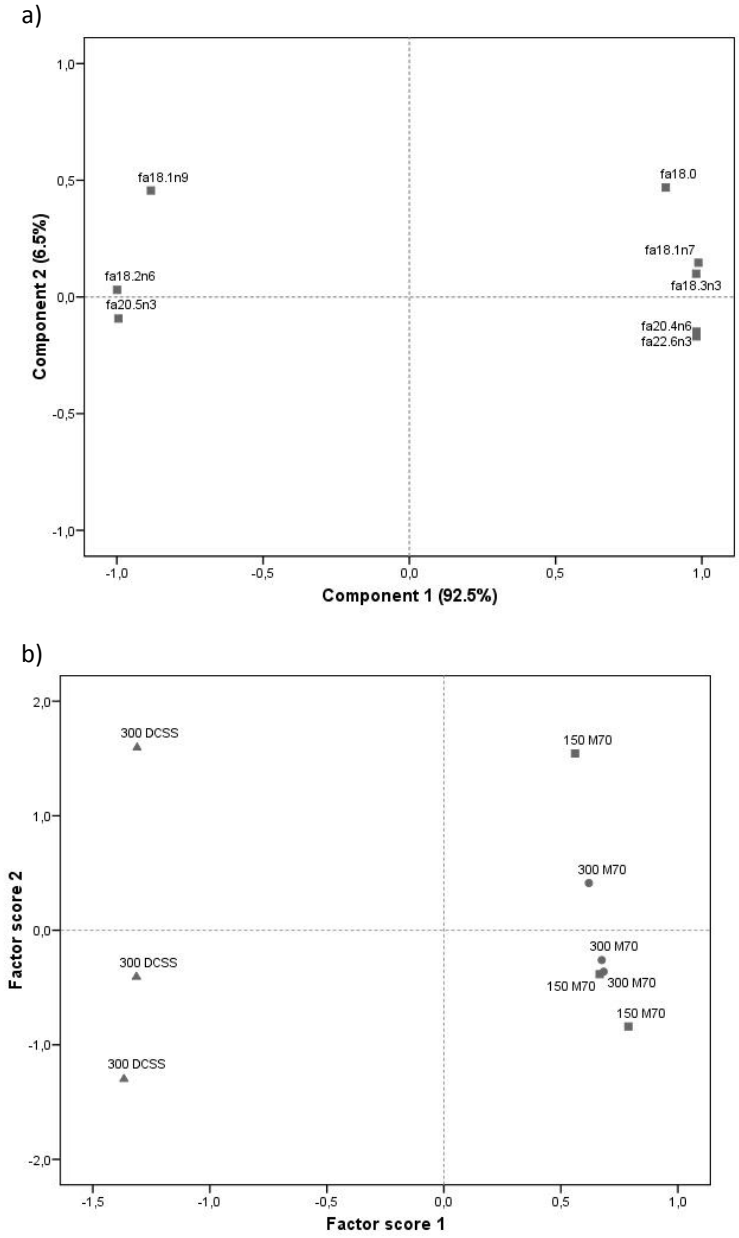

Figure 4.1. Component plot (a) and factor score plot (b) of the multivariate Principal Components Analysis of selected FA from total lipids of enriched *Artemia* nauplii from Experiment 6.

Our experiments investigating the effects of the M70 concentration and the nauplial density on *Artemia* bioencapsulation efficiency evidenced that M70 behaved similarly to other enrichment products, and no special specification derived from its particularly high-DHA content needs to be considered. Thus, a dose of 0.8 g L^{-1} of M70, equivalent in dry weight basis to the recommended 0.6 g L^{-1} for commercial products (McEvoy *et al*. 1995, 1996, 1997; Smith *et al*. 2002; Villalta *et al*. 2005a, b; Sui *et al*. 2007), resulted in increased DHA bioencapsulation into *Artemia* compared to lower concentration enrichments. At first glance, no significant differences in DHA contents between *Artemia* enriched at M70 concentrations of 0.6 and 0.8 g L^{-1} would have indicated that a concentration of 0.6 g M70 L^{-1} was sufficient to produce as much DHA enrichment as 0.8 g L^{-1}. However, the high variability observed in the FA profiles from *Artemia* enriched at concentration of 0.6 g L^{-1} could have certainly masked a lowered bioencapsulation efficiency in comparison with that obtained at 0.8 g L^{-1}. Indeed we did observe significant higher DHA/EPA ratio, as well as higher average contents of individual DHA for *Artemia* enriched at 0.8 g M70 L^{-1}. These results enabled us to choose 0.8 g L^{-1} of the M70 emulsion as a more adequate enricher concentration than 0.6 g L^{-1}. Additionally, our experiment assessing the effect of nauplial density on HUFA enrichment revealed that M70 can be used at nauplial densities of 300 nauplii mL^{-1}, with the potential limitation of DO not

causing any apparent effect on nauplial motility (Southgate & Lou 1995). Qualitative (FA profile) enrichment efficiency of M70 did not vary between 150 and 300 nauplii mL^{-1}. However, from a quantitative point of view (lipid %), a higher uptake of enrichment product was achieved at 150 nauplii mL^{-1}. Thus, any positive cost-effectiveness aspects related, among others, to a more restricted utilisation of enrichment product, pointing at 300 nauplii mL^{-1} as the recommended nauplial density for M70 enrichments, has to be counterbalanced by the higher uptake achieved at lower nauplial density.

The DHA/EPA ratio is a common biochemical parameter used to evaluate the nutritional suitability of diets for marine finfish larviculture (Reitan *et al*. 1994; Evjemo *et al*. 1997). For instance, the yolk of marine fish eggs and the polar lipids of copepodites, natural preys of marine fish larvae in the wild, have DHA/EPA ratios around 2.0 (Fraser *et al*. 1989; McEvoy *et al*. 1997; Sargent *et al*. 1997; Sorgeloos *et al*. 2001; Evjemo *et al*. 2003; Van der Meeren *et al*. 2008). Generally the results from the present study showed that the emulsion M70 allowed us to obtain in most cases *Artemia* nauplii with DHA/EPA ratios above 1.3 and only suboptimal experimental conditions (i.e., excessive aeration or relatively low temperature) resulted in lower values. It is worth mentioning that the DHA/EPA ratios of *Artemia* nauplii were particularly high (1.8-1.9) for Experiment 6, supporting that the final (optimised) experimental conditions

developed for a more efficient use of M70 as enrichment diet had been adequately established. Moreover, M70-enriched nauplii had higher DHA individual contents and DHA/EPA ratios than nauplii enriched with commercial enrichment products like the one utilised in this study (DC Super Selco) and previous studies (Léger *et al*. 1986; Woods 2003; Lund *et al*. 2007; Naz 2008). Interestingly, Haché *et al*. (2011) recently reported DHA/EPA ratios of 3.6 in *Artemia* nauplii enriched with Algamac 3050, a commercial product based on spray-dried cells of the marine protist *Schizochytrium* sp. (Barclay & Zeller 1996). Since DHA concentration of Algamac 3050 (~40 % of total FA) is lower than that of M70 (~70 %), factors possibly related to the physical and biochemical nature of Algamac 3050 might account for such unexpectedly high HUFA bioencapsulation. Nevertheless, the results obtained from the present study clearly show that M70 emulsion was able to consistently produce DHA/EPA ratios above 1 in *Artemia* lipids, with particularly high values when optimised conditions are used.

In summary, the results from the present study indicated that an efficient utilisation of the emulsion M70 as enrichment diet for *Artemia* nauplii is achieved with incubation temperatures of 28ºC, low/moderate aeration (1 L min^{-1}) and nauplial densities of 150 ind mL^{-1}. Moreover, the emulsion M70 can be administered to newly hatched nauplii through a single

dose of 0.8 g L^{-1}, with no detrimental effects such as DHA autooxidation and nauplial mortalities becoming apparent.

4.5. References

Anger, K., 2001. The biology of decapod crustacean larvae. In: Vonk, R. (Ed.), Crustacean Issues, vol. 14. A. A. Balkema Publishers, Lisse, 419 p.

Aragão, C., Conceição, L.E.C., Dinis, M.T., Hans-Jørgen, F., 2004. Amino acid pools of rotifers and *Artemia* under different conditions: nutritional implications for fish larvae. Aquaculture 234, 429-445.

Barclay, W., Zeller, S., 1996. Nutritional enhancement of n-3 and n-6 fatty acids in rotifers and *Artemia* nauplii by feeding spray-dried *Schizochytrium* sp. J. World Aquac. Soc. 27, 214-322.

Bell, M.V., Batty, R.S., Dick, J.R., Fretwell, K., Navarro, J.C., Sargent, J.R., 1995. Dietary deficiency of docosahexaenoic acid impairs vision at low light intensities in juvenile herring (*Clupea harengus* L.). Lipids 30, 443-449.

Bell, J.G., McEvoy, L.A., Estévez, A., Shields, R.J., Sargent, J.R., 2003. Optimising lipid nutrition in first-feeding flatfish larvae. Aquaculture 227, 211-220.

Bell. M.V., Dick, J.R., 2004. Changes in capacity to synthesise 22:6n-3 during early development in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 235, 393-409.

Boglino, A., Darias, M.J., Ortiz-Delgado, J.B., Özcan, F., Estévez, A., Andree, K.B., Hontoria, F., Sarasquete, C., Gisbert, E., 2012. Commercial products for *Artemia* enrichment affect growth performance, digestive system maturation, ossification and incidence of skeletal deformities in Senegalese sole (*Solea senegalens*is) larvae. Aquaculture 324, 290-302

Buzzy, M., Henderson, R.J., Sargent, J.R., 1996. The desaturation and elongation of linolenic acid and eicosapentaenoic acid by hepatocytes and liver microsomes from rainbow trout (*Oncorhynchus mykiss*) fed diets containing fish oil or olive oil. Biochim. Biophys. Acta 1299, 235-244.

Christie, W.W., 2003. Lipid analysis, third ed. Oily Press, Bridgwater.

Cosgrove, J.P., Church, D.F., Pryor, W.A., 1987. The kinetics of the autoxidation of polyunsaturated fatty acids. Lipids 22, 299–304.

Coutteau, P., Sorgeloos, P., 1997. Manipulation of dietary lipids, fatty acids and vitamins in zooplankton cultures. Freshwater biology 38, 501-512.

Estévez, A., McEvoy, L.A., Bell, J. G., Sargent, J. R., 1998. Effects of temperature and starvation time on the pattern and rate of loss of essential fatty acids in *Artemia* nauplii previously enriched using archidonic acid and eicosapentaenoic acid-rich emulsions. Aquaculture 165, 295-311.

Estévez, A., McEvoy, L.A., Bell, J.G., Sargent, J.R., 1999. Growth, survival, lipid composition and pigmentation of turbot (*Scophthalmus maximus*) larvae fed live-prey enriched in Arachidonic and Eicosapentaenoic acids. Aquaculture 180, 321-343.

Evjemo, J.O., Coutteau, P., Olsen, Y., Sorgeloos, P., 1997. The stability of docosahexaenoic acid in two *Artemia* species following enrichment and subsequent starvation. Aquaculture 155, 135-148.

Evjemo, J.O., Danielsen, T.L., Olsen, Y., 2001. Losses of lipid, protein and n-3 fatty acids in enriched *Artemia franciscana* starved at different temperatures. Aquaculture 193, 65-80.

Evjemo, J.O., Reitan, K.I., Olsen, Y., 2003. Copepods as live food organisms in the larval rearing of halibut larvae (*Hippoglossus hippoglossus* L.) with special emphasis on the nutritional value. Aquaculture 227, 191–210.

Figueiredo, J., van Woesik, R., Lin, J., Narciso, L., 2009. *Artemia franciscana* enrichment model – How to keep them small, rich and alive? Aquaculture 294, 212-220.

Folch, J., Lees, M., Sloane Stanley, H., 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem.. 226, 497-509.

Fraser, A.J., Sargent, J.R., Gamble, J.C., 1989. Lipid class and fatty acid composition of *Calanus finmarchicus* (Gunnerus), *Pseudocalanus* sp. and *Temora longicornis* Muller from a nutrient-enriched seawater enclosure. J. Exp. Mar. Biol. Ecol. 130, 81-92.

Furuita, H., Takeuchi, T., Toyota, M., Watanabe, T., 1996. EPA and DHA requirements in early juvenile sea bream using HUFA enriched *Artemia* nauplii. Fish. Sci. 62, 246-251.

Garcia, A.S., Parrish, C.C., Brown, J.A., 2008. Growth and lipid composition of Atlantic cod (*Gadus morhua*) larvae in response to differently enriched *Artemia franciscana*. Fish. Physiol. Biochem. 34, 77-94.

Giner, E., 2005. Desarrollo de emulsiones de alto contenido en ácidos grasos poliinsaturados para complementar el valor nutritivo de presas vivas en acuicultura. Universidad Politécnica de Valencia (Valencia). pp 75.

Hamre, K., Harboe, T., 2008. *Artemia* enriched high n-3 HUFA may give a large improvement in perfomance of Atlantic halibut (*Hippoglossus* L.) larvae. Aquaculture 277, 239-243.

Haché, R., Plante, S., 2011. The relationship between enrichment, fatty acid profiles and bacterial load in cultured rotifers (*Brachionus plicatillis* L-strain) and *Artemia* (*Artemia salina* strain *franciscana*). Aquaculture 311, 201-208.

Han, K., Geurden, I., Sorgeloos, P., 2000. Enrichment strategies for *Artemia* using emulsions providing differents levels of n-3 highly unsaturated fatty acids. Aquaculture 183, 335-347.

Han, K., Geurden, I., Sorgeloos, P., 2001. Fatty acid changes in enriched and subsequently starved *Artemia franciscana* nauplii enriched with different essential fatty acids. Aquaculture 199, 93-105.

Han, K., Geurden, I., Van der Meeren, P., Bai, S.C., Sorgeloos, P., 2005. Particle size distribution in two lipid emulsion used for the enrichment of *Artemia* nauplii as a function of their preparation method and storage time. J. World Aquac. Soc. 36, 196-202.

Harel, M., Ozkizilcik, S., Lund, E., Behrens, P., Place, A.R., 1999. Enhanced absorption of docosahexaenoic acid (DHA, 22:6n3) in *Artemia* nauplii using a dietary combination of DHA-rich phospholipids and DHA-sodium salts. Comp. Biochem. Physiol. B 124, 169-176.

Hontoria, F., Crowe, J.H., Crowe, L.M., Amat, F., 1994. Potential use of liposomes in larviculture as a delivery system through Artemia nauplii. Aquaculture 127, 255-264.

Koven, W.M., Tandler, A., Kissil, G.W., Sklan, D., Friezlander, O., Harel, M., 1990.The effect of dietary (*n*-3) polyunsaturated fatty acids on growth, survival and swim bladder development in *Sparus aurata* larvae. Aquaculture 91, 131-141.

Léger, P., Bengtson, D.A., Simpson, K.L., Sorgeloos, P., 1986. The use and nutritional value of *Artemia* as a food source. Oceanogr. Mar. Biol. Annu. Rev. 24, 521-623.

Léger, P., Bengtson, D.A., Sorgeloos, P., Simpson, K.L., Beck, A.D., 1987. The nutritional value of *Artemia*: a review: p.357-372. In: Sorgeloos, P., Bengtson, D.A., Declier, W. and Jaspers, E. (Eds.), *Artemia* Research and its applications. Vol. 3. Universa Press, Wetteren, Belgium, 556 p.

Lund, I., Steenfeldt, S.J., Hanses, B.W., 2007. Effect of dietary arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid on survival, growth and pigmentation in larvae of common sole (*Solea solea* L.). Aquaculture 273, 532-544.

McEvoy, L.A., Navarro, J.C., Bell, J.G., Sargent, J.R., 1995. Autoxidation of oil emulsions during the *Artemia* enrichment process. Aquaculture 134, 101-112.

McEvoy, L.A., Navarro, J.C., Hontoria, F., Amat, F., Sargent, J.R., 1996. Two novel *Artemia* enrichment diets containing polar lipid. Aquaculture 144, 339-352.

McEvoy, L.A., Navarro, J.C., Amat, F., Sargent, J.R., 1997. Application of soya phosphatidylcholine in tuna orbital oil enrichment emulsions for *Artemia*. Aquaculture international 5, 517-526.

Monroig, O., Navarro, J.C., Amat, I., González, P., Amat, F., Hontoria, F., 2003. Enrichment of *Artemia* nauplii in PUFA, phospholipids, and water-soluble nutrients using liposomes. Aquaculture international 11, 151-161.

Monroig. O., 2006. Diseño y optimización de liposomas para su uso como sistema de suministro de nutrientes a larvas de peces marinos. PhD thesis. University of Valencia, Spain, 239 p.

Monroig, O., Navarro, J.C., Amat, F., González, P., Bermejo, A., Hontoria, F., 2006a. Enrichment of *Artemia* nauplii in essential fatty acids with different types of liposomes and their use in the rearing of gilthead sea bream (*Sparus aurata*) larvae. Aquaculture 251, 491-508.

Monroig, O., Navarro, J.C., Amat, F., Gonzalez, P., Hontoria, F., 2006b. Effect of aeration on the efficiency of *Artemia* enrichment with EFA-rich emulsion and liposomes. Aquaculture 257, 382-392.

Monroig, O., Navarro, J.C., Amat, F., Gonzalez, P., Hontoria, F., 2006c. Effects of nauplial density, product concentration and product dosage on the survival of the nauplii and EFA incorporation during *Artemia* enrichment with liposomes. Aquaculture 261, 659-669.

Monroig, O., Navarro, J.C., Amat, F., Hontoria, F., 2007. Enrichment of *Artemia* nauplii in vitamin A, vitamin C and methionine using liposomes. Aquaculture 269, 504-513.

Monroig, O., Webb, K., Ibarra-Castro, L., Holt, G.J., Tocher, D.R., 2011. Biosynthesis of long-chain polyinsaturated fatty acids in marine fish: Characterization of an Elovl4-like elongase from cobia *Rachycentron canadum* and activation of the pathway during early life stages. Aquaculture 312, 145-153.

Morais, S., Mourente, G., Ortega, A., Tocher, J.A., Tocher, D.R., 2011. Expression of fatty acyl desaturase and elongase genes, and evolution of DHA:EPA ratio during development of unfed larvae of Atlantic bluefin tuna (*Thunnus Thynnus* L.). Aquaculture 313, 129-139.

Navarro, J.C., Henderson, R.J., McEvoy, L.A., Bell, M.V., Amat, F., 1999. Lipid conversions during enrichment of *Artemia* . Aquaculture 174, 155-166.

Naz, M., 2008. The changes in the biochemical composition and enzymatic activities of rotifer (*Brachionus plicatillis*, Müller) and *Artemia* during the enrichment and starvation periods. Fish Phys. and Biochem. 34, 391-404.

Reitan, K.I., Rainuzzo, J.R., Olsen, Y., 1994. Influence of lipid composition of live feed on growth, survival and pigmentation of turbot larvae. Aquaculture international 2, 22-48.

Ries, D., 2009. Studies on the antioxidant activity of milk proteins in model oil-in-water emulsions. PhD thesis. Riddet Institute (Massey University) Palmerston North, New Zealand, 256 pp.

Sargent, J.R., McEvoy, L.A., Bell, J.G., 1997. Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. Aquaculture 155, 117-127.

Sargent, J.R., Tocher, D.R., Bell, J.G., 2002. The lipids. In: Halver, J.E., Hardly, R.W. (Eds.). Fish Nutrition Academic Press, San Diego, CA, USA. Pp: 181-257.

Smith, G.G., Ritar, A.J., Phleger, C.F., Nelson, M.M., Mooney, B., Nichols, P.D., Hart, P.R., 2002. Changes in gut content and composition of juvenile *Artemia* after oil enrichment and during starvation. Aquaculture 208, 137-158.

Sokal, R.R. and Rohlf, F.J., 1981. Biometry. New York. 859 pp.

Sorgeloos, P., Dhert, P., Candreva, P., 2001. Use of the brine shrimp, *Artemia* spp., in marine fish larviculture. Aquaculture 200, 147-159.

Southgate, P.C. and Lou, D.C., 1995. Improving the *n* - 3 HUFA composition of *Artemia* using microcapsules containing marine oils. Aquaculture 134, 91-99.

Sui, L., Wille, M., Cheng, Y., Sorgeloos, P., 2007. The effect of dietary n-3 HUFA levels and DHA/EPA ratios on growth, survival and osmotic stress tolerance of Chinese mitten crab *Eriocheir sinensis* larvae. Aquaculture 273, 139-150.

Tocher, D.R., 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Rev. Fish. Sci. 11, 107-184.

Van der Meeren, T., Olsen, R.E., Hamre, K.,Fyhn, H., 2008. Biochemical composition of copepods for evaluation of feed quality in production of juvenile marine fish. Aquaculture 274, 375-397.

Van Stappen, G., 1996. Introduction , biology and ecology of *Artemia*. In: Lavens, P., Sorgeloos, P., (Eds.), Manual on the production and use of live food for aquaculture. FAO Fisheries Technical Paper (361), Roma.

Villalta, M., Estévez, A., Bransden, M.P., 2005a. Arachidonic acid enriched live prey induces albinism in Senegal sole (*Solea senegalensis*) larvae. Aquaculture 245, 193-209.

Villalta, M., Estévez, A., Bransden, M.P., Bell, J.G., 2005b. The effect of graded concentrations of dietary DHA on growth, survival and tissue fatty acid profile os Senegal Sole (*Solea senegalensis*) larvae during the *Artemia* feeding period. Aquaculture 249, 353-365.

Watanabe, T., Ohta, M., Kitajima, C., Fujita, S., 1982. Improvement of dietary value of brine shrimp *Artemia salina* for fish larvae by feeding them on w3 highly unsaturated fatty acids. Bull. Jpn. Soc. Sci. Fish. 48, 1775-1782.

Watanabe, T., Kitajima, C, Fujita, S., 1983. Nutritional values of live organisms used in Japan for mass propagation of fish: A review. Aquaculture 34, 115-143.

Woods, C.M.C., 2003. Effects of varying *Artemia* enrichment on growth and survival of juvenile seahorses, *Hippocampus abdominalis*. Aquaculture 220, 537-548.

Yamamoto, Y., Kabeya, N., Takeuchi, Y., Alimuddin, Haga, Y., Satoh, S., Takeuchi, T., Yoshizaki, G., 2010. Cloning and nutritional regulation of polyunsaturated fatty acid desaturase and elongase of a marine teleost, the nibe croaker *Nibea mitsukurii*. Fisheries science 76, 463-472.

CAPÍTULO 5

Antioxidant activity and lipid peroxidation in *Artemia* nauplii enriched with a new DHA-rich emulsion and the effect of adding an external antioxidant based on hydroxytyrosol

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Aquaculture Research, Aceptado. 2015

17-Oct-2015

Dear Miss Viciano:

It is a pleasure to accept your manuscript entitled "Antioxidant activity and lipid peroxidation in *Artemia* nauplii enriched with a DHA-rich oil emulsion and the effect of adding an external antioxidant based on hydroxytyrosol" in its current form for publication in Aquaculture Research.

Thank you for your fine contribution. We look forward to your continued contributions to the Journal.

Yours Sincerely,

Prof. Lindsay Ross
Editor, Aquaculture Research
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Abstract

Artemia nauplii catabolise polyunsaturated fatty acids (PUFA); in particular, they retroconvert docosahexaenoic acid (DHA, 22:6n-3), so enrichment is a continuous quest towards increasing PUFA through the use of PUFA-rich enrichment products. However, optimal conditions during enrichment (aeration, illumination and temperatures around 28 ºC) tend to accelerate autoxidation of PUFA, and the formation of potentially toxic oxidation products. Water-soluble antioxidants like the polyphenolic compound hydroxytyrosol (3,4-dihydroxyphenylethanol), a polar molecule found in the water fraction resulting after the milling process of olives, arise as promising compounds to prevent oxidation during *Artemia* enrichments. We investigated the antioxidant activity and lipid peroxidation in *Artemia* nauplii during enrichment, and the effect of adding an external antioxidant based on hydroxytyrosol during the enrichment with a PUFA-rich emulsion (M70). For this purpose, the activity of antioxidant enzymes (catalase, superoxide dismutase, glutathione-S-transferase, glutathione peroxidase), as well as lipid peroxidation, were determined in enriched and unenriched *Artemia* nauplii. To validate antioxidant activity and lipid peroxidation, in a first experiment, nauplii were enriched with microalgae (*Tetraselmis suecica*), yeast (*Saccharomyces cerevisiae*) and M70 emulsion. In a second experiment, enrichment with a commercial emulsion (DC Super Selco), M70, and a combination of M70 and

hydroxytyrosol (Hytolive, HYT) added as an external antioxidant was performed. The combination of M70 with HYT produced the best results, in terms of activity of antioxidant enzymes. The analysis of the fatty acids from total lipids showed that the addition of hydroxytyrosol preserved the DHA percentage of enriched nauplii.

Resumen

Los nauplios de Artemia *catabolizan ácidos grasos poliinsaturados (PUFA); en concreto, retroconvierten el ácido docosahexaenoico (DHA, 22:6n-3), por lo que el enriquecimiento es una búsqueda contínua para incrementar los PUFA a través de productos ricos en estos ácidos grasos. Sin embargo, las condiciones óptimas durante el enriquecimiento (aireación, iluminación y temperaturas de 28 ºC) tienden a acelerar la autoxidación de los PUFA, y la formación de productos de oxidación potencialmente tóxicos. Los antioxidantes solubles en agua, como el compuesto polifenólico hidroxitirosol (3,4-dihidroxifeniletanol), molécula polar encontrada en la fracción de agua resultante del proceso de molienda de las olivas, se postulan como prometedores para prevenir la oxidación durante el enriquecimiento de* Artemia. *Se investigó la actividad antioxidante y la peroxidación lipídica durante el enriquecimiento de nauplios de* Artemia, *así como el efecto de añadir un antioxidante externo basado en hidroxitirosol durante el enriquecimiento con una emulsión rica en PUFA (M70). Para ello, se determinó la actividad de los enzimas antioxidantes (catalasa, superóxido dismutasa, glutatión-S-transferasa, glutatión peroxidasa), así como la peroxidación lipídica, en nauplios de* Artemi*a enriquecidos y sin enriquecer. Para validar la actividad antioxidante y la peroxidación lipídica, en un primer experimento, los nauplios*

*fueron enriquecidos con microalgas (*Tetraselmis suecica*), levadura (*Saccharomyces cerevisi*ae) y la emulsión M70. En un segundo experimento, se llevó a cabo un enriquecimiento con una emulsión comercial (DC Super Selco), M70, y una combinación de M70 e hidroxitirosol (Hytolive, HYT) añadido como antioxidante externo. La combinación de M70 con HYT produjo los mejores resultados, en términos de la actividad de los enzimas antioxidantes. El análisis de los ácidos grasos de lípidos totales mostró que la adición de hidroxitirosol preservaba el porcentaje de DHA de los nauplios enriquecidos.*

5.1. Introduction

Adequate provision of polyunsaturated fatty acids (PUFA) is important in the diet of marine organisms, in particular during early life stages, where these compounds accumulate in rapidly developing tissues such as brain and eye (Bell et al., 1995, 2003; Benitez-Santana et al., 2007; Tocher, 2010). Thus, enrichment of live preys like *Artemia*, which are naturally deficient of essential PUFA such as docosahexaenoic acid (22:6n-3, DHA), is critical for the survival and normal development of larval stages of marine organisms, and thus the production of good quality fingerlings (Hamre & Harboe, 2008; Tocher, 2010).

McEvoy et al. (1995) investigated the stability of PUFA in lipid emulsions throughout the enrichment process since optimal enrichment conditions such as vigorous aeration, illumination and temperature of 28 ºC, tend to accelerate autoxidation of PUFA and the formation of potentially toxic oxidation products. It was found that, at later stages of the enrichment process, all the emulsions tested showed autoxidation associated to notable decreases in the concentration of PUFA in the lipids of *Artemia* nauplii, particularly for eicosapentaenoic acid (EPA, 20:5n-3) and DHA. Interestingly, it was reported that autoxidation of enrichment diets occurred even in commercial emulsions that are formulated with antioxidants. Beyond the decrease in the enrichment efficiency described above, the study of autoxidation in enrichment diets is also important because it implies that

Artemia nauplii can accumulate potentially toxic oxidation products that can compromise the health and survival of fish and crustacean larvae. In fact, McEvoy et al. (1995) recommended that *Artemia* enrichments, particularly those with DHA-rich emulsions, should be shortened so that the alluded to accumulation of potentially toxic compounds in nauplii could be reduced.

We have recently investigated the efficiency of a DHA-rich oil emulsion (M70) as enrichment diet for *Artemia* nauplii (Viciano et al., 2015). M70 consists of a 1:1 (v/v) oil/water suspension, based on the synthetic oil DHA Algatrium (Brudy Technology, Barcelona, Spain) that contains 70% of total fatty acids as DHA in the form of ethyl esters (Viciano et al., 2015). Additionally, the emulsion M70 is also formulated with Tocobiol Plus (BTSA Biotecnologías Aplicadas, Madrid, Spain), a liposoluble antioxidant. Considering the particular aspects derived from its DHA-rich nature, a series of experiments were carried out to optimise the use of M70 under different enrichment conditions including oxygenation regime, incubation temperature, nauplial density and enrichment product concentration and dosage mode, which have been regarded as important factors determining the enrichment efficiency (Van Stappen, 1996; Han et al., 2000, 2001; Monroig et al., 2006a, b; Sui et al., 2007; Hamre & Harboe, 2008; Figueiredo et al., 2009). Importantly, our results suggested that, despite the likely

preservative action of the liposoluble antioxidant Tocobiol Plus, oxidation of DHA contained in M70 could not be ruled out as suggested by relatively low DHA incorporation in *Artemia* nauplii (Viciano et al., 2015).

Pro-oxidative conditions during enrichment could partly explain massive mortalities events reported in the literature during enrichments (Monroig et al., 2006b), since they can alter the capacity of live preys for catalyzing oxidative reactions, leading to the production of reactive oxygen species (ROS) as a general pathway of toxicity causing oxidative stress (Livingstone, 2001; Barata et al., 2005b). ROS include superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) (Correia et al., 2003), compounds that are involved in cellular damage and with the capacity to promote morphological and physiological failures and peroxidation of PUFA, all this leading to pathologies and compromising normal development (Tovar-Ramirez et al., 2010). To minimize oxidative damage to cellular components, organisms have developed key antioxidant enzyme defences that can be used as biomarkers (Barata et al., 2005a, b).

Water-soluble antioxidants appear to have potential for preventing DHA oxidation during *Artemia* enrichments. In a number of studies, the polyphenolic compound hydroxytyrosol (3,4-dihydroxyphenylethanol) (Hao et al., 2010), has been shown to be more efficient than antioxidant vitamins and

synthetic antioxidants (Gordon et al., 2001; González, 2005; Deiana et al., 2008; Fernández-Bolaños et al., 2008). Hydroxytyrosol is a polar molecule found in the water fraction resulting after the milling process of olives (Schaffer et al., 2007). Several studies have investigated the potent activity of hydroxytyrosol to neutralize free radicals and slow oxidative stress (Chimi et al., 1991; Visioli et al., 1998, 2000). To the best of our knowledge, there are no data available concerning its potential role as protective agent of oil emulsions used for live prey enrichments.

The global aim of the present study was to investigate the protective effects of the addition of an external water-soluble natural olive fruit extract, rich in the polyphenol hydroxytyrosol (HYT, Hytolive Protect, Genosa I+D S.I., Malaga, Spain), to the enrichment of *Artemia* nauplii with a DHA-rich oil emulsion (M70). For this purpose, key enzymes (superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase) as biomarkers of antioxidant activity, and the lipid peroxidation of enriched *Artemia* nauplii were determined. To the best of our knowledge, such an approach has never been used before to study the effects of oxidation during enrichment. Two experiments were performed. Experiment 1 aimed at establishing the baseline of lipid oxidation in the enrichment of *Artemia* nauplii. We analyzed the antioxidant activity and lipid peroxidation of *Artemia* nauplii enriched with M70, and

compared them with those of nauplii enriched with yeast *Saccharomyces cerevisiae* or the green microalgae *Tetraselmis suecica*, as well as those of non-enriched nauplii. Yeast and green microalgae are known to have antioxidant properties (Greetham & Grant, 2009; Lee et al., 2009). Experiment 2, aimed at studying the effect of the external antioxidant HYT during enrichment with high PUFA emulsions. For this purpose we determined the antioxidant activity, lipid peroxidation and fatty acid composition of: a) non-enriched nauplii, used as a control; b) nauplii enriched with a commercial emulsion (DC Super Selco, DCSS, Inve, Dendermonde, Belgium), as reference of conventional enrichment; c) nauplii enriched with M70; and d) nauplii enriched with M70 plus the water-soluble antioxidant HYT.

5.2. Materials and methods

5.2.1. Experimental design

Artemia nauplii were obtained from the hatching of EG grade cysts (Inve, Dendermonde, Belgium). After an incubation period of 23 h at 28 ºC, nauplii were collected and rinsed with tap water to remove the hatching debris and metabolites. Newly hatched nauplii were thereafter placed in 1 L cylinder-conical vessels containing seawater.

The *Artemia* enrichments were performed for 21 h with incubation temperatures of 28 ± 1 ºC, low/moderate aeration (1 L min^{-1}), the air diffusion system consisting of a 25 cm long and 0.5 cm-diameter section glass tube applied from the bottom of the vessel. Nauplial densities were 300 nauplii mL^{-1} (Monroig et al., 2006a, b), and illumination was 1500 lux (Viciano et al., 2015). The temperature was kept (±1 ºC) constant by placing the hatching and enrichment vessels in a thermostatic bath. The enrichment product was dispensed in a single dose at the beginning of the incubation.

All the enrichment treatments were run in triplicates (n=3). After an enrichment period, samples of *Artemia* nauplii were collected by filtering the enrichment medium through a 100 µm mesh carefully washed with tap water and subsequently rinsed with distilled water.

Experiment 1: Baseline of M70 oxidation

Antioxidant activity and lipid peroxidation levels were determined in *Artemia* nauplii enriched with: a) 0.6 g L^{-1} *Saccharomyces cerevisiae* (fresh yeast (Levital, Lesaffre Ibérica S.A., Valladolid, Spain) dispersed in sea water using a mixer for 2 minutes), b) *Tetraselmis suecica* (algal culture stock from IATS facilities, at 300000 ± 20000 cells mL^{-1}), c) 0.8 g L^{-1} M70 emulsion (dispersed in sea water using a mixer for 2 minutes in a single dose at the beginning of the enrichment period), and d)

a control group consisting of unenriched *Artemia* nauplii kept in the same conditions.

Experiment 2: External antioxidant

Antioxidant activity, fatty acid profile and lipid peroxidation of *Artemia* nauplii enriched with 0.6 g L^{-1} commercial emulsion DCSS, 0.8 g L^{-1} M70 emulsion, 0.8 g L^{-1} M70 in combination with 0.04 g L^{-1} of exogenously added water-soluble antioxidant HYT (hereafter named M70+HYT treatment) and a control group of unenriched *Artemia* nauplii were compared. All enrichment media were dispersed in sea water using a domestic blender for 2 min.

5.2.2. Sample preparation for antioxidant enzymes

After enrichment, nauplii were filtered through a 100 µm mesh, washed with distilled water and kept at -80 ºC until analysis. Samples were processed as soon as possible and always within 3 h of the end of the enrichment period to avoid possible oxidation during the storage. *Artemia* samples were homogenized in a 1:4 wet weight: buffer volume ratio in 100 mM phosphate buffer, pH 7.4 at 4 ºC, containing 100 mM KCl and 1 mM ethylenediaminetetrasacetic acid (EDTA). Homogenates were further centrifuged at 10000 g for 10 min, and supernatants were immediately used as enzyme sources. Supernatant proteins were measured by the Bradford method (Bradford, 1976) using bovine serum albumin as standard.

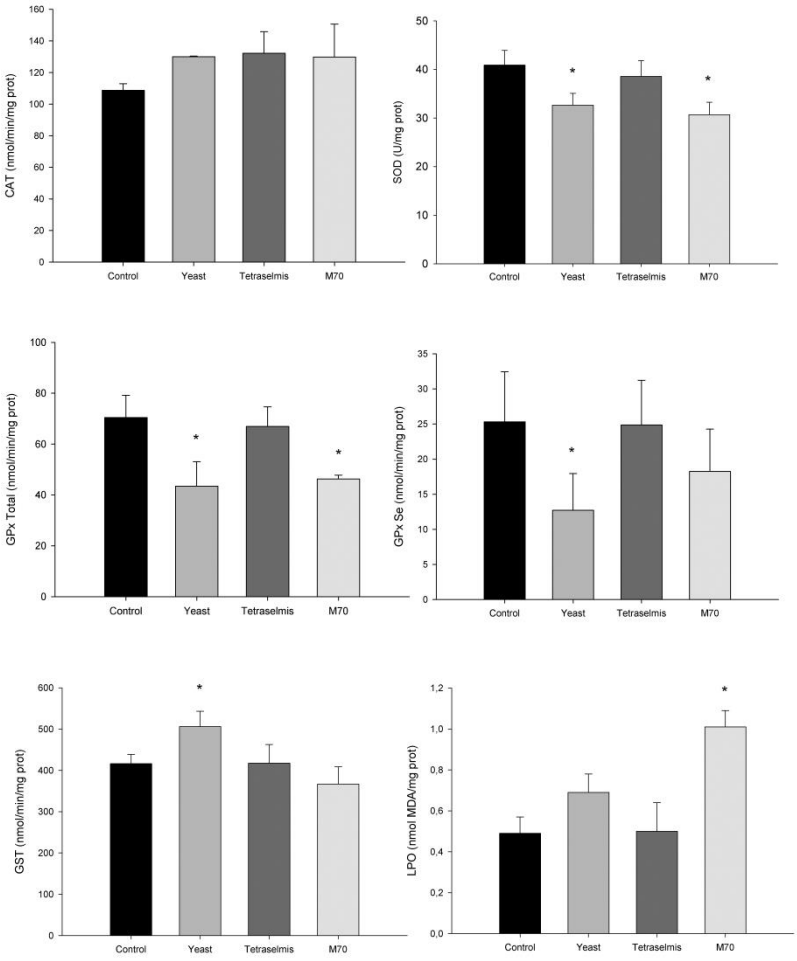

Figure 5.1. Activity of Catalase (CAT), superoxide dismutase (SOD), total glutathione peroxidase (GPx Total), Selenium dependent glutathione peroxidase (GPx Se-dep), glutathione-s-transferase (GST) and lipid peroxidation (LPO) in whole body of *Artemia* nauplii enriched with M70 emulsion, *Tetraselmis suecica* algae, yeast (*Saccharomyces cerevisiae*) and non-enriched *Artemia*. Asterisk shows statistical differences between the different treatments and the control group (Dunnett test).

5.2.3. Enzymatic activities

The following enzymatic activities were measured in *Artemia* homogenates: Superoxide dismutase, SOD (EC 1.15.1.1, converts O_2^- to H_2O_2); catalase, CAT (EC 1.11.1.6, reduces H_2O_2 to water); glutathione peroxidise, GPx (EC 1.11.1.9, detoxifies H_2O_2 or organic hydroperoxides produced e.g. by lipid peroxidation); and Glutathione-S-transferase, GST (EC 2.5.1.18, plays a role preventing oxidative damage by conjugating breakdown products of lipid peroxides to glutathione (GSH)) (Barata et al., 2005a, b; Tovar-Ramirez et al., 2010).

All the enzymatic assays were measured as described by Barata et al. (2005a, b). The final results for enzymatic activities were normalized by protein content. Assays were run at least in duplicate.

5.2.4. Lipid peroxides

Another factor affecting the potential oxidative damage is the level of target molecules like PUFA, which are easily oxidized by ROS in lipid peroxides (Di Giulio et al., 1995). Lipid peroxidation was evaluated analysing the lipid peroxidation levels (LPO). Malondialdehyde (MDA) is a common substance produced in lipid peroxidation. LPO method is based in the reaction of MDA with N-methyl-2-phenylindole

(NMPI) to form a coloured complex with known absorbance (586 nm) (Esterbauer et al., 1991). By this method the interference from other lipid peroxidation products (such as 4-hydroxyalkenals) is minimized.

5.2.5. Fatty acid analysis

Fatty acid analyses were performed to check any protective effect of the external antioxidant (mainly on PUFA). Thus, in Experiment 2, two samples of each replicate of every treatment (*Artemia* enriched with the different diets) were obtained, one was assigned to the analysis of enzymatic activity (processed as specified above), and the other was freeze-dried to proceed with the analysis of total lipids and fatty acids. Total lipids were extracted following the method of Folch et al. (1957), and analyses of fatty acids were carried out following the methods described in Viciano et al. (2015).

5.2.6. Statistical analysis

Analytical data were expressed as means ± standard deviation (SD) (n=3). When more than two means were compared, differences with control group were analysed by Dunnett test (Dunnett, 1964). Dunnett test is a multiple comparison procedure and is performed by computing a Student t-statistic for each experimental or treatment group, where the statistic compares the treatment group to a single control group.

When significance was $P \leq 0.05$, means were considered statistically different. For pair-wise comparisons a Student t test was used.

Enzyme activities analytical data, and highly unsaturated fatty acids (HUFA), fatty acids of 20 or more atoms of carbon and two or more double bonds, which are considered to have the highest oxidative potential (Barata et al., 2005c), were included as variables in multivariate Principal Component Analyses (PCA), to highlight the effects of the different treatments on the patterns of enzyme activities and on the fatty acid profiles composition. With such a parsimonious approach, the dataset of variables is reduced into a smaller set of factors or components. Parsimony is achieved by explaining the maximum amount of common variance in a correlation matrix using the smallest number of explanatory concepts. Factors are statistical entities that can be visualized as classification axes along which measurement variables can be plotted, providing an idea of their correlation with the corresponding factor (loading). Score plots are a graphical representation of individual (treatment groups) scores in the new subset of measurement variables (factors). They illustrate the relationship among individual cases (treatment groups), and the variables, and help in the analysis of data by showing graphical display. Statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA).

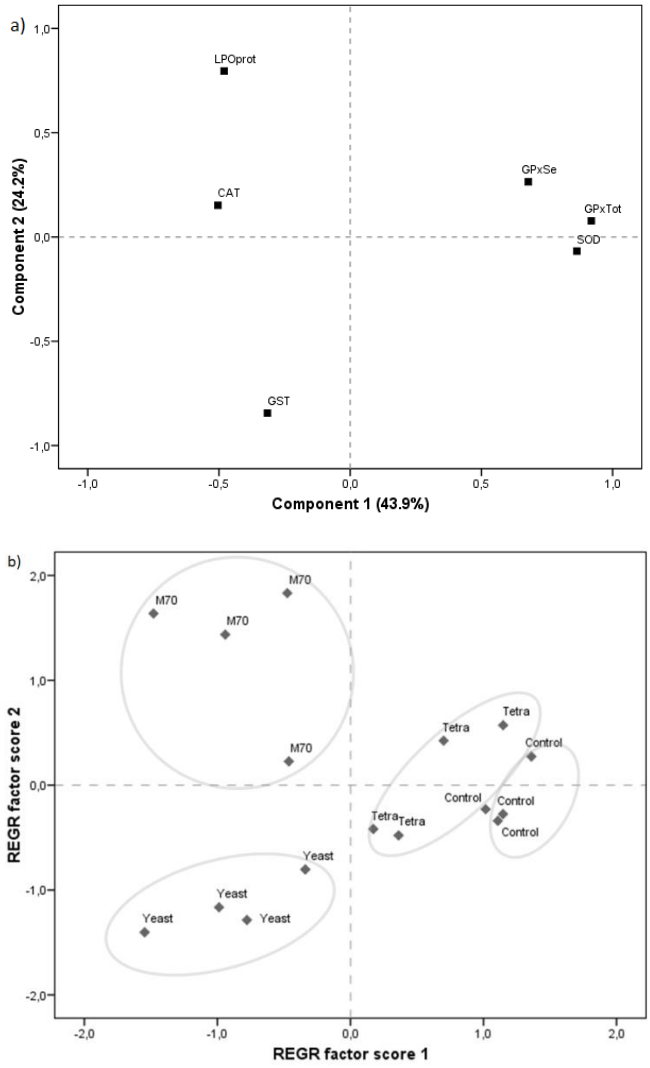

Figure 5.2. Component plot (a) and factor score plot (b) of the multivariate Principal Components Analysis of lipid peroxidation (LPOprot) and antioxidant enzymes (Catalase (CAT), glutathione-s-transferase (GST), superoxide dismutase (SOD), total glutathione peroxidase (GPxTot), Selenium dependent glutathione peroxidase (GPxSe)) of enriched *Artemia* nauplii with M70 emulsion, *Tetraselmis suecica* algae (Tetra), yeast (*Saccharomyces cerevisiae*) and non-enriched *Artemia* from Experiment 1.

5.3. Results

Experiment 1: Baseline of M70 oxidation

Figure 5.1 illustrates the levels of enzymatic activities and LPO measured in the control group and in the *Artemia* nauplii enriched with different treatments. The *Tetraselmis* group was not significantly different from control group in any of the enzymatic activities or LPO measured. Yeast treatment presented lower activities of SOD, GPx-Tot and GPx-Se, but a high GST activity. M70 group showed a decreased activity of SOD and GPx-Tot and a significant ($P<0.05$) increase, near two-fold relatively to control animals, in LPO.

The component plot (Figure 5.2a) obtained from PCA shows variables that were responsible for separation along the two principal components. The first two components of the PCA explained more than 68% of total variance (43.9% and 24.2% for the first and second component, PC1 and PC2, respectively). The factor score plot (Figure 5.2b) showed groups separated in the second component on the basis of lipid peroxidation. M70 treatment appeared to be related with high loads of LPO and clearly separated from the other treatments. Control and *Tetraselmis* treatments appeared very close to each other and on the opposite side of M70 treatment, indicating less lipid peroxidation, and higher activities of GPx-Tot, GPx-Se and SOD than M70. The Yeast treatment was related to high GST

activity. This graph allowed us to establish the baseline of antioxidant activity for the M70 emulsion, and to investigate the effect of including an external antioxidant into the enrichment media.

Experiment 2: External antioxidant

Figure 5.3 shows the enzyme activities and levels of LPO of the control and enriched *Artemia* nauplii with and without the antioxidant HYT. Observed patterns of antioxidant enzyme activities and LPO levels for M70 versus controls were quite similar to those reported in Figure 1, except for GST. *Artemia* nauplii incubated with the two tested enriched media (DCSS and M70) showed similar enzymatic activity and LPO patterns. The addition of the antioxidant HYT decreased the activities of CAT and GST, and increased those of GPx-Tot of *Artemia* nauplii enriched with M70.

LPO were significantly ($P<0.05$) higher in nauplii enriched with DCSS and M70 treatments. Adding an external antioxidant (HYT) dramatically reduced LPO.

Table 5.1 lists the main fatty acids (% of total fatty acids) from total lipids of *Artemia* nauplii enriched with different treatments. EPA values were significantly ($P<0.05$) higher in DCSS treatment (12.2 ± 2.6) compared to all other treatments. The n-3 HUFA increased in all treatments when compared with

Table 5.1. Selected fatty acids (percentage of total fatty acids) from enriched *Artemia* nauplii from Experiment 2. Data represent means ± SD (n=3). Treatments with asterisk are significantly different ($P \leq 0.05$) from control treatment (Dunnett test). If no superscript appears, values are not different. T-Student test was performed between M70 and M70+HYT treatments, and statistical differences were marked with a capital letter superscript (A, B)

% Fatty acids	Control	DCSS	M70	M70+HYT
14:0	1.1±0.1	1.0±0.1	1.2±0.2	1.1±0.1
16:0	12.2±0.7	10.0±0.5*	10.8±0.3*	10.8±0.4*
16:1n-9	0.6±0.1	0.5±0.1	0.7±0.1	0.7±0.2
16:1n-7	2.4±0.1	2.3±0.2	2.4±0.1	2.3±0.1
18:0	6.7±0.0	5.0±0.1*	5.0±0.1*	5.0±0.1*
18:1n-9	17.5±0.9	16.4±0.8	15.7±0.7*	15.5±0.3*
18:1n-7	8.6±0.4	6.7±0.5*	7.1±0.5*	6.8±0.2*
18:2n-6	5.4±0.1	5.9±0.4	5.1±0.3	4.9±0.1
18:3n-3	25.1±0.6	22.0±0.8*	25.5±0.7^{A}	23.8±0.4^{B}
18:4n-3	3.2±0.1	2.8±0.2	3.1±0.3	3.0±0.0
20:0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
20:2n-6	0.2±0.0	0.3±0.0	0.2±0.0	0.2±0.0
20:3n-6	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
20:4n-6	0.8±0.1	1.0±0.2	1.1±0.1*	1.2±0.0*
20:3n-3	0.7±0.0	0.6±0.0	0.7±0.1	0.6±0.0
20:4n-3	0.6±0.0	0.8±0.0*	0.6±0.0	0.6±0.0
20:5n-3	2.2±0.4	12.0±2.6*	4.3±0.7	4.6±0.2
22:0	0.4±0.0	0.3±0.0*	0.3±0.0*	0.3±0.0*
22:5n-3	0.0±0.0	0.4±0.1*	0.4±0.1*	0.5±0.0*
22:6n-3	0.1±0.0	2.4±0.3*	5.1±0.8^{*A}	8.4±0.5^{*B}
Saturated	22.8±0.8	18.1±0.8*	19.5±0.4*	19.0±0.6*
Monounsaturated	31.0±0.9	28.6±1.0*	27.4±1.2*	26.7±0.5*
Polyunsaturated	40.0±1.4	48.0±2.5*	47.9±0.9*	49.1±1.0*
HUFA n-3	3.6±0.5	15.5±5.2*	12.0±3.0*	14.3±1.3*
HUFA n-6	1.4±0.3	1.6±0.2	1.9±0.2	2.2±0.2*
DHA/EPA ratio	0.0±0.0	0.2±0.1*	1.2±0.1^{*A}	1.8±0.0^{*B}
Total FAME (mg g^{-1})	43.7±7.9	138.3±12.1*	105.7±17.1*	121.3±9.2*
% Lipid (DW)	20.9±0.4	27.8±1.3	27.4±0.5	26.2±0.4

HUFA n-3: ≥20:3n-3; HUFA n-6: ≥20:2n-6; DHA/EPA: docosahexaenoic/eicosapentaenoic fatty acid ratio

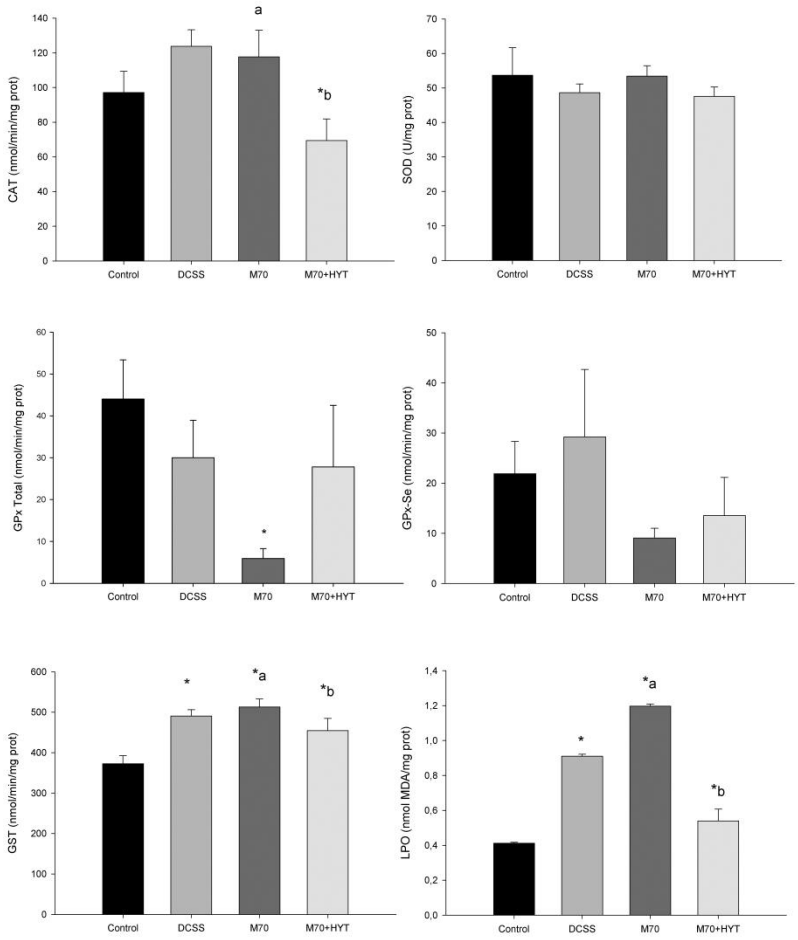

Figure 5.3. Activity of Catalase (CAT), superoxide dismutase (SOD), total glutathione peroxidase (GPx Total), Selenium dependent glutathione peroxidase (GPx Se-dep), glutathione-s-transferase (GST) and lipid peroxidation (LPO) in whole body of non-enriched *Artemia* nauplii and *Artemia* nauplii enriched with commercial emulsion DCSS, M70 emulsion and M70 emulsion in combination with an external antioxidant HYT, M70+HYT treatment. Asterisk shows statistical differences between the treatment and the control group (Dunnett test) and lowercase letter superscript shows statistical differences between M70 and M70+HYT treatment (Student t-test).

the control group, but n-6 HUFA were only significantly ($P<0.05$) higher in M70+HYT treatment. DHA levels were significantly ($P<0.05$) higher in M70+HYT treatment compared to other treatments, and this result is reflected in the highest DHA/EPA ratio among all treatments.

Figure 5.4a illustrates the component plot for the enzymatic activity and HUFA data. The two first components of PCA accounted for the 66.9 % of variation, PC1 explaining 45.0% and PC2 explaining 21.9% of the variation of the data set. Figure 5.4b shows the factor score plot based on enrichment and antioxidant scores from the constituent variables. All treatments were clearly separated from each other, with control treatment to the left, DCSS and M70+HYT in the middle, and M70 to the right. The PCA grouping indicates that M70 treatment has higher LPO than the other treatments. Figure 4 also shows that the scores of M70+HYT treatment were associate to both n-3 and n-6 HUFA variables pointing at the protective role of HYT during enrichment.

Analysing the PCA results of both experiments, the M70 group is always linked to lipid peroxidation as evidenced by the association of the scores to the location of the LPO variable in the components graph. The association is lost by adding HYT to the enrichment. The separation of the two groups (M70 and M70+HYT) was achieved, with the LPO values of M70+HYT

treatment approaching those of the commercial emulsion DCSS and control group.

5.4. Discussion

High levels of DHA are important in the development of nervous and sensory system during early development of vertebrates, including fish (Hamre & Harboe, 2008; Tocher, 2010). For that reason, enrichment of live preys including *Artemia* that naturally lack DHA is critical for the successful rearing of fish larvae, particularly those from marine species (Tocher, 2010). DHA levels achieved in *Artemia* nauplii with the M70 and M70+HYT treatments were different. The DHA/EPA ratio is a common biochemical parameter used to evaluate the nutritional suitability of diets for marine finfish larviculture (Evjemo et al., 1997), and high DHA/EPA ratios were also reported as important to promote growth, stress resistance and pigmentation (Mourente et al., 1993; Reitan et al., 1994; Sorgeloos et al., 2001). Rodríguez et al. (1997) observed significantly higher growth rates in sea bream larvae fed rotifers with a DHA/EPA ratio of 1.5 compared to those fed DHA/EPA ratio < 0.6. In natural preys such as copepods, this ratio is around 2.0 (Fraser et al., 1989; Sorgeloos et al., 2001; Van der Meeren et al., 2011). Currently satisfactory results in an enrichment process with *Artemia* nauplii is achieved with DHA/EPA ratios of 2 and higher (Dhert et al., 1993; Sorgeloos et al., 2001) but, on the other hand, this has been acknowledged

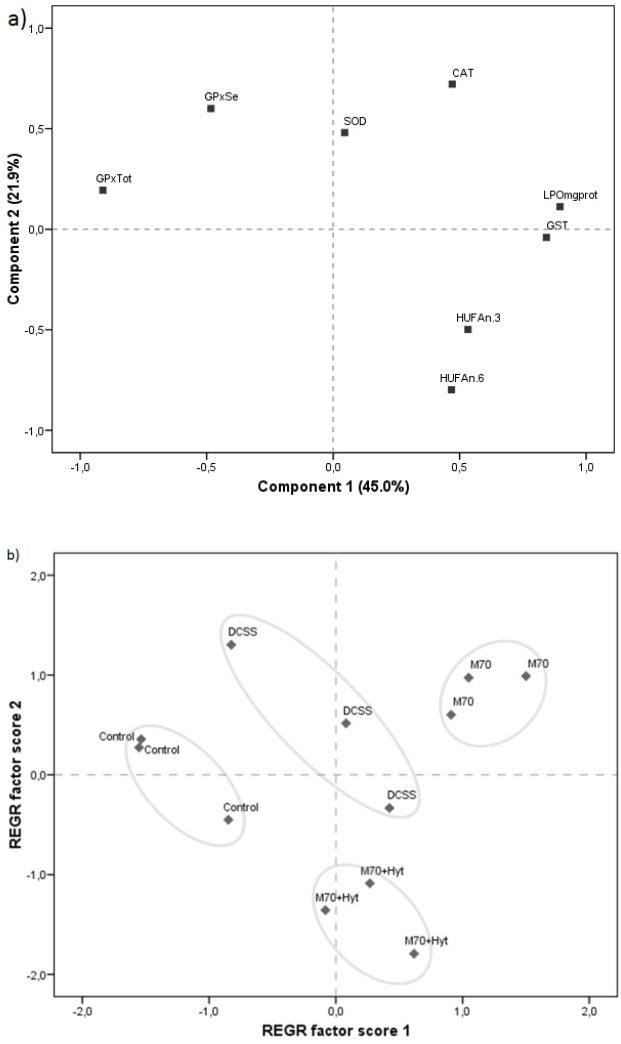

Figure 5.4. Component plot (a) and factor score plot (b) of the multivariate Principal Components Analysis of lipid peroxidation (LPOprot), antioxidant enzymes (Catalase (CAT), glutathione-s-transferase (GST), superoxide dismutase (SOD), total glutathione peroxidase (GPxTot), Selenium dependent glutathione peroxidase (GPxSe)) and total HUFA (n-3 and n-6 series) of enriched *Artemia* nauplii with commercial emulsion DCSS, M70 emulsion, M70 emulsion in combination with an external antioxidant, HYT (M70+HYT treatment) and non-enriched *Artemia* from Experiment 2.

as a major challenge due to an apparent inability of *Artemia* nauplii to retain DHA (Evjemo et al., 1997). In fact, *Artemia* nauplii actively retroconvert DHA to EPA during and after enrichment (Navarro et al., 1999). Thus, enrichment of *Artemia* nauplii is a continuous quest towards increasing PUFA and DHA, and despite the use of high DHA emulsions like M70 which consistently produces *Artemia* DHA/EPA ratios higher than 1 (Viciano et al., 2015), the fact that it only produces moderate levels of DHA in the enriched nauplii (5-8 % of total fatty acids) illustrates the problems of DHA retroconversion in *Artemia*.

Artemia nauplii possess antioxidant enzymes required to metabolize O_2^- (SOD), H_2O_2 (CAT and GPx) and organic hydroperoxides activity (GPx-Se) (Nunes et al., 2006). These enzymes allow the organism to prevent, intercept and repair the damage caused by the free radicals (Olsen et al., 2013). However, during live feed enrichments, it is possible that these endogenous protective mechanisms are not sufficient to prevent oxidative damage as HUFA contained in the enrichment diet are very prone to oxidation, particularly under physical-chemical conditions set up during the enrichment procedures (McEvoy et al., 1995; Monroig et al., 2007), thus contributing to the low efficiency in HUFA enrichment in general and DHA in particular.

SOD is the first defence mechanism in the detoxification process and, along with CAT, is regarded as playing an

important antioxidant role in aquatic invertebrates (Livingstone, 1991; Barata et al., 2005b). In Experiment 1, SOD activity was inhibited in the nauplii enriched with yeast and M70, in comparison with those enriched with *Tetraselmis*, and the control group (unenriched nauplii). These differences can be explained by SOD *modus operandi*, with SOD activity increasing in early stages of oxidative stress when ROS abundance increases. If the oxidative stress persists, SOD becomes depleted and unable to cope with free radicals (Lukaszewicz-Hussan & Moniuszko-Jakoniuk, 2004). This is consistent with increased LPO levels achieved in *Artemia* nauplii enriched with M70 and yeast in Experiment 1. In Experiment 2 differences in SOD activity were not observed, although lipid peroxidation in M70 treatment was very high. Inter experiment difference in SOD supports the previous argument that antioxidant enzyme activities are transient (Livingstone, 1991; Barata et al., 2005b).

The response to oxidative stress has been related to increased CAT activities (Tovar-Ramirez et al., 2010) and therefore, the activity of this enzyme is expected to increase with the presence of substrates that enhance or produce ROS (Barata et al., 2005a; Tovar-Ramirez et al., 2010). In this study, CAT activity in *Artemia* nauplii from the control group remained at low levels, increasing in those nauplii enriched with yeast, *Tetraselmis*, DCSS or M70 emulsion. Nauplii enriched with a combination of the DHA-rich emulsion M70 and the

antioxidant Hytolive (HYT) treatment showed significantly lower CAT activities than those enriched with the same emulsion (M70) without antioxidant, and a commercial emulsion (DCSS). Clearly, these results strongly suggest the protective effect of Hytolive added to the enrichment diet. CAT and GPx have complementary roles in the detoxification of hydrogen peroxides, but have different subcellular localization (peroxisomal and cytosolic, respectively) and affinity for H_2O_2 levels. Thus, high levels of H_2O_2 produce an increased affinity for CAT, whereas low levels of H_2O_2 produce an increased affinity for GPx (Orbea et al., 2000; Barata et al., 2005b). Interestingly, GPx activity (GPx-Tot and GPx-Se) reached the highest values in unenriched nauplii (control). On the contrary, *Artemia* nauplii enriched with M70 showed low activity values for both GPx enzymes, suggesting high levels of H_2O_2 and oxidative stress. Addition of the antioxidant HYT increased the GPx activity in nauplii enriched with M70 and, together with the decreased CAT activity mentioned above, made the nauplii enriched with M70+HYT exhibit the best antioxidant status among all enrichment diets tested and most closely resembled the control *Artemia*. These results clearly indicate that the Hytolive exerts an effective antioxidant effect during *Artemia* enrichment and, in addition to lipophilic antioxidants that are commonly used in the formulation of enrichment diets, inclusion of water-soluble antioxidants may also help live preys to cope with exposure to toxic oxidized compounds.

GSTs are a family of Phase II detoxification enzymes that catalyze the conjugation of glutathione (GSH) to a wide variety of endogenous and exogenous electrophilic compounds (Townsend & Tew, 2003). GST activity detoxifies endogenous compounds such as peroxidized lipids and enables the breakdown of xenobiotics (Sherrat & Hayes, 2001). GST activity was higher than the experimental control in nauplii enriched with yeast, DCSS, M70 (Expt. 2) and M70+HYT treatment. This is consistent with previous reports showing that GST activity increases in response to exogenous chemical sources including those promoting ROS (Barata et al., 2005b). Increased GST activity indicates indirect conjugation to its endogenous substrate during Phase II detoxification, which would help eliminate toxic metabolites (Townsend & Tew, 2003). Phenolic compounds from olive oil can restore redox balance by free radical quenching (El-Azem, 2013). By neutralizing the free radicals formed, the amount of antioxidant enzymes such as GSH is preserved in the body (Masella et al., 2004; El-Azem, 2013). The previous effect may explain the slight but significant reduction of GST in *Artemia* nauplii co-exposed to M70 and HYT.

Because ROS have extremely short half-lives, they are difficult to measure directly and therefore oxidative stress can be inferred through measurement of several products of the damage produced (Pryor, 1991). Lipid peroxidation (LPO) can be considered an index of oxidative damage to lipids produced by

toxic compounds (Fernández et al., 2012). Results from this study showed that *Artemia* nauplii enriched with M70 and DCSS emulsions presented significantly higher LPO levels than those supplemented with HYT and the control group.

It is reasonable to assume that lipid peroxides and other potentially toxic oxidation products are filtered by *Artemia* nauplii during the enrichment procedure (McEvoy et al., 1995). *Artemia* enrichment is largely regarded as a "bioencapsulation" process whereby the *Artemia* ingest enrichment diet particles until the gut is full (Figueiredo et al., 2009). The supplementation of M70 enrichments with HYT was hypothesised to decrease the lipid peroxidation and therefore the presence of oxidized lipid available in the enrichment media. Consequently, the PCA results strongly suggest that such a protective action from HYT took place, as the HYT-supplemented treatment clearly separated from the M70 group, the latter containing higher levels of the oxidation biomarker LPO. There are no previous studies using hydroxytyrosol on live prey enrichment. In humans, hydroxytyrosol is rapidly absorbed in a dose-dependent manner in the small intestine and colon by bidirectional passive diffusion (El-Azem, 2013), and then is mostly conjugated as glucuronoconjugates, the most abundant form found in plasma. From this conjugated form, it displays its antioxidant potential. Hydroxytyrosol is rapidly metabolized and, after five minutes of administration, it is possible to find derivatives in plasma (Miro-Casas et al., 2003). McEvoy et al.

(1995) demonstrated autoxidation in three enrichment emulsions when *Artemia* were present, however the oil emulsions in the control media without *Artemia* did not show autoxidation within the experimental period. It is expected that once introduced into the enrichment media, with the brine shrimp present, the protective effect of hydroxytyrosol begins due to its water soluble nature, protecting against oxidation of the fatty acids. Thereby oxidation may be reduced before the fatty acids entrance in the digestive tract of *Artemia*, ensuring that the enzymatic system is not oversaturated and remains active. This is shown in the improvement of the enzymatic activity when hydroxytyrosol was added in the enrichment medium.

In conclusion, the addition of a natural water-soluble olive fruit extract with a high amount of hydroxytyrosol to the lipid emulsion enrichment improves the antioxidant defence of *Artemia* nauplii and decreases the levels of the oxidative biomarker LPO. Moreover, the addition of this olive fruit extract makes the activity of all antioxidant enzymes in *Artemia* enriched with a DHA-rich oil emulsion resemble those of homeostasis as defined by unenriched nauplii. Such an antioxidant protective role of hydroxytyrosol is reflected in the lipid profile of *Artemia*, since higher levels of DHA and a higher DHA/EPA ratio were found in nauplii enriched in the presence of hydroxytyrosol. As a first approach, here, only a concentration of 0.04 g L^{-1} was tested and, although the benefits prove significant, probably higher concentrations would produce

even better results, since there is evidence of an inhibitory effect of HYT in LPO at increasing concentrations (Gargouri et al., 2011). Thus further dose dependent studies are advisable in order to establish if the antioxidant potential can be further enhanced and to verify how this improvement may be applicable in large-scale enrichments.

5.5. References

Barata, C., Lekumberri, I., Vila-Escalé, M., Prat, N., Porte, C., 2005a. Trace metal concentration, antioxidant enzyme activities and susceptibility to oxidative stress in the tricoptera larvae *Hydropsyche exocellata* from the Llobregat river basis (NE Spain). Aquatic Toxicology 74, 3-19.

Barata, C., Varo, I., Navarro, J.C., Arun, S., Porte, C., 2005b. Antioxidant enzyme activities and lipid peroxidation in the freshwater cladoceran *Daphnia magna* exposed to redox cycling compounds. Comparative Biochemistry and physiology Part C: Toxicology & pharmacology 140, 175-186.

Barata, C., Navarro, J.C., Varo, I., Riva, M.C., Arun, S., Porte, C., 2005c. Changes in antioxidant enzyme activities, fatty acid composition and lipid peroxidation in *Daphnia magna* during the aging process. Comparative Biochemistry and Physiology Part B 140, 81-90.

Bell, M.V., Batty, R.S., Dick, J.R., Fretwell, K., Navarro, J.C., Sargent, J.R., 1995. Dietary deficiency of docosahexaenoic acid impairs vision at low light intensities in juvenile herring (*Clupea harengus* L.). Lipids 30, 443-449.

Bell, J.G., McEvoy, L.A., Estévez, A., Shields, R.J., Sargent, J.R., 2003. Optimising lipid nutrition in first-feeding flatfish larvae. Aquaculture 227, 211-220.

Benítez-Santana, T., Masuda, R., Juárez Carrillo, E., Ganuza, E., Valencia, A., Hernández-Cruz, C.M, Izquierdo, M.S., 2007. Dietary n-3 HUFA deficiency induces a reduced visual response in gilthead seabream *Sparus aurata* larvae. Aquaculture 264, 408-417.

Bradford, M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry 72, 248-254.

Chimi, H., Cillard, J., Cillard, P., Rahmani, M., 1991. Peroxyl and hydroxyl radical scavenging activity of some natural phenolic antioxidants. JAOCS 68, 307-312.

Correia, A.D., Costa, M.H., Luis, O.J., Livingstone, D.R., 2003. Age-related changes in antioxidant enzyme activities, fatty acid composition and lipid peroxidation in whole body *Gammarus locusta* (Crustacea: Amphipoda). J. Exp. Mar. Biol. and Ecol. 289, 83-101.

Deiana, M., Incani, A., Rosa, A.,Corona, G., Atzeri, A., Loru, D., Melis, M.P., Dessi, A., 2008. Protective effect of hydroxytyrosol and its metabolite homovanillic alcohol on H_2O_2 induced lipid peroxidation in renal tubular epitelial cells. Food and Chem. Toxixology 46, 2984-2990.

Dhert, P., Sorgeloos, P., Devresse, B., 1993. Contributions towards a specific DHA enrichment in the live food *Brachionus plicatilis* and *Artemia* sp. In: Reinertsen, H., Dahle, L.A., Jorgensen, L., Tvinnereim, K. (Eds.), Fish Farming Technology. Balkema, Rotterdam, Netherlands, pp. 109-115.

Di Giulio, R.T., Benson, W.H., Sanders, B.M., Van Veld, P.A., 1995. Biochemical mechanisms: metabolism, adaptation and toxicity. In: Rand, G. (Ed.), Fundamentals of aquatic toxicology, effects, environmental fate and risk assessment, Taylor & Francis, London, pp. 523-561.

Dunnett, C.W., 1964. New tables for multiple comparisons with a control. Biometrics 20, 482-491.

El-azem, N., 2013. Efecto del hidroxitirosol sobre la cardiotoxicidad producida por doxorubicina. PhD. Universidad de Granada. Pp. 202.

Esterbauer, H., Schaur, R.J., Zollner, H., 1991. Chemistry and biochemistry of 4-hydroxynonenal malonaldehyde and related aldehydes. Free radic. Biol. Med. 11, 81-128.

Evjemo, J.O., Coutteau, P., Olsen, Y., Sorgeloos, P., 1997. The stability of docosahexaenoic acid in two *Artemia* species following enrichment and subsequent starvation. Aquaculture 155, 135-148.

Fernández, B., Campillo, J.A., Martínez-Gómez, C., Benedicto, J., 2012. Assessment of the mechanisms of detoxification of chemical compounds and antioxidant enzymes in the digestive gland of mussels, *Mytilus galloprovincialis*, from Mediterranean coastal sites. Chemosphere 87, 1235-1245.

Fernández-Bolaños, J.G., López, Ó., Fernández-Bolaños, J., Rodríguez-Guitiérrez, G., 2008. Hydroxytyrosol and derivates: Isolation, synthesis, and biological properties. Curr. Org. Chem. 12, 442-463

Figueiredo, J., Van Woesik, R., Lin, J., Narciso, L., 2009. *Artemia franciscana* enrichment model – How to keep them small, rich and alive? Aquaculture 294, 212-220.

Folch, J., Lees, M., Sloane Stanley, H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509.

Fraser, A.J., Sargent, J.R., Gamble, J.C., 1989. Lipid class and fatty acid composition of *Calanus finmarchicus* (Gunnerus), *Pseudicalanus* sp. and *Temora longicornis* Muller from a nutrient-enriched seawater enclosure. J. Exp. Mar. Biol. Ecol. 130, 81-92.

Gargouri, B., ben Mansour, R., Jmal, M.M., Boujelben, R.N., Allouch, N., Lassoued, S., Hamden, K. 2011. Hydroxytyrosol supplementation inhibits oxidative DNA damage, suppresses protein-lipid oxidation and modulates antioxidant enzymes in human peripheral blood lymphocytes. J. Medicinal Plants Research 5, 4863-4869.

González, M.P., 2005. Estudio de los efectos cardiovasculares y la absorción oral del hidroxitirosol en modelos animales y humanos. Ph D. University of Granada, Spain. 201 pp.

Gordon, M.H., Paiva-Martins, F., Almeida, M., 2001. Antioxidant activity of hydroxytyrosol acetate compared with that of other olive oil polyphenols. J. Agric. Food Chem. 49, 2480-2485.

Greetham, D. & Grant, C.M., 2009. Antioxidant activity of the yeast mitochondrial one-cys peroxidation is depedent on thioredoxin reductase and glutathione in vivo. Mol. Cell. Boil. 29: 3229-40

Hamre, K. & Harboe, T., 2008. *Artemia* enriched with high n-3 HUFA may give a large improvement in performance of Atlantic halibut (*Hippoglossus hippoblossus* L.) larvae. Aquaculture 277, 239-243

Han, K., Geurden, I., Sorgeloos, P., 2000. Enrichment strategies for *Artemia* using emulsions providing different levels of n-3 highly unsaturated fatty acids. Aquaculture 183, 335-347.

Han, K., Geurden, I., Sorgeloos, P., 2001. Fatty acid changes in enriched and subsequently starved *Artemia franciscana* nauplii enriched with different essential fatty acids. Aquaculture 199, 93-105.

Hao, J., Shen, W., Yu, G., Jia, H.,Li, X., Feng, Z., Wang, Y., Weber, P., Wertz, Z., Sharman, E, Liu, J., 2010. Hydroxytyrosol promotes mitochondrial biogenesis and mitochondrial function in 3T3-L1 adypocites. J. Nutr. Biochem. 21: 634-44.

Lee, S., Chang, D., Lee, B., Jeon, Y., 2009. Antioxidant activity of solubilized *Tetraselmis suecica* and *Chlorella ellipsoidea* by enzymatic digest. J. Food Sci. and Nutrition 14, 21-28.

Livingstone, D.R., 1991. Organic xenobiotics metabolism in marine invertebrates. Adv. Comp. Environ. Physiol. 7, 45-185.

Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Marine pollution bulletin 42, 656-666.

Lukaszewicz-Hussain, A. & Moniuszko-Jakoniuk, J., 2004. Liver catalase, glutathione peroxidase and reductase activity, reduced glutathione and hydrogen peroxide levels in acute intoxication with chlorfenviphos, an organophosphate insecticide. Pol. J. Environ. Studies 13, 303-309.

Masella, R., Varí, R., D'Archivio, M., Di Benedetto, R., Matarrese, P., Malorni, W., Scazzocchio, B., Giovannini, C., 2004. Extra virgin olive oil biophenols inhibit cell-mediated oxidation of LDL by increasing the mRNA transcription of glutathione-related enzymes. J. Nutr. 134, 785-791.

McEvoy, L.A., Navarro, J.C., Bell, J.G., Sargent, J.R., 1995. Autoxidation of oil emulsions during the *Artemia* enrichment process. Aquaculture 134, 101-112.

Miro-Casas, E., Covas, M.I., Farre, M., Fito, M., Ortuño, J., Weinbrenner, T., Roset, P., de la Torre, R., 2003. Hydroxytyrosol disposition in humans. Clin. Chem. 49, 945-952.

Monroig, Ó., Navarro, J.C., Amat, F., González, P., Bermejo, A., Hontoria, F., 2006a. Enrichment of *Artemia* nauplii in essential fatty acids with different types of liposomes and their use in the rearing of gilthead sea bream (*Sparus aurata*) larvae. Aquaculture 251, 491-508.

Monroig, Ó., Navarro, J.C., Amat, F., González, P., Hontoria, F., 2006b. Effect of aeration on the efficiency of *Artemia* enrichment with EFA-rich emulsion and liposomes. Aquaculture 257, 382-392.

Monroig, Ó, Navarro, J.C., Amat, F., González, P., Hontoria, F., 2007. Oxidative stability and changes in the particle size of liposomes used in the *Artemia* enrichment. Aquaculture 266, 200-210.

Mourente, G., Rodríguez, A., Sargent, J.R., 1993. Effects of dietary docosahexaenoic acid (DHA; 22:6*n*-3) on lipid and fatty acid composition and growth in gilthead sea bream (*Sparus aurata* L.) larvae during first feeding. Aquaculture 112, 79-98.

Navarro, J.C., Henderson, R.J., McEvoy, L.A., Bell, M.V., Amat, F., 1999. Lipid conversion during enrichment of *Artemia*. Aquaculture 174, 155-166.

Nunes, B., Carvalho, F., Guilhermino, L., 2006. Effects of widely used pharmaceuticals and a detergent on oxidative stress biomarkers of the crustacean *Artemia parthenogenetica*. Chemosphere 62, 581-594.

Olsen, E.K., Hansen, E., Isaksson, J., Andersen, J.H., 2013. Cellular antioxidant effect of four bromophenols from the red algae, *Vertebrata lanosa*. Mar. Drugs 11, 2769-2784.

Orbea, A., Fahimi, H.D., Cajaraville, M.P., 2000. Immunolocalization of four antioxidant enzymes in digestive glands of mollusks and crustaceans and fish liver. Histochem. Cell. Biol. 114, 393-404.

Pryor, W., 1991. The antioxidant nutrients and disease prevention – What do we know and what do we need to find out?. Am. J. Clin. Nutr 53, 391S-393S.

Reitan, K.I., Rainuzzo, J.R., Olsen, Y., 1994. Influence of lipid composition of live feed on growth, survival and pigmentation of turbot larvae. Aquacult. Int. 2, 33-48.

Rodríguez, C., Perez, J.A., Diaz, M., Izquierdo, M.S, Fernández-Palacios, H., Lorenzo, A., 1997. Influence of EPA/DHA ratio in rotifers on gilthead seabream (*Sparus aurata*) larval development. Aquaculture 150, 77-89.

Schaffer, S., Podstawa, M., Visioli, F., Bogani, P., Müller, W.E., Eckert, G.P., 2007. Hydroxytyrosol-rich olive mill wastewater extract protects brain cells in vitro and ex vivo. J. Agric. Food Chem. 55, 5043-5049.

Sherrat, P.J & Hayes, J.D., 2001. Glutathione-S-transferases. In: Ioannides, C. (Ed.), Enzyme system that metabolise drugs and other xenobiotics. John Wiley & Sons Ltd. pp. 319-352.

Sorgeloos, P., Dhert, P., Candreva, P., 2001. Use of the brine shrimp *Artemia* spp., in marine fish larviculture. Aquaculture 200, 147-159.

Sui, L., Wille, M., Cheng, Y., Sorgeloos, P., 2007. The effect of dietary n-3 HUFA levels and DHA/EPA ratios on growth, survival and osmotic stress tolerance of Chinese mitten crab *Eriocheir sinensis* larvae. Aquaculture 273, 139-150.

Tocher, D., 2010. Fatty acid requirements in ontogeny of marine and freshwater fish. Aquacult. Res. 41, 717-732.

Tovar-Ramirez, D., Mazurais, D., Gatesoupe, J.F., Quazuguel, P., Cahu, C.L., Zambonino-Infante, J.L., 2010. Dietary probiotic live yeast modulates antioxidant enzyme activities and gene expression of sea bass (*Dicentrarchus labrax*) larvae. Aquaculture 300, 142-147.

Townsend, D.M. & Tew, K.D., 2003. The role of glutathione-S-transferase in anti-cancer drug resistance. Oncogene 22, 7369-7375.

Van der Meeren, T., Olsen, R.E., Hamre, K., Fyhn, H., 2011. Biochemical composition of copepods for evaluation of feed quality in production of juvenile marine fish. Aquaculture 274, 375-397.

Van Stappen, G., 1996. Introduction, biology and ecology of *Artemia*. In: Manual on the production and use of live food for Aquaculture, (ed. by P. Lavens & P. Sorgeloos), FAO Fisheries Technical Paper (361), Roma.

Viciano, E., Monroig, Ó., Salvador, A., Amat, J., Fiszman, S, Navarro; J.C., 2015. Enriching *Artemia* nauplii with a high DHA-containing lipid emulsion: search for an optimal protocol. Aquaculture Research 46, 1066-1077.

Visioli, F., Bellomo, G., Galli, C., 1998. Free radical-scavenging properties of olive oil polyphenols. Biochem. Biophys. Res. Commun. 247, 60-64.

Visioli, F., Caruso, D., Galli, C, Viappiani, S, Galli, G., Sala, A., 2000. Olive oils rich in natural catecholic phenols decrease isoprostane excretion in humans. Biochem. Biophys. Res. Commun. 278, 797-799.

CAPÍTULO 6

Fatty acid composition of polar and neutral lipid fractions of *Octopus vulgaris* Cuvier, 1797 paralarvae reared with enriched on-growth *Artemia*

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Abstract

Rearing of common octopus *Octopus vulgaris* is limited by the lack of success during the paralarval stage, with generalized mortalities occurring before the settlement of the juveniles. The use of on-grown *Artemia* cultured with the microalga *Isochrysis galbana* and further enriched with *Nannochloropsis* sp. has led to a certain degree of success.

The present work aims at studying the effects of this rearing protocol (Nanno) on the fatty acid composition of paralarvae, by comparison with a diet based on on-grown Artemia further enriched with a high polyunsaturated fatty acid oil emulsion (M70). After 28, survival was estimated at 3% for M70 and 22.5% for Nanno, whereas the average dry weight was not significantly different (Nanno: 1.76 ± 0.28 mg; M70: 1.88 ± 0.22 mg). Although apparently no clear association between the fatty acid composition of the enriched preys and that of the total lipids of paralarvae could be established, further fractionation and fatty acid analysis of the total lipids into polar and neutral classes, followed by principal components analysis, revealed that irrespective of the diet, both lipid fractions showed distinct fatty acid patterns. Besides, the fatty acid composition of the polar lipids was more conservative, whereas that of the neutral lipids was more influenced by the diet, showing more variation among dietary treatments.

Keywords: Octopus vulgaris, paralarvae, lipids, fatty acids, Artemia

Resumen

La cría del pulpo común Octopus vulgaris *está limitada por la falta de éxito durante la fase de paralarva, con mortalidades generalizadas que se producen antes del asentamiento de los juveniles. El uso de* Artemia *cultivada con el microalga* Isochrysis galbana *y luego enriquecida con* Nannochloropsis sp. *ha proporcionado cierto grado de éxito.*

El presente trabajo tiene como objetivo estudiar los efectos de este protocolo de cultivo (Nanno) en la composición de ácidos grasos de las paralarvas, comparándolos con los de una dieta basada en Artemia *enriquecida con una emulsión lipídica rica en ácidos grasos poliinsaturados (M70). Tras 28 días, la supervivencia se estimó en un 3% para M70 y un 22,5% para Nanno, mientras que la media de peso seco no fue significativamente diferente (Nanno: 1,76 ± 0,28 mg; M70: 1,88 ± 0,22 mg). Aunque no se pudo establecer una asociación clara entre la composición de ácidos grasos de las presas enriquecidas y la de los lípidos totales de las paralarvas, el fraccionamiento adicional y el análisis de ácidos grasos de los lípidos totales en clases polares y neutras, seguidas de un Análisis de Componentes Principales, revelaron que independientemente de la dieta, ambas fracciones lipídicas tenían diferentes patrones de ácidos grasos. Además, la composición de ácidos grasos de los lípidos polares fue más conservadora, mientras que la de los lípidos neutros estuvo más influida por la dieta, mostrando una mayor variación entre los tratamientos dietéticos.*

6.1. Introduction

In recent years, common octopus *Octopus vulgaris* has been targeted as a promising candidate for aquaculture but, to date, paralarvae rearing is severely limited by the lack of success during the planktonic stage, with generalized mortalities occurring before the settlement of the juveniles.

The life cycle of *O.vulgaris* under conditions of captivity was completed for the first time in 2001 (Iglesias, Otero, Moxica, Fuentes & Sánchez 2004), using *Artemia* and *Maja* zoeae as prey. Although some successful cultures of a reduced number of paralarvae up to juveniles and even sub-adults have been achieved (Moxica, Linares, Otero, Iglesias & Sánchez 2002; Iglesias *et al*., 2004; Carrasco, Arronte & Rodríguez 2006), these experiences have been mainly occasional, anecdotic and, needless to say, economically profitless.

According to Navarro and Villanueva (2000, 2003), among others, a lack of balance in the lipid and fatty acid composition of the food could be responsible for the high mortalities encountered in the rearing of the paralarval stages of *O. vulgaris*. These authors studied the lipid requirements of early stages of cephalopods to conclude that a nutritional imbalance in the lipid and fatty acid profile of the artificial feeding protocol, based on *Artemia*, may be responsible for the high mortalities encountered. *Octopus vulgaris* require feeding

on low-lipid preys rich in polar lipids, long-chain polyunsaturated fatty acids (PUFA) and possibly cholesterol (Navarro & Villanueva 2000, 2003; Okumura, Kurihara, Iwamoto & Takeuchi 2005; Kurihara, Okumura, Iwamoto & Takeuchi 2006; Seixas 2009; Seixas, Rey-Méndez,Valente & Otero 2010).This closely resembles the composition of a 'natural' diet (totally unpractical from an aquaculture approach) based on crustacean larvae (e.g. *M. zoeae*) and other marine planktonic forms like copepods, but is considerably different from the typical composition of enriched *Artemia* in any of its forms. In fact, early stages of cephalopods are particularly rich in polar lipids and cholesterol, and artificial feeding under culture conditions increases their triacylglyceride content drastically (Navarro & Villanueva 2000, 2003).

When using only *Artemia* as food, the only experiment that has attained *Octopus* adult stages was reported by Moxica, Fuentes, Hernández, Iglesias, Lago, Otero and Sánchez (2006), who obtained 67% survival and 1.89mg of dry weight for 1-month-old paralarvae using on-grown *Artemia* (>1.5 mm) cultured with the microalga *Isochrysis galbana* and further enriched with *Nannochloropsis* sp. Imamura (1990) and Hamazaki, Fukunaga, Yoshida and Maruyama (1991) reported a limited production of presettlement individuals on adding *Nannochloropsis* sp. to the culture tanks. Hamasaki and Takeuchi (2000) and Hamasaki and Morioka (2002) also reported success (although also limited), on adding

Nannochloropsis sp. to the culture tanks and as food for *Artemia* respectively. The reasons for the improvements obtained with the rearing protocols involving the use of *Nannochloropsis* sp. remain obscure, but a striking feature of this microalga is its high eicosapentaenoic acid (EPA, 20:5n-3) content (Sukenik, Zmora & Carmeli1993), an essential fatty acid for marine animals (Sargent, McEvoy, Estévez, Bell, Bell, Henderson & Tocher 1999). Other hypotheses about the beneficial effects of *Nannochloropsis* sp. relate to their potentiality to inhibit the microflora growth and improve the culture conditions (Iglesias personal observation).

The present work aims at studying the effects of a rearing protocol based on *Artemia* on-grown with *Isochrysis galbana* and enriched with *Nannochloropsis* sp. on the fatty acid composition of paralarvae. It will be carried out by comparison with the effects of a diet based on the same on-grown *Artemia* further enriched with a high PUFA oil emulsion especially rich in docosahexaenoic acid (DHA, 22:6n-3). Thus, the work will ultimately focus on the dietary effects of DHA-rich and EPA-rich diets on the total, polar and neutral lipid fatty acid composition of the paralarvae.

6.2. Materials and methods

Octopus vulgaris paralarvae were obtained from broodstock kept in captivity using the technology described by Moxica *et al*. (2002). They were fed for 28 days with on-grown *Artemia* (1.5-2mm) cultured with *I. galbana* and further enriched for 24 h with either *Nannochloropsis* sp. (Nanno treatment) or a PUFA-rich oil emulsion based on a speciality oil containing approximately 70% of DHA, 50/50 oil/water, xanthan gum as a stabilizer and Tween 80 as an emulsifier (M70 treatment).

Cultures were carried out in1000-L black, circular tanks (diameter 130 cm), at a larval density of 5 individuals L^{-1} (5000 paralarvae per tank). The mean water temperature was 21-22 ºC and salinity was 34-35 g L^{-1}; a continuous illumination of 800-1000 lx provided by two 36 W day-light fluorescent tubes was supplied to the tanks. During the first week, the rearing tanks were maintained under standing conditions, with gentle central aeration, and a concentration of 1 million cells $mL^{-1}$of *Nannochloropsis* sp. From day 8, a water flow of 200 L h^{-1} was partially opened (4 h d^{-1}) every 2 days.

Enriched *Artemia* was added (three to four times per day) at a density of 0.5 prey mL^{-1} until day 11 and at 0.2 prey mL^{-1} onwards. *Artemia* was on-grown with 300 000 cells mL^{-1} of *I.*

galbana for 4 days, and further enriched for 24 h with 10 million cells mL^{-1} of *Nannochloropsis* sp.

Dissolved oxygen, nitrites and ammonium were measured daily. The dry weights of ten paralarvae after washing with distilled water and drying at 90 ºC 24 h were recorded at the end of the experiment. Survival was recorded at day 28.

At the end of the trial, the lipids of diets and paralarvae (triplicate pools) were extracted from freeze-dried samples using the method of Folch, Lees and Sloane-Stanley (1957). The total lipids were determined gravimetrically (0.0001 g, Mettler Toledo, Barcelona, Spain) and stored in chloroform/methanol (2:1, v/v) with 0.01% BHT as an antioxidant. An aliquot of total lipids was transmethylated after the addition of 19:0 as an internal standard. Fatty acid methyl esters (FAME) were purified by thin-layer chromatography (Silica gel G 60, Merck, Darmstadt, Germany) and injected on-column in a Fisons 8000 gas chromatograph equipped with a fused silica 30m x 0.25mm open tubular column (Tracer, TR-WAX, film thickness: 0.25 mm, Teknokroma, Barcelona, Spain). Helium was used as a carrier gas, and the analyses were run in a 50-220 ºC thermal gradient. Peaks were recorded and integrated in a personal computer using AZUR software (Azur, Datalys, France), and identified by comparison with well-characterized standards. A further aliquot of the total lipids was fractionated into polar and neutral lipids by thinlayer chromatography, and the fatty acids

were transmethylated and analysed as described for the total lipids.

The fatty acid profiles thus obtained were subsequently analysed chemometrically by principal component analysis (PCA). The score plot obtained after the generation of the two principal components was used to identify patterns of similarity among the cases.

For each lipid class, the mean values of the fatty acid composition of the paralarvae from the two dietary treatments were compared using Student's t-tests. Statistics were carried out using SPSS 17.0 software (SPSS).

6.3. Results and discussion

The average dry weight of 28-day paralarvae from both dietary groups was not significantly different (Nanno: 1.76 ± 0.28 mg; M70: 1.88 ± 0.22 mg) and is similar to the values reported by Moxica *et al*. (2006) using the same microalgal enrichment. They are clearly lower than those reported for paralarval of the same age (30 days post hatch) using crustacean zoeae as complementary food by Moxica *et al*. (2002): 2.42 mg; Iglesias *et al*. (2004): 3.33 mg; Carrasco *et al*. (2006): 2.83 mg; and higher than those obtained by other authors using different feeding regimes: Villanueva, Koueta, Riba and Boucaud-Camou

(2002) reported weights from 0.79 to 1.49 mg using enriched *Artemia* co-fed with millicapsules; Seixas (2009) obtained 0.83 mg paralarvae at day 25 using on-grown *Artemia* enriched with a mixture of *I. galbana* and *Rhodomonas lens*; Fuentes, Sánchez, Otero, Lago and Iglesias (2009) reported values between 0.96 and 1.12 mg with *Artemia* and frozen wild zooplankton as food; and Estévez, Gairín and Berger (2009) produced 0.33 mg paralarvae using live zooplankton as food.

These differences can be explained by the different culture conditions and foods. It is evident that crustacean zoeae seem to be a superior food, and the data reported here are better than those obtained with other experimental feeding protocols.

Survival was roughly estimated at 3% for M70 and 22.5% for Nanno. Culture of this last group was followed until 35 days, reaching an average paralarval weight of 1.83 ± 0.28 mg (n=10) and a 3% survival.

These data indicate that the biometrical outcome of both dietary treatments was very similar and that any hypothetical advantage of the Nanno group was lost1week later.

The lipid content of diets [% dry weight (DW)] was significantly different ($P<0.05$): 21.01 ± 0.84 for Nanno and 18.23 ± 0.70 for M70. Analysis of the main fatty acids (Table 6.1) showed that the main differences were due to the Nanno treatment being higher in 16:1n-7 (which resulted in a higher

monoene content) and 20:5n-3, whereas M70 showed higher18:2n-6 (thus increasing the total n-6 content) and 22:6n3 contents, which was not detected in the other diet (Table 6.1).

Differences in the lipid content of diets did not translate ($P>0.05$) into the lipid content of the paralarvae [Total lipid (% DW): M70 = 16.31 ± 1.04; Nanno = 17.5 ± 0.23; total FAME (mg g^{-1} DW): M70 = 45.71 ± 1.82; Nanno = 47.29 ± 1.51]. Similarly, a first glance at the fatty acid profiles of the total lipids of both dietary groups did not reveal streaking differences between them (Table 6.2). Especially notable is the case of 16:1n7, very different in the diets. Besides, the paralarvae seem to be able to cope with a 22:6n-3-deficient diet, although the poor performance of the two dietary groups could also point towards a dietary deficiency of both treatments.

A closer look at the polar and neutral lipid fatty acid composition showed that the polar lipids were much richer in 20:5n-3 and 22:6n-3 (absent in the neutrals), which was reflected in a higher polyunsaturated, n-3 and highly unsaturated fatty acid (HUFA) n-3 content. Neutrals in turn were richer in monounsaturated fatty acids. Within the polar lipids, M70 treatment generally increased the polyunsaturated, n-3 and HUFA n-3 contents with respect to Nanno. Within the neutral lipids, Nanno treatment could be associated with higher n-6 content, whereas M70 seemed to increase HUFA n-3.

Table 6.1. Selected fatty acids (% of total lipid fatty acids) and total FAME of enriched on-growth *Artemia* (n=3)

	M70		Nanno	
Fatty acid	Mean	SD	Mean	SD
14:0	1.43	0.15	1.13	0.01
16:0	10.54	0.47	15.71	0.04
16:1n-7	5.19	0.13	23.69	0.62
18:0	7.26	0.38	5.40	0.12
18:1n-9	16.22	0.79	12.31	0.37
18:1n-7	9.27	0.70	7.89	0.37
18:2n-6	12.15	1.06	2.34	0.04
18:3n-3	5.05	0.13	0.38	0.01
20:4n-6	2.75	0.09	2.88	0.04
20:3n-3	0.07	0.07	ND	
20:4n-3	0.25	0.04	ND	
20:5n-3	11.99	0.43	22.55	0.52
22:5n-3	0.52	0.26	0.02	0.03
22:6n-3	8.10	1.10	ND	
Sat	20.38	0.70	23.33	0.30
Mono	31.58	0.80	44.19	0.18
Poly	44.42	1.76	28.96	0.50
n-3	27.92	1.15	23.04	0.54
n-6	16.67	1.03	5.62	0.13
HUFA n-3	20.93	0.99	22.57	0.55
HUFA n-6	3.83	0.09	3.16	0.02
FAME	60.49	4.35	116.97	12.89

Sat, saturates; Mono, monoenes; Poly, polyunsaturated; HUFA, higly unsaturated fatty acids (>20C); SD, standard deviation; FAME, total fatty acids methyl esters (mg g^{-1} DW); ND, not detected

Table 6.2. Selected fatty acids (% of total fatty acids) of the total, polar and neutral lipid of 28 days *Octopus vulgaris* paralarvae fed two enriched on-grown *Artemia* diets (n=3)

	Total lipid				Polar lipid				Neutral lipid			
	M70		Nanno		M70		Nanno		M70		Nanno	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
14:0	0.74	0.06	1.62*	0.05	0.58	0.13	1.09*	0.17	2.56	0.93	5.19*	0.23
16:0	21.09	1.12	21.23	0.07	18.53	0.67	18.25	0.65	20.21	3.18	18.22	1.50
16:1n-7	5.02	0.64	4.31	0.45	2.07	0.42	2.47	0.29	16.22	4.27	13.01	1.08
18:0	12.61	0.28	12.51	0.29	14.21	0.28	13.56	0.42	10.50	3.35	8.19	1.25
18:1n-9	6.93	1.17	8.32	0.22	3.94	0.35	5.33*	0.34	12.36	1.78	17.25*	1.08
18:1n-7	5.58	0.11	5.28*	0.06	4.08	0.21	3.88	0.18	6.27	2.57	6.10	0.14
18:2n-6	1.50	0.32	2.95*	0.06	1.11	0.02	2.08*	0.21	2.52	0.63	7.06*	0.48
18:3n-3	0.94	1.20	0.77	0.04	0.22	0.11	0.56*	0.03	0.24	0.21	1.81*	0.31
20:4n-6	6.35	0.33	5.50*	0.08	7.69	0.34	6.31*	0.22	1.19	0.53	0.49	0.12
20:3n-3	1.25	0.04	1.37*	0.02	1.51	0.13	1.58	0.05	ND		ND	
20:4n-3	0.03	0.06	ND		0.01	0.02	0.05	0.05	ND		ND	
20:5n-3	21.89	1.54	20.33	0.36	25.73	0.43	23.31*	0.64	5.33	1.48	2.31*	0.14
22:5n-3	1.20	0.08	1.09	0.05	1.48	0.06	1.43	0.05	ND		ND	
22:6n-3	5.55	0.63	5.21	0.07	6.44	0.74	5.63	0.35	ND		ND	
Sat	34.57	1.28	35.54	0.15	33.73	0.59	33.35	0.59	34.62	6.19	31.94	1.96
Mono	20.20	1.26	20.45	0.66	12.81	1.02	14.55	0.82	36.41	7.43	37.19	1.76
Poly	41.57	0.54	40.48	0.89	46.06	1.66	43.42	1.11	11.96	3.83	13.99	1.20
n-3	30.98	0.62	29.09*	0.57	35.45	0.83	32.75*	0.94	5.57	1.67	4.56	0.78
n-6	9.73	0.08	10.72*	0.12	10.75	0.28	10.56	0.36	4.16	1.51	9.25*	1.29
HUFA n-3	29.92	0.87	28.00*	0.46	35.16	0.75	32.01*	0.94	5.33	1.48	2.31*	0.14
HUFA n-6	7.02	0.29	6.35*	0.14	8.38	0.25	7.12*	0.39	1.64	1.06	1.60	1.13

For abbrevations see Table 6.1.

*Significant difference between means within lipid class (t-test P<0.05).

These trends were confirmed by the results of the PCA. The first component explained 76% of the variance and was associated with variables14:0, 18:1n-9, 18:2n-6 and16:1n-7 on the positive side and variables 20:5n-3, 20:4n-6, 22:6n-3 and 18:0 on the negative side. The second component explained only 11% of the variance. The results of the score plot (Fig. 6.1) showed two main clouds of scores corresponding to polar (associated with variables on the negative side) and total lipids on one side, clearly distinct from neutral lipids (associated with variables on the positive side).Within the polar and neutral lipids, the scores corresponding to the different dietary treatments were distinguishable, whereas those corresponding to the total lipids did not separate the two dietary groups. The scores of the polar lipids were more grouped than those of the neutral lipids, indicating a higher similarity in the fatty acid patterns, which indicates the structural role of this lipid class (Gurr & Harwood 1991). Recently, Quintana (2009) have reported on the effects of the broodstock diets on the fatty acid composition of 3-day-old paralarvae, fed and starved, to conclude that the neutral lipids of the paralarvae reflect better than the polars any difference in the fatty acid profile of the breeders'diets. This is a further indication of the importance of the polar lipids as a structural and conservative lipid class for this species. In fact, the mantle being essentially protein and membranes, any changes in the neutral lipid fraction may be indicative of the contribution of this lipid class to the lipid

composition of the digestive gland and ultimately of the lipid metabolism and turnover linked to the dietary fatty acid composition.

To a certain point, the above result seems to contradict the results of Navarro and Villanueva (2003), who found that the main dietary changes were already very evident in the total lipids of *O. vulgaris* paralarvae, although they were also clear in the polar lipids (unfortunately, the neutral lipid composition was not analysed). Thus, the dietary changes induced and their influence on the structural lipids may be dependent on the composition of the diets and on the coverage of the essential requirements. From this point of view, both Nanno and M70 diets fulfil the essential requirements of the species, and the presence of DHA in Nanno fed paralarvae may be indicative of a certain degree of synthetic capacity. Alternatively, the levels of DHA may be a remnant from the original content at hatching, a result of a conservative strategy for the retention of an essential lipid.

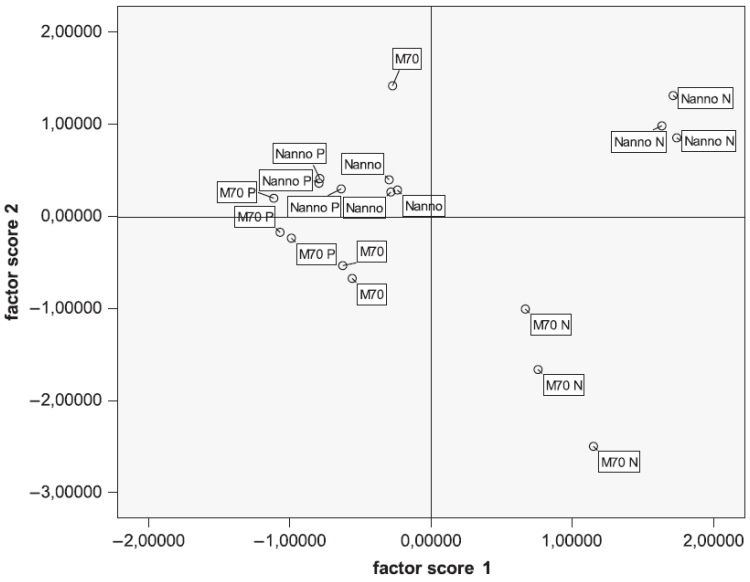

Figure 6.1. Score plot generated after principal component analysis of the fatty acid pattern of 28-day Octopus vulgaris paralarvae fed two enriched on-grown Artemia diets. Nanno, M70, total lipid; Nanno N, M70 N, neutral lipid; Nanno P, M70 P, polar lipid.

Paralarval DHA levels not correlating with the amount in food have been reported previously (Navarro & Villanueva 2000; Seixas 2009; Seixas *et al*. 2010). In fact, Seixas *et al*. (2010) have recently pointed out the importance of an adequate ratio protein/lipid before the essential lipid requirements of the species may be considered. Given the generalized low performances of the paralarval cultures, it is difficult to focus on a single source of variation, i.e. lipids, in a cause-effect design. Obviously, factors other than lipids may be affecting the final

outcome, and even the fine-tuning of the lipid requirements may have a drastic influence. It should suffice to recall that for example, in larval sea bream (*Sparus aurata*), Rodríguez, Pérez, Díaz, Izquierdo, Fernández-Palacios and Lorenzo (1997) reported that the essential PUFA requirements were considerably dependent on the supply of an adequate DHA/EPA ratio. In this sense, it is interesting to note that EPA requirements of *O. vulgaris* may be particularly important, because this fatty acid has been found in very high amounts (and in 1:1 ratios with DHA) in the phosphatidylcholine fraction of the early stages (Quintana 2009).

6.4. Conclusions

Although apparently no clear association between the fatty acid composition of the enriched preys and that of the total lipids of paralarvae could be established, further fractionation and fatty acid analysis of the total lipids of the paralarvae into polar and neutral classes, followed by PCA, revealed that irrespective of the diet, both lipid fractions showed distinct fatty acid patterns. Besides, the fatty acid composition of the polar lipids was more conservative, whereas that of the neutral lipids was more influenced by the diet and showed more variation among dietary treatments. Further research is needed to determine the lipid requirements of this species.

6.5. References

Carrasco J.F., Arronte J.C. & Rodríguez C. (2006) Paralarval rearing of the common octopus, *Octopus vulgaris* (Cuvier). Aquaculture Research 37, 1601-1605.

Estévez A., Gairín J.I. & Berger E. (2009) Wild zooplankton for *Octopus vulgaris* larval rearing. In: *Larvi '09 - Fish and Shelfish Larvivulture Symposium*. European Aquaculture Society Special Publication No. 38 (ed. by C.I. Hendry, G. Van Stappen, M. Wille & P. Sorgeloos), pp. 228–229. European Aquaculture Society, Oostende, Belgium.

Folch J., Lees N. & Sloane-Stanley G.H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry 226, 497-509.

Fuentes L., Sánchez F.J., Otero J.J., Lago M.J. & Iglesias J. (2009) Utilización de zooplancton y artemia en el cultivo larvario de pulpo *Octopus vulgaris*. In: *Actas del XII Congreso Nacional de Acuicultura* (ed. by D. Beaz, M. Villarroel & S. Cárdenas), pp. 122–123. CICEGRAF, Madrid, Spain.

Gurr M.I. & Harwood J.L. (1991) Lipid Biochemistry. An introduction. Chapman and Hall, London, UK.

Hamasaki K. & Morioka T. (2002) Effects of temperature on egg incubation period, paralarval survival and growth of common octopus, *Octopus vulgaris* reared in the laboratory. Suisanzoshoku 50, 407-413.

Hamasaki K. & Takeuchi T. (2000) Effects of the addition of *Nannochloropsis* to the rearing water on survival and growth of planktonik larvae of *Octopus vulgaris*. Saibai-Giken 28, 65-68.

Hamazaki K., Fukunaga K.,YoshidaY. &Maruyama K. (1991) Effects of marine microalgae *Nannochloropsis* sp., on survival and growth of rearing pelagic paralarvae of *Octopus vulgaris*, and results of mass culture in the tank of 20 metric tons. Saibai-Giken19, 75-84.

Iglesias J., Otero J.J., Moxica C., Fuentes L. & Sánchez F.J. (2004) The completed life cycle of the octopus (*Octopus vulgaris*, Cuvier) under culture conditions: paralarval rearing using *Artemia* and Zoeae, and first data on juvenile growth up to 8 months of age. Aquaculture International 12, 481-487.

Imamura S. (1990) Larval rearing of octopus (*Octopus vulgaris* Cuvier). The progress of technological development and some problems remained. Collecting and Breeding 52, 339-343.

Kurihara A., Okumura S., IwamotoA. & Takeuchi T. (2006) Feeding pacific sandeel enhances DHA level in common octopus paralarvae. Aquaculture Science 54, 413-420.

Moxica C., Linares F., Otero J.J., Iglesias J. & Sánchez F.J. (2002) Cultivo intensivo de paralarvas de pulpo, *Octopus vulgaris* Cuvier, 1797, en tanques de 9 m3. Boletín del Instituto Español de Oceanografía 18, 31-36.

Moxica C., Fuentes L., Hernández J., Iglesias J., Lago M.J., Otero J.J. & Sánchez F.J. (2006) Efecto de *Nannochloropsis* sp. en la supervivencia y crecimiento de paralarvas de pulpo *Octopus vulgaris*. In: *Libro de Resúmenes del IX Foro dos Recursos Mariños e da Acuicultura das Rías Galegas* (ed. by M. Rey-Méndez, J. Fernández & M. Izquierdo), pp. 255–260. IX Foro Marino, O Grove, Spain.

Navarro J.C. & Villanueva R. (2000) Lipid and fatty acid composition of early stages of cephalopods: an approach to their lipid requirements. Aquaculture183, 161-177.

Navarro J.C. & Villanueva R. (2003) The fatty acid composition of *Octopus vulgaris* paralarvae reared with live and inert food: deviation from their natural fatty acid profile. Aquaculture 219, 613-631.

Okumura S., Kurihara A., Iwamoto A. & Takeuchi T. (2005) Correlations among arm sucker count, wet and dry weight of reared common octopus paralarvae. Aquaculture Science 53, 329-330.

Quintana D. (2009) Valoración de los requerimientos nutricionales de reproductores de pulpo común (*Octopus vulgaris*).Tesis doctoral, Universidad de La Laguna.

Rodríguez C., Pérez J.A., Díaz M., Izquierdo M.S., Fernández-Palacios H. & Lorenzo A. (1997) Influence of the EPA/DHA ratio in rotifers on gilthead seabream (*Sparus aurata*) larval development. Aquaculture150, 77-89.

Sargent J.R.,McEvoy L., Estévez A., Bell J.G., Bell M., Henderson J. & Tocher D.R. (1999) Lipid nutrition of marine fish during early development: current status and future directions. Aquaculture179, 217-230.

Seixas P. (2009) Composición bioquímica y crecimiento de paralarvas de pulpo (*Octopus vulgaris* Cuvier, 1797), alimentadas con juveniles de *Artemia* enriquecidos con microalgas y otros suplementos nutricionales. Tesis doctoral, Universidad de Santiago de Compostela, p. 255.

Seixas P., Rey-Méndez M., Valente L.M.P. & Otero A. (2010) High DHA content in *Artemia* is ineffective to improve *Octopus vulgaris* paralarvae rearing. Aquaculture 300, 156-162.

Sukenik A., Zmora O. & Carmeli Y. (1993) Biochemical quality of marine unicellular algae with special emphasis on lipid composition. II. *Nannochloropsis* sp. Aquaculture 117, 313-326.

Villanueva R., Koueta N., Riba J. & Boucaud-Camou E. (2002) Growth and proteolitic activity of *Octopus vulgaris* paralarvae with different food rations during first feeding, using *Artemia* nauplii and compound diets. Aquaculture 205, 269-286.

CAPÍTULO 7

Discusión general

Los organismos marinos, en especial sus estadios larvarios, necesitan de ciertos ácidos grasos altamente insaturados (HUFA, *Highly Unsaturated Fatty Acids*) entre los que se incluyen el ácido araquidónico (ARA, 20:4n-6), el ácido eicosapentaenoico (EPA, 20:5n-3) y el ácido docosahexaenoico (DHA, 22:6n-3) (Tocher, 2010). Estos compuestos se consideran esenciales (EFA, *Essential Fatty Acids*) para la mayor parte de especies de organismos marinos cultivadas debido a que, en general, no pueden ser biosintetizados en cantidades suficientes para satisfacer las necesidades fisiológicas y por lo tanto, deben de ser suministrados en la dieta para asegurar su normal desarrollo y crecimiento (Sargent y cols., 2002; Tocher, 2003, 2010, 2015).

Los nauplios de *Artemia* se utilizan habitualmente como presa viva en el cultivo larvario de organismos marinos. Sin embargo, la calidad nutricional de los nauplios es pobre para alimentar a larvas de organismos marinos ya que contienen niveles muy bajos de EFA, siendo especialmente deficientes en EPA y sobre todo, DHA (Evjemo y cols., 1997; Navarro y cols., 1999). El uso de procedimientos de enriquecimiento o bioencapsulación de nutrientes esenciales como los HUFA señalados anteriormente, es una práctica ineludible para el éxito del cultivo de larvas de organismos vivos, pero dista de ser completamente satisfactorio desde el punto de vista de niveles de EFA como el DHA, y además lleva asociados efectos

secundarios como la potencial autoxidación de los HUFA del producto enriquecedor (McEvoy y cols., 1995; Sargent y cols., 1997) y la consiguiente bioacumulación de peróxidos lipídicos tóxicos en los nauplios de *Artemia* enriquecidos (Monroig, 2006).

El éxito en la modificación del perfil de ácidos grasos de los nauplios está influido por el tipo de dieta enriquecedora, las condiciones físico-químicas en las que se lleva a cabo el enriquecimiento y la cepa de *Artemia* utilizada (Han y cols., 2000). Se han estudiado una gran variedad de productos enriquecedores destinados a aumentar el contenido en EFA en *Artemia*: microalgas (Watanabe y cols., 1982; Arãgao y cols., 2004), microcápsulas (Southgate y Lou, 1995), liposomas (Hontoria y cols., 1994; McEvoy y cols., 1996; Monroig y cols., 2003, 2006a, 2006b, 2006c) y emulsiones lipídicas (Léger y cols., 1986; Han y cols., 2000, 2001, 2005). Las emulsiones lipídicas son una de las formas más extendidas de uso de dieta enriquecedora y existen productos disponibles comercialmente.

Uno de los mayores problemas, aún sin resolver, de los enriquecimientos de presas vivas es la dificultad de aumentar el contenido en DHA de los nauplios de *Artemia*, debido al rápido catabolismo de este ácido graso por los nauplios de *Artemia* (Evjemo y cols., 1997), más específicamente a la retroconversión del DHA en EPA (Navarro y cols., 1999). Para

intentar solucionar estos efectos adversos se necesita diseñar nuevas estrategias.

En este contexto se desarrolla la presente tesis doctoral. La información recogida en los capítulos precedentes, describe cómo se investigó la eficacia enriquecedora de una nueva emulsión lipídica (M70) especialmente rica en DHA (70% de su contenido). Teniendo en cuenta su naturaleza rica en DHA, se llevaron a cabo experimentos para establecer el uso óptimo de la emulsión M70 durante el enriquecimiento de *Artemia*, modificando diferentes regímenes de oxigenación, temperaturas de incubación, concentración y dosis de producto enriquecedor y densidades naupliares. También se investigó el efecto protector de un antioxidante externo natural, soluble en agua y basado en hidroxitirosol (potente antioxidante obtenido del extracto de oliva) al medio de enriquecimiento. Por último, se utilizó la mencionada emulsión enriquecedora a escala semi-industrial, en un experimento de cría paralarvaria de pulpo (*Octopus vulgaris*), con el fin, entre otros, de determinar si su alto contenido en DHA podía mejorar la baja tasa de supervivencia en el cultivo de estadios tempranos de desarrollo de esta especie.

La elaboración de un nuevo producto enriquecedor como M70 conlleva el establecimiento de las condiciones óptimas de enriquecimiento, máxime cuando dicho producto es particularmente rico en DHA. Esta tarea es acometida cada vez que se diseña un producto enriquecedor destinado a maximizar

los resultados en cuanto a enriquecimiento de ácidos grasos, vitaminas, aminoácidos, etc., en especies auxiliares de acuicultura (rotíferos y *Artemia*). Los fabricantes de estos productos enriquecedores han desarrollado sus propios protocolos con el fin de optimizar su rendimiento, aunque las condiciones físicas suelen variar alrededor de lo siguiente: temperaturas entre 25 y 28 ºC (Van Stappen, 1996), iluminación alrededor de 1500 lux para la eclosión previa, y aireación suficiente para mantener el oxígeno disuelto por encima de 4 mg L^{-1} con el fin de minimizar la mortalidad naupliar (Van Stappen, 1996).

La nueva emulsión lipídica M70 tiene la particularidad de ser especialmente rica en DHA (aproximadamente un 70% de los ácidos grasos de la emulsión son DHA), con el fin de incrementar especialmente el contenido de este ácido graso en los nauplios de *Artemia*, tras el enriquecimiento. La adición de un compuesto relativamente polar como el DHA en tan elevada proporción ocasiona problemas físicos a la emulsión, que se resumen en una tendencia hacia la separación de sus fases, por lo que el establecimiento de las condiciones óptimas de enriquecimiento cobra aquí especial protagonismo y, así, se probaron diferentes condiciones de enriquecimiento, entre ellas: temperatura (24 y 28 ºC), flujo de aireación (1, 2 y 3 L min^{-1}), tipo de aireación (aire comprimido, oxígeno y una mezcla de ambas), dosis de la emulsión (0,6 y 0,8 g L^{-1}), distribución de la

emulsión (1 o 2 dosis) y densidad naupliar (150 y 300 nauplios mL^{-1}).

La disponibilidad de oxígeno en el medio de cultivo es un factor crítico para la supervivencia de *Artemia* durante el enriquecimiento, al igual que mantener el oxígeno disuelto por encima de 4 mg L^{-1} (Van Stappen, 1996). Los procedimientos de enriquecimiento estándar implican el uso de aire comprimido para proporcionar niveles adecuados de oxígeno disuelto, pero el uso de oxígeno puro como sistema de aireación es especialmente recomendable cuando los niveles de oxígeno disuelto pueden estar comprometidos. Este podría ser nuestro caso, ya que el DHA existente en M70 es altamente susceptible a procesos de peroxidación lipídica y por tanto el oxígeno disuelto podría descender (McEvoy y cols., 1995; Ries, 2009). Al comparar el uso de oxígeno puro *versus* aire comprimido utilizando la emulsión M70 como enriquecedor, se observó que no había diferencias estadísticas en cuanto a bioencapsulación de EFA dentro de *Artemia* y que el uso de aire comprimido era un sistema de difusión lo suficientemente efectivo como para suplir las demandas de oxígeno disuelto en el enriquecimiento con M70. De este modo, al menos en las condiciones ensayadas, el uso de otras alternativas más caras, como el oxígeno puro, no parecen necesarias.

Otros factores como el flujo de aire y la temperatura del proceso de enriquecimiento también han de ser consideradas, ya

que existe un compromiso entre la peroxidación lipídica, el suministro de oxígeno y la eficiencia del enriquecimiento. Además de la solubilidad del oxígeno y de sus posibles consecuencias en la peroxidación de los lípidos, el flujo de aire también condiciona la turbulencia general en el medio de enriquecimiento y puede determinar en última instancia la eficacia bajo la cual los nauplios incorporan las partículas de enriquecedor (Monroig y cols., 2006a). Así, existen estudios que remarcan la necesidad de encontrar un sistema apropiado de aireación que sea capaz de proporcionar oxígeno a los nauplios y que al mismo tiempo permita una distribución uniforme por el medio de enriquecimiento (Babu y cols., 2001; Kolkovski y cols., 2004) evitando su acumulación en la base de los recipientes de enriquecimiento (Monroig y cols., 2006a).

En estudios anteriores (Babu y cols., 2001; Monroig y cols, 2006a), se ha visto que los niveles más elevados de DHA y EPA se obtuvieron con un flujo de aireación bajo (1 L min^{-1}), lo que implica niveles menores de turbulencia en el medio de cultivo. La turbulencia es un factor importante en el proceso de enriquecimiento porque interfiere con la natación normal de los nauplios. Turbulencias fuertes y prolongadas pueden dañar sus estructuras de filtración (Monroig, 2006b) y obstaculizar la incorporación de partículas de producto de enriquecimiento (Navarro y cols., 1999; Monroig y cols., 2006a). Así, Monroig y cols. (2006a) demostraron que elevados niveles de turbulencia

en un medio de enriquecimiento con liposomas causaban bajas tasas de incorporación de ácidos grasos en los nauplios.

La temperatura ambiental es un factor determinante sobre la tasa metabólica de organismos poiquilotermos como los crustáceos (Hernandorena, 1976). Así, más allá de controlar los mecanismos de solubilidad de oxígeno y la peroxidación lipídica, la temperatura también determina la capacidad metabólica de los nauplios de *Artemia* (Van Stappen, 2002) y por lo tanto importantes hitos de desarrollo, tales como la apertura de la boca y la absorción del vitelo, así como la aparición de apéndices de filtrado/natatorios (Hochanchka y Somero 1984; Anger 2001). La temperatura también influye en la sincronía y eficiencia de eclosión de los quistes de *Artemia*. La temperatura favorece que el tiempo transcurrido hasta la apertura de la boca se acorte en los nauplios recién eclosionados, aumentando paralelamente el periodo filtrador. Así, unas adecuadas sincronías y eficiencias de eclosión facilitan la obtención de nauplios abundantes y de desarrollo y enriquecimiento homogéneo. Esto es particularmente importante en un organismo como *Artemia* capaz de crecer muy rápidamente tras la eclosión hasta casi doblar su talla a las 24 h de vida.

Los experimentos realizados en el **Capítulo 4** de la presente Tesis Doctoral muestran que los mayores niveles de EFA se obtuvieron al llevar a cabo el enriquecimiento a 28 ºC en

comparación con 24 ºC, siendo consistente con las condiciones publicadas en la literatura (Han y cols., 2000, 2001; Sui y cols., 2007; Boglino y cols., 2012). Este hecho sugiere que la disminución en la tasa metabólica de *Artemia* a bajas temperaturas, se traduce también en una menor tasa de ingestión de partículas que resulta, por lo tanto, en un menor contenido en HUFA a los nauplios. Dado que la eficiencia de enriquecimiento de M70 no parece estar comprometida por temperaturas potencialmente más pro-oxidantes (28 ºC *vs.* 24 ºC) se procedió con la investigación de otros parámetros experimentales que pudiera afectar a la estabilidad oxidativa de M70 y en consecuencia a su eficacia en la bioencapsulación de HUFA.

Otra estrategia para reducir la oxidación del DHA existente en los productos enriquecedores, implica el establecimiento adecuado de dosis (posología) a lo largo del protocolo de enriquecimiento. Mientras que una dosis al comienzo del enriquecimiento parece ser la práctica más extendida (Estévez y cols., 1999; Villalta y cols., 2005a, 2005b), dos dosis repartidas a lo largo del mismo, una al comienzo y otra a mitad el proceso (Evjemo y cols., 1997; Estévez y cols., 1998; Han y cols., 2000, 2001; Sui y cols., 2007; Hamre y Harboe, 2008) pueden contribuir a reducir la peroxidación lipídica, ya que el tiempo que los ácidos grasos están expuestos a condiciones pro-oxidantes (aireación, elevada temperatura) se reduce. La autoxidación de compuestos del producto

enriquecedor provoca un aumento de los lípidos rancios en el medio de cultivo, lo que puede provocar toxicidad en los organismos expuestos a éstos (presas vivas y larvas alimentadas con ellas), que incluso puede afectar a proteínas y vitaminas (McEvoy y cols., 1995).

Teóricamente, el suministro del producto enriquecedor en dos dosis, parece la estrategia más aconsejable según lo expuesto anteriormente. Según McEvoy y cols. (1995), las emulsiones como Super Selco (Inve, Dendermonde, Bélgica), que llevan añadido un antioxidante en su formulación, conservan durante más tiempo su porcentaje de HUFA en el medio de cultivo. En dicho estudio, a las 19 horas desde el inicio del enriquecimiento no se hallaron signos de autoxidación. Sin embargo a las 23 horas, se observó un aumento de los niveles de especies reactivas al ácido tiobarbitúrico (TBARS, *Thiobarbituric Acid Reactive Substances*). Esto indica que la autoxidación, en emulsiones formuladas con antioxidante, ocurre en las últimas fases del proceso, a pesar de la presencia de antioxidantes. El reparto de la dosis de emulsión en dos dosis, puede tener sentido si la autoxidación tiene lugar desde el primer momento en que se introducen los nauplios de *Artemia* al medio de cultivo. El estudio de McEvoy y cols. (1995) confirma que, para los productos enriquecedores estudiados, tal estrategia no ofrece una ventaja evidente con respecto a la monodosis. Nuestra experiencia con M70, está en consonancia con los

resultados de McEvoy y cols. (1995), ya que, como se presenta en el **Capítulo 4**, no se encontraron diferencias en cuanto a la bioencapsulación de EFA a lo largo del periodo de enriquecimiento. La estabilidad oxidativa de M70 permite dispensar dicha emulsión en una única dosis al comienzo del enriquecimiento sin ningún detrimento evidente en la incorporación de HUFA durante el enriquecimiento.

Teóricamente, la dosis de emulsión determina la disponibilidad de ácidos grasos para los nauplios de *Artemia*. La adición de una cantidad insuficiente de emulsión, supone que no se enriquecen adecuadamente y, por tanto, no alcanzan los niveles idóneos de EFA. La incorporación al medio de enriquecimiento de una cantidad excesiva de emulsión, puede implicar, al margen de su derroche, que se incremente innecesariamente el riesgo de contaminación del medio de cultivo por microorganismos (Verdonck y cols., 1994).

Las dosis de enriquecedor referidas en la bibliografía oscilan entre 0,4 y 0,6 g L^{-1} (McEvoy y cols., 1996; Van Stappen, 1996; Han y cols., 2000, 2001; Villalta y cols., 2005a, 2005b). Estas dosis se han establecido en función del contenido en lípidos totales de los productos enriquecedores. Teniendo en cuenta este hecho, y debido a su mayor contenido en agua, la dosis de M70 se fijó en 0,8 g L^{-1}. Los resultados de los ensayos realizados indican que la dosis de 0,6 g M70 L^{-1} es suficiente para producir un enriquecimiento equiparable en DHA al

conseguido 0,8 g L^{-1}. Sin embargo, la elevada variabilidad observada en el perfil de ácidos grasos de nauplios de *Artemia* enriquecidos a 0,6 g L^{-1} puede enmascarar una baja eficiencia de incorporación en comparación a los obtenidos con 0,8 g L^{-1}. Asimismo, se observó una mayor relación DHA/EPA en *Artemia* enriquecida a 0,8 g L^{-1} (1,8), siendo este un parámetro diferencial de esta emulsión enriquecedora.

Además, los resultados muestran también que M70 se puede utilizar tanto con una densidad de nauplios en el medio de cultivo de 150 o de 300 nauplios mL^{-1}. Desde un punto de vista cuantitativo (% lípido) la mayor incorporación de producto se consiguió con 150 nauplios mL^{-1}, pero desde un punto de vista coste-efectivo y teniendo en cuenta que no se observaron diferencias significativas en los EFA ni en la relación DHA/EPA entre enriquecer a 150 o a 300 nauplios mL^{-1}, la opción de utilizar 300 nauplios mL^{-1} parece la más recomendable.

Como se ha mencionado anteriormente, el uso de procedimientos de enriquecimiento conlleva efectos secundarios nocivos como la autoxidación de los HUFA de la emulsión (McEvoy y cols., 1995; Sargent y cols., 1997), y la bioacumulación de peróxidos lipídicos tóxicos en los nauplios de *Artemia* enriquecidos (Monroig, 2006). En este sentido, la emulsión M70, debido a su elevado contenido en DHA, y por tanto su alto grado de insaturación, es más proclive a la oxidación durante el enriquecimiento. La peroxidación lipídica

está causada por sustancias pro-oxidantes que pueden alterar la capacidad del organismo para catalizar las reacciones y aumentar la presencia de especies reactivas de oxígeno (ROS, *Reactive Oxygen Species*) (Livingstone, 2001; Barata y cols., 2005). Las ROS están involucradas en el daño celular y tienen capacidad para provocar fallos morfológicos, fisiológicos y peroxidación de los HUFA, lo que comprometería el normal desarrollo del organismo (Tovar-Ramirez y cols., 2010). Un factor importante que afecta al potencial del daño oxidativo es la cantidad de moléculas diana, como los EFA, que son fácilmente oxidados por ROS en peróxidos lipídicos. Como se ha mencionado, este es un punto crucial en la presente tesis doctoral, ya que la emulsión M70 contiene un 70% de DHA. En este contexto y por lo tanto, bajo la suposición de que la peroxidación lipídica en el enriquecimiento con la emulsión M70 sería elevada debido a su alto contenido en EFA, se optó por la adición de un antioxidante externo hidrosoluble, al medio de enriquecimiento. El antioxidante elegido fue Hytolive protect (HYT, Genosa I+D S.I., Málaga, España) basado en hidroxitirosol (3,4-dihidroxifeniletanol). El hidroxitirosol es un antioxidante natural obtenido de la fracción acuosa resultante tras el proceso de molienda de las olivas (Schaffer y cols., 2007). El hidroxitirosol es un potente antioxidante natural (Hao y cols., 2010) con actividad para neutralizar radicales libres y minimizar el estrés oxidativo, comparable incluso a los

antioxidantes sintéticos (Gordon y cols., 2001; González, 2005; Deiana y cols., 2008; Fernández-Bolaños y cols., 2008).

En humanos, el hidroxitirosol es rápidamente absorbido de manera dosis-dependiente en el intestino delgado y colon mediante difusión pasiva bidireccional (El-Azem, 2013). La mayor parte del hidroxitirosol se conjuga en glucuronoconjugados, que es la forma más abundante en plasma y con un gran potencial antioxidante. La utilización de un antioxidante externo, basado en hidroxitirosol, para tratar de reducir la peroxidación lipídica en el medio de enriquecimiento, es una práctica totalmente novedosa ya que no existen estudios previos usando hidroxitirosol en el enriquecimiento de presas vivas.

McEvoy y cols. (1995) demostraron que existía autoxidación en enriquecimientos con emulsiones lipídicas cuando *Artemia* estaba presente, mientras que las emulsiones en el medio de enriquecimiento, pero sin *Artemia*, no produjeron autoxidación en el periodo experimental. Por tanto es esperable que una vez introducido en el medio de enriquecimiento, con los nauplios de *Artemia* presentes, el efecto protector del hidroxitirosol actúe debido a su naturaleza hidrosoluble, protegiendo a los ácidos grasos de la oxidación. De este modo, cabe esperar que la oxidación se reduzca antes de que los ácidos grasos sean ingeridos por *Artemia*, asegurando que su sistema enzimático antioxidante no se sature y permanezca activo.

Estudios previos han confirmado que los nauplios de *Artemia* poseen los enzimas antioxidantes necesarios para metabolizar el O_2^- (SOD), H_2O_2 (CAT y GPx) y la actividad de hidroperóxidos orgánicos (GPx) (Nunes y cols., 2006). Estos enzimas permiten al organismo prevenir, interceptar y reparar el daño causado por los radicales libres (Olsen y cols., 2013). Sin embargo, durante el enriquecimiento es posible que este mecanismo protector sea insuficiente para prevenir el daño oxidativo que se puede producir en los HUFA de la emulsión lipídica los cuales son muy propensos a la oxidación, sobre todo bajo las condiciones físicas de un enriquecimiento (McEvoy y cols., 1995; Monroig y cols., 2007).

El primer experimento del **Capítulo 5**, en el que se analizaron los principales enzimas antioxidantes de nauplios de *Artemia* enriquecidos con varios tratamientos (*Tetraselmis suecica*, *Saccharomyces cerevisiae*, M70 y un grupo control sin enriquecer), corroboró la hipótesis antes esbozada, el empleo de M70 con nauplios de *Artemia* produce una peroxidación lipídica notable y activa toda la maquinaría enzimática antioxidante de los nauplios con el fin de contrarrestar este estrés oxidativo. En el segundo experimento del **Capítulo5**, se introdujo el antioxidante externo (Hytolive), y se midió la actividad antioxidante de nauplios sin enriquecer y enriquecidos con M70, con y sin Hytolive añadido, comparándola con la de nauplios

enriquecidos con una emulsión comercial, concretamente DC Super Selco (DCSS, Inve, Dendermonde, Bélgica).

Los resultados muestran que los nauplios de *Artemia* enriquecidos con M70 y DCSS tienen niveles significativamente mayores de peroxidación lipídica (LPO) que los nauplios enriquecidos con M70 suplementados con Hytolive y los del grupo control. El enriquecimiento de *Artemia* se considera como un proceso de "bioencapsulación" en el que *Artemia* ingiere partículas de enriquecimiento del medio hasta que su intestino está lleno (Figueiredo y cols., 2009). Por tanto, los lípidos oxidados y otros productos tóxicos potenciales pueden ser asimismo filtrados por los nauplios de *Artemia* en los últimos estadios del proceso de enriquecimiento (McEvoy y cols., 1995). Al añadir Hytolive al tratamiento con M70, se disminuye la peroxidación lipídica y por tanto, la presencia de lípidos oxidados disponibles en el medio, asegurando aparentemente que la actividad de las enzimas antioxidantes no se sobresature y provocando que el nivel de LPO se asemeje a la del grupo control. Concretamente los niveles de LPO en el tratamiento con la emulsión M70 suplementada con Hytolive se redujeron a la mitad cuando se compararon con los niveles alcanzados al utilizar la emulsión lipídica DCSS o M70 sin el antioxidante externo. Estas diferencias se corroboran quimiométricamente al analizar el gráfico de puntuaciones del Análisis de Componentes Principales (PCA, *Principal Component Analysis*) en el que el

grupo de nauplios suplementados con Hytolive se separa claramente del grupo de nauplios enriquecidos sólo con M70 (Figura 5.4).

La disminución de la peroxidación lipídica en el enriquecimiento de nauplios de *Artemia* al utilizar Hytolive como antioxidante externo fue claramente notable, por lo que también se estudió el efecto que podría ejercer la presencia de este antioxidante sobre los niveles de ácidos grasos en los nauplios enriquecidos. Previamente ya se ha expuesto la importancia del DHA para el correcto desarrollo del sistema nervioso en vertebrados, incluyendo peces (Hamre y Harboe, 2008, Tocher, 2010), y de ahí que el enriquecimiento de las presas vivas como *Artemia*, que de forma natural carece de DHA, sea crítico para el éxito del cultivo de larvas de peces, particularmente de especies marinas (Tocher, 2010), e incluso de otros organismo marinos. Claramente, la disminución en la peroxidación lipídica al utilizar M70 como enriquecedor y suplementado con Hytolive, también se traduce en un aumento de los niveles de DHA y en una mayor relación DHA/EPA.

Los resultados del **Capítulo 5** resaltan el efecto protector obtenido tras la adición de un antioxidante al medio de cultivo y el incremento potencial concomitante de la relación DHA/EPA. Como primera aproximación, sólo se ensayó la concentración de 0,04 g L^{-1}, y aunque los beneficios fueron ya significativos, probablemente concentraciones mayores podrían producir

mejores resultados, ya que hay evidencias de un efecto inhibidor del Hytolive en la LPO a concentraciones mayores (Gargouri y cols., 2011). Estudios sucesivos probando mayores concentraciones son pues aconsejables para establecer si el potencial antioxidante puede mejorar aún más.

A lo largo del periodo de realización de la presente tesis doctoral, se tuvo la oportunidad de probar la nueva emulsión lipídica M70 a escala semi-industrial, en la cría larvaria de pulpo común (*Octopus vulgaris*), con el fin, entre otros, de determinar si su alto contenido en DHA podía mejorar de alguna forma la supervivencia en la cría de fases tempranas (paralarvas) de esta especie.

El pulpo (*Octopus vulgaris*) es un molusco cefalópodo presente en casi todos los mares del mundo (Caddy, 1983), que se ha postulado en los últimos años como una de las especies con mayor futuro en el campo de la acuicultura marina gracias a su elevado valor comercial y a sus características biológicas: rápido crecimiento, alta fecundidad, resistencia a la manipulación y adaptación a la vida en cautividad. A pesar de que se ha avanzado bastante en el conocimiento de los aspectos biológicos y técnicos relacionados con su cría, el desarrollo industrial del pulpo está aún limitado debido a la imposibilidad de obtener masivamente subadultos en cautividad, así como, en menor medida, a la ausencia de piensos artificiales con la

apropiada composición nutritiva que garanticen unos rendimientos satisfactorios.

Entre los muchos factores que pueden afectar al fracaso en el cultivo del pulpo (fracaso entendido como elevadas mortalidades en el paso de paralarvas a juveniles), Navarro y Villanueva (2000, 2003) apuntaron a que un desequilibrio en los lípidos y composición de los ácidos grasos podían ser los responsables de estas elevadas mortalidades. Su dieta "natural" (larvas de crustáceos, copépodos, etc) tiene una composición lipídica notablemente diferente a la de *Artemia* enriquecida. Mientras que los estadios tempranos de cefalópodos, al igual que sus presas naturales, son bajos en lípidos totales y ricos en lípidos polares (ricos en HUFA) y colesterol, el alimento larvario artificial, entendiendo como tal *Artemia* en cualquiera de sus formas (nauplios y metanauplios enriquecidos o no), es rico en lípidos totales, y en particular en triacilglicéridos (TAG) (Navarro y Villanueva, 2000, 2003).

Para intentar atenuar el posible desequilibrio entre lípidos y composición de ácidos grasos señaladas por Navarro y Villanueva (2000, 2003), especialmente en lo que a la concentración de HUFA se refiere, se consideró la utilización de metanauplios de *Artemia* enriquecidos con M70 como dieta de paralarvas en un cultivo piloto de pulpo atendiendo especialmente al elevado contenido en DHA de esta emulsión. Como se ha expuesto anteriormente, gracias a colaboraciones

desarrolladas con el Instituto Español de Oceanografía en su sede de Vigo, en el ámbito de proyectos del equipo de investigación, se tuvo la oportunidad de probar esta emulsión enriqueciendo metanauplios de *Artemia* destinados al cultivo de paralarvas de pulpo a escala semi-industrial (acuarios de 1000 L). El **Capítulo 6** recoge los resultados obtenidos al alimentar paralarvas de *O. vulgaris* con *Artemia* enriquecida con varios tratamientos: las microalgas *Isochrisis galbana y Nannochloropsis* sp., y con la emulsión M70. Se estudiaron especialmente las diferencias en los perfiles lipídicos de las paralarvas de pulpo alimentadas con estos tratamientos, haciendo un seguimiento del crecimiento y la supervivencia durante la cría paralarvaria.

El peso seco de las paralarvas de pulpo fue similar en ambos tratamientos (1,8 ± 0,3 mg para *Isochrisis* y *Nannochloropsis* y 1,9 ± 0,2 mg para M70), sin embargo la supervivencia a los 30 días fue mayor con el tratamiento de *Isochrisis* y *Nannochloropsis* (3% con M70 y del 22,5% con *Isochrisis* y *Nannochloropsis*). En las paralarvas que sobrevivieron con el tratamiento con *Isochrisis* y *Nannochloropsis* se prolongó su cría hasta los 35 días, pero su supervivencia cayó hasta el 3%, con lo que el aparente beneficio que obtenido con este tratamiento se perdió una semana más tarde.

El análisis de las clases de lípidos de las paralarvas, alimentadas tanto con *Isochrisis* y *Nannochloropsis* como con M70, se reveló que los lípidos polares (LP) fueron más ricos en EPA y DHA, lo que se tradujo en unos HUFA n-3 más elevados. Los lípidos neutros (LN) fueron más ricos en ácidos grasos monoinsaturados. Estas diferencias en composición de LP y LN se corroboran gráficamente al interpretar los resultados del PCA. En el gráfico de puntuaciones (Figura 6.1) se distinguen tres grupos claramente diferenciados correspondientes a los perfiles de ácidos grasos de los lípidos totales, LN y LP de las paralarvas. Las puntuaciones para los LP aparecen más juntas que las de los LN, indicando que su patrón de ácidos grasos es más semejante y remarcando el papel estructural de esta clase lipídica (Gurr y Harwood, 1991).

Shchervakova (2012) observó que la composición en lípidos de la presa influía en la composición lipídica de las paralarvas de pulpo cultivadas. A fecha de hoy, la correlación entre la supervivencia y la composición cualitativa de los lípidos de la dieta de las paralarvas de pulpo está por esclarecer (Okumura y cols., 2005; Almansa y Cerezo, 2013; Reis y cols., 2014). Este experimento no fue una excepción, pero sirvió para poner de manifiesto que la elevada cantidad de DHA de la emulsión lipídica M70, se reflejó en las paralarvas de pulpo alimentadas con este tratamiento, tanto en los lípidos totales como en los polares. A pesar de ello, aparentemente, los

resultados parecen indicar que las necesidades esenciales de las paralarvas están suplidas tanto con *Artemia* enriquecida con *Isochrisis* y *Nannochloropsis* como con M70, y que la presencia de DHA en las paralarvas alimentadas con *Isochrisis* y *Nannochloropsis* indica cierto grado de bioconversión por parte de éstas, o de retención selectiva de este componente esencial. Seixas y cols. (2010) indican que, más importante que los requerimientos en los lípidos esenciales, lo es la adecuada relación proteína/lípido de las presas usadas como alimento en la cría de paralarvas de pulpo común. De cualquier modo las masivas mortalidades obtenidas en los distintos experimentos impiden sacar conclusiones claras.

Estudios recientes (Roura y cols., 2012; Jin y cols., 2014; Li y Olsen, 2015) muestran que la distribución de los n-3 HUFA en LP y TAG de los nauplios de copépodos, alimento natural tanto de paralarvas de pulpo como de larvas de peces marinos, y de las presas vivas utilizadas en acuicultura, como *Artemia* y rotíferos, son muy diferentes. Los nauplios de copépodos tienen cerca del 50 % del DHA de los ácidos grasos totales en sus LP, concretamente en los fosfolípidos (Li y Olsen, 2015), mientras que los niveles de DHA en LP de *Artemia* y rotíferos son bastante pobres incluso tras un enriquecimiento en n-3 HUFA (Guinot y cols., 2013; Jin y cols., 2014; Li y Olsen, 2015). En las presas vivas enriquecidas, el contenido total de n-3 HUFA puede ser relativamente elevado, pero la mayoría del DHA y del

EPA está en forma de TAG (Coutteau y cols., 1997). Cada vez más estudios sugieren que el DHA asociado a los LP de la dieta, está más disponible metabólicamente para las larvas de organismos marinos (Olsen y cols., 2014) ya que éstas tienen una limitada capacidad de biosíntesis *de novo* de fosfolípidos (Tocher y cols., 2008). Reis y cols. (2014) establecieron que no solo el perfil de ácidos grasos es crucial para el normal desarrollo de paralarvas de *O. vulgaris*, sino que el modo en que se presentan estos ácidos grasos también es importante, ya que influye en la biosíntesis de clases lipídicas o en el intercambio de ácidos grasos entre fosfolípidos y TAG.

Fuentes y cols. (2011) señalan que las propuestas para mejorar la cría de paralarvas de *O. vulgaris* se han realizado en experimentos de laboratorio, y que su implementación a escala comercial es complicada. Actualmente siguen existiendo dificultades para llevar a cabo la cría del pulpo en cautividad. Este experimento se propuso en el contexto de que el bajo contenido de DHA en dietas para las paralarvas de pulpo podía ser una explicación plausible a su elevada mortalidad. Iglesias y cols. (2007) establecieron que unos de los principales factores que influyen en la mortalidad de las paralarvas de pulpo son aspectos nutricionales como los contenidos en fosfolípidos, colesterol y ácidos grasos. El contenido en HUFA, especialmente DHA y EPA, constituye uno de los requerimientos básicos nutricionales para esta especie (Iglesias y

cols, 2007; Reis y cols., 2014). Los resultados de Iglesias y cols. (2007) demostraron que, aunque el contenido en DHA de la alimentación empleada puede ser un factor determinante en la viabilidad de las paralarvas, no es el único que explique las diferencias en supervivencia. Ello no excluye que el uso de emulsiones como M70 sea un medio eficaz de incrementar el contenido en DHA en la dieta (y en la composición corporal final) de estadios iniciales de desarrollo de pulpo común y también de otras especies marinas.

7.4. Bibliografía

Almansa, E. y Cerezo, J., 2013. Hacia el cultivo integral del pulpo de roca (*Octopus vulgaris*). Revista AquaTIC 39, 23-26.

Anger, K., 2001. The biology of decapod crustacean larvae. In: Crustacean Issues, vol. 14 (ed. By R. Vonk). Pp. 149. A. A. Balkema Publishers, Lisse.

Aragão, C., Conceição, L.E.C., Dinis, M.T., Hans-Jørgen, F., 2004. Amino acid pools of rotifers and *Artemia* under different conditions: nutritional implications for fish larvae. Aquaculture 234, 429-445.

Babu, M.M., Marian, P.M., Kitto, M.R., 2001. A cradle aeration system for hatching *Artemia*. Aquacult. Engin. 24, 85-89.

Barata, C., Varo, I., Navarro, J.C., Arun, S., Porte, C., 2005. Antioxidant enzyme activities and lipid peroxidation in the freshwater cladoceran *Daphnia magna* exposed to redox cycling compounds. Comparative Biochemistry and physiology Part C: Toxicology & pharmacology 140, 175-186.

Boglino, A., Darias, M.J., Ortiz-Delgado, J.B., Özcan, F., Estévez, A., Andree, K.B., Hontoria, F., Sarasquete, C., Gisbert, E., 2012. Commercial products for *Artemia* enrichment affect growth performance, digestive system maturation, ossification and incidence of skeletal deformities in Senegalese sole (*Solea senegalensi*) larvae. Aquaculture 324-325, 290-302.

Caddy, J.F., 1983. Advances in assessment of world cephalopods resources. FAO Fish. Tech. Pap. Nº 231: 452 pp.

Coutteau, P., Geurden, I., Camara, M.R., Bergot, P., Sorgeloos, P., 1997. Review on the dietary effects of phospholipids in fish and crustacean larviculture. Aquaculture 155, 149-164.

Deiana, M., Incani, A., Rosa, A.,Corona, G., Atzeri, A., Loru, D., Melis, M.P., Dessi, A., 2008. Protective effect of hydroxytyrosol and its metabolite homovanillic alcohol on H_2O_2 induced lipid peroxidation

in renal tubular epitelial cells. Food and Chemical Toxixology 46, 2984-2990.

El-azem, N., 2013. Efecto del hidroxitirosol sobre la cardiotoxicidad producida por doxorubicina. PhD. Universidad de Granada. Pp. 202.

Estévez, A., McEvoy, L.A., Bell, J.G., Sargent, J.R., 1998. Effects of temperature and starvation time on the pattern and rate of loss of essential fatty acids in *Artemia* nauplii previously enriched using arachidonic acid and eicosapentaenoic acid-rich emulsions. Aquaculture 165, 295-311.

Estévez, A., McEvoy, L.A., Bell, J.G., Sargent, J.R., 1999. Growth, survival, lipid composition and pigmentation of turbot (*Scophthalmus maximus*) larvae fed live-prey enriched in arachidonic and eicosapentaenoic acids. Aquaculture 180, 321-343.

Evjemo, J.O., Coutteau, P., Olsen, Y., Sorgeloos, P., 1997. The stability of docosahexaenoic acid in two *Artemia* species following enrichment and subsequent starvation. Aquaculture 155, 135-148.

Fernández-Bolaños, J.G., López, Ó., Fernández-Bolaños, J., Rodríguez-Guitiérrez, G., 2008. Hydroxytyrosol and derivates: Isolation, synthesis, and biological properties. Current Organic Chemistry 12, 442-463

Figueiredo, J., Van Woesik, R., Lin, J., Narciso, L., 2009. *Artemia franciscana* enrichment model – How to keep them small, rich and alive? Aquaculture 294, 212-220.

Fuentes, L., Sánchez, F.J., Lago, M.J., Iglesias, J., Pazos, G., Linares, F., 2011. Growth and survival of *Octopus vulgaris* (Cuvier 1797) paralarvae fed on three *Artemia*-based diets complemented with frozen fish flakes, crushed zooplankton and marine microalgae. Scientia Marina 75, 771-777.

Gargouri, B., ben Mansour, R., Jmal, M.M., Boujelben, R.N., Allouch, N., Lassoued, S., Hamden, K. 2011. Hydroxytyrosol supplementation inhibits oxidative DNA damage, suppresses protein-lipid oxidation

and modulates antioxidant enzymes in human peripheral blood lymphocytes. Journal of Medicinal Plants Research 5, 4863-4869.

Gonzalez, M.P., 2005. Estudio de los efectos cardiovasculares y la absorción oral del hidroxitirosol en modelos animales y humanos. Tesis doctoral. Universidad de Granada, España. 201 pp.

Gordon, M.H., Paiva-Martins, F., Almeida, M., 2001. Antioxidant activity of hydroxytyrosol acetate compared with that of other olive oil polyphenols. Journal of Agricultural and Food Chemistry 49, 2480-2485.

Guinot, D., Monroig, Ó., Hontoria, F., Amat, F., Varó, I., Navarro, J.C., 2013. Enriched on-growth *Artemia* metanauplii actively metabolise highly unsaturated fatty acid-rich phospholipids. Aquaculture 412-413, 173-178.

Gurr, M.I. y Harwood, J.L., 1991. Lipid biochemistry. An introduction. Chapman and Hall, London, UK.

Hamre, K. y Harboe, T., 2008. *Artemia* enriched with high n-3 HUFA may give a large improvement in performance of Atlantic halibut (*Hippoglossus hippoblossus* L.) larvae. Aquaculture 277, 239-243.

Han, K., Geurden, I., Sorgeloos, P., 2000. Enrichment strategies for *Artemia* using emulsions providing differents levels of n-3 highly unsaturated fatty acids. Aquaculture 183, 335-347.

Han, K., Geurden, I., Sorgeloos, P., 2001. Fatty acid changes in enriched and subsequently starved *Artemia franciscana* nauplii enriched with different essential fatty acids. Aquaculture 199, 93-105.

Han, K., Geurden, I., Van der Meeren, P., Bai, S.C., Sorgeloos, P., 2005. Particle size distribution in two lipid emulsion used for the enrichment of *Artemia* nauplii as a function of their preparation method and storage time. Journal of World Aquaculture Society 36, 196-202.

Hao, J., Shen, W., Yu, G., Jia, H.,Li, X., Feng, Z., Wang, Y., Weber, P., Wertz, Z., Sharman, E, Liu, J., 2010. Hydroxytyrosol promotes

mitochondrial biogenesis and mitochondrial function in 3T3-L1 adypocites. Journal of Nutritional Biochemistry 21, 634-44.

Hernandorena, A., 1976. Effects of temperatura on the nutritional requirements of the *Artemia salina* (L.). Biological Bulletin 151, 314-321.

Hochachka, P.W. y Somero, G.N. (ed.) 1984. Temperature adaptation. In: Biochemical Adaptations. Pp 355-449. Princeton University Press, Princeton, NJ.

Hontoria, F., Crowe, J.H., Crowe, L.M., Amat, F., 1994. Potential use of liposomes in larviculture as a delivery system through *Artemia* nauplii. Aquaculture 127, 255-264.

Iglesias, J., Sánchez, F.J., Bersano, J.G.F., Carrasco, J.F., Dhont, J., Fuentes, L., Linares, F., Muñoz, J.L., Okumura, S., Roo, J., van der Meeren, T., Vidal, E.A.G., Villanueva, R., 2007. Rearing of *Octopus vulgaris* paralarvae: Present status, bottlenecks and trends. Aquaculture 266, 1-15.

Jin, Y., Li, K., Olsen, Y., 2014. Time kinetics of fatty acids changes in phospholipids following enrichment and starvation of *Artemia franciscana* with a main focus on docosahexaenoic acid. Aquaculture Nutrition. http://dx.doi.org/10.1111/anu.12224.

Kolkovski, S., Curnow, J., King, J., 2004. Intensive rearing system for fish larvae research II *Artemia* hatching and enriching system. Aquacultural Engineering 31, 309-317.

Léger, P., Bengtson, D.A., Simpson, K.L., Sorgeloos, P., 1986. The use and nutritional value of *Artemia* as a food source. Oceanography and Marine Biology: An Annual Review 24, 521-623.

Li, K. y Olsen, Y., 2015. Effect of enrichment time and dietary DHA and non-highly unsaturated fatty acid composition on the efficiency of DHA enrichment in phospholipid of rotifer (*Brachionus* Cayman). Aquaculture 446, 310-317.

Livingstone, D.R., 1991. Organic xenobiotics metabolism in marine invertebrates. Advances in Comparative and Environmental Physiology 7, 45-185.

McEvoy, L.A., Navarro, J.C., Bell, J.G., Sargent, J.R., 1995. Autoxidation of oil emulsions during the *Artemia* enrichment process, Aquaculture 134, 101-112.

McEvoy, L.A., Navarro, J.C., Hontoria, F. Amat, F., Sargent, J.R., 1996. Two novel *Artemia* enrichment diets containing polar lipid. Aquaculture 144, 339-352.

Monroig, O., 2006. Diseño y optimización de liposomas para su uso como sistema de suministro de nutrientes a larvas de peces marinos. Tesis doctoral. Universidad de Valencia. pp. 240.

Monroig, O., Navarro, J.C., Amat, I., González, P., Amat, F., Hontoria, F., 2003. Enrichment of *Artemia* nauplii in PUFA, phospholipids, and wáter-soluble nutrients using liposomes. Aquacult. Intern. 11, 151-161.

Monroig, O., Navarro, J.C., Amat, F., González, P., Hontoria, F., 2006a. Effect of aeration on the efficiency of *Artemia* enrichment with EFA-rich emulsion and liposomes. Aquaculture 257, 382-392.

Monroig, O., Navarro, J.C., Amat, F., González, P., Bermejo, A., Hontoria, F., 2006b. Enrichment of *Artemia* nauplii in essential fatty acids with different types of liposomes and their use in the rearing of gilthead sea bream (*Sparus aurata*) larvae. Aquaculture 251, 491-508.

Monroig, O., Navarro, J.C., Amat, F., González, P., Hontoria, F., 2006c. Effects of nauplial density, product concentration and product dosage on the survival of the nauplii and EFA incorporation during *Artemia* enrichment with liposomes. Aquaculture 261, 659-669.

Monroig, O, Navarro, J.C., Amat, F., González, P., Hontoria, F., 2007. Oxidative stability and changes in the particle size of liposomes used in the *Artemia* enrichment. Aquaculture 266, 200-210.

Navarro, J.C., Henderson, R.J., McEvoy, L.A., Bell, M.V., Amat, F., 1999. Lipid conversion during enrichment of *Artemia*. Aquaculture 174, 155-166.

Navarro, J.C. y Villanueva, R., 2000. Lipid and fatty acid composition of early stages of cephalopods: an approach to their lipid requirements. Aquaculture 183, 161-177.

Navarro, J.C. y Villanueva, R., 2003. The fatty acid composition of *Octopus vulgaris* paralarvae reared with live and inert food: deviation from their natural fatty acid profile. Aquaculture 219, 613-631.

Nunes, B., Carvalho, F. Guilhermino, L., 2006. Effects of widely used pharmaceuticals and a detergent on oxidative stress biomarkers of the crustacean *Artemia parthenogenetica*. Chemosphere 62, 581-594.

Okumura, S., Kurihara, A., Iwamoto, A., Takeuchi, T., 2005. Improved survival and growth in *Octopus vulgaris* paralarvae by feeding large type *Artemia* and Pacific sandeel, *Ammodytes personatus*. Aquaculture 244, 147-157.

Olsen, E.K., Hansen, E., Isaksson, J., Andersen, J.H., 2013. Cellular antioxidant effect of four bromophenols from the red algae, *Vertebrata lanosa*. Marine Drugs 11, 2769-2784.

Olsen, Y., Evjemo, J.O., Kjorsvik, E., Larsen, H., Li, K.S, Overrein, I., Rainuzzo, J., 2014. DHA content in dietary phospholipids affects DHA content in phospholipids of cod larvae and larval performance. Aquaculture 428, 203-214.

Reis, D.B., Acosta, N.G., Almansa, E., Navarro, J.C., Tocher, D.R., Monroig, Ó., Andrade, J.P., Sykes, A.V., Rodríguez, C., 2014. *In vivo* metabolism of unsaturated fatty acids in *Octopus vulgaris* hatchlings determined by incubation with ^{14}C-labelled fatty acids added directly to seawater as protein complexes. Aquaculture 431, 28-33.

Ries, D., 2009. Studies on the antioxidant activity of milk proteins in model oil-in-water emulsions. PhD thesis, Riddet Institute (Massey University), Palmerston North, New Zealeand, 256 pp.

Roura, Á., González, Á., Redd, K., Guerra, Á., 2012. Molecular prey identification in wild *Octopus vulgaris* paralarvae. Marine Biology 159, 1335-1345.

Sargent, J.R., McEvoy, L.A., Bell. J.G., 1997. Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. Aquaculture 155, 177-127.

Sargent, J.R., Tocher, D.R., Bell, J.G., 2002. The lipids. In: Fish nutrition (ed. By J.E. Halver & R.W. Hardly) pp 181-257. Academic Press, San Diego, CA, USA.

Schaffer, S., Podstawa, M., Visioli, F., Bogani, P. Müller, W.E., Eckert, G.P., 2007. Hydroxytyrosol-rich olive mil wastewater extract protects brain cells *in vitro* and *ex vivo*. Journal of Agricultural and Food Chemistry 55, 5043-5049.

Seixas, P., Rey-Méndez, M., Valente, L.M.P., Otero, A., 2010. High DHA content in *Artemia* is ineffective to improve *Octopus vulgaris* paralarvae rearing. Aquaculture 300, 156-162.

Shcherbakova, A., 2012. The effect of tank types on the growth and survival of *Octopus vulgaris* paralarvae fed the crab *Grapsus adscensionis* zoeae. Master in Aquaculture and fisheries. Universidade do Algarve, Faro, Portugal.

Southgate, P.C. y Lou, D.C., 1995. Improving the n-3 HUFA composition of *Artemia* using microcapsules containing marine oils. Aquaculture 134, 91-99.

Sui, L., Wille, M., Cheng, Y., Sorgeloos, P., 2007. The effect of dietary n-3 HUFA levels and DHA/EPA ratios on growth, survival and osmotic stress tolerance of Chinese mitten crab *Eriocheir sinensis* larvae. Aquaculture 273, 139-150.

Tocher, D.R., 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Reviews in Fisheries Science 11, 107-184.

Tocher, D.R., Bendiksen, E.A., Campbell, P.J.,Bell, J.G., 2008. The role of phospholipids in nutrition and metabolism of teleost fish. Aquaculture 280, 21-34.

Tocher, D.R., 2010. Fatty acid requirements in ontogeny of marine and freshwater fish. Aquaculture research 41, 717-732.

Tocher, D.R., 2015. Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. Aquaculture, *in press*.

Tovar-Ramirez, D., Mazurais, D., Gatesoupe, J.F., Quazuguel, P., Cahu, C.L., Zambonino-Infante, J.L., 2010. Dietary probiotic live yeast modulates antioxidant enzyme activities and gene expression of sea bass (*Dicentrarchus labrax*) larvae. Aquaculture 300, 142-147.

Van Stappen, G., 1996. Introduction, biology and ecology of *Artemia*. En: Lavens, P., Sorgeloos, P., (Eds.), Manual on the production and use of live food for aquaculture (FAO). Fisheries Technical Paper Nº 361. Roma, Italia, pp. 101-121.

Van Stappen, G., 2002. Zoogeography; p. 171-215. In T. J. Abatzopoulos, J.A. Beardmore, J.S. Cleeg and P. Sorgeloos (Ed.). *Artemia* Basic and Applied biology. Netherlands/Dordrecht: Kluwer Academic Publishing.

Verdonck, L., Swings, J., Kersters, K., Dehasque, M., Sorgeloos, P., Leger, P., 1994. Variability of the microbial environment of rotifer *Brachionus plicatilis* and *Artemia* production systems. Journal of World Aquaculture Society 25, 55-59.

Villalta, M., Estévez, A., Bransden, M.P., Bell, J.G., 2005a. The effect of graded concentrations of dietary DHA on growth, survival and tissue fatty acid profile on Senegal Sole (*Solea senegalensis*) larvae during the *Artemia* feeding period. Aquaculture 249, 353-365.

Villalta, M., Estévez, A., Bransden, M.P., 2005b. Arachidonic acid enriched live prey induces albinism in Senegal Sole (*Solea senegalensis*) larvae. Aquaculture 245, 193-209.

Watanabe, T., Ohta, M., Kitajima, C., Fujita, S., 1982. Improvement of dietary value of brine shrimp *Artemia salina* for fish larvae by feeding them on w3 highly unsaturated fatty acids. Bul. Jap. Soc. Scient. Fish. 48, 1775-1782.

CONCLUSIONES

1. M70 es una emulsión lipídica formulada a partir de DHA-Algatrium, con un 70 % de DHA en su composición, destinada a enriquecer nauplios de *Artemia*. Este elevado porcentaje de DHA permite que durante dicho enriquecimiento se pueda revertir la relación DHA/EPA hasta valores superiores a 1, más cercanos a los que presenta el alimento natural (zooplancton).

2. Las condiciones óptimas que se han establecido para llevar a cabo el enriquecimiento con la emulsión M70 son: flujos de aire comprimido de 1 L min^{-1}, temperatura de cultivo de 28 ºC, una única dosis de 0,8 g L^{-1} al comienzo de dicho enriquecimiento, y una densidad de 300 nauplios mL^{-1}.

3. El enriquecimiento de nauplios de *Artemia* utilizando la emulsión M70, y otras ricas en ácidos grasos poliinsaturados, aumenta la peroxidación lipídica y ello se refleja en la actividad de las principales enzimas antioxidantes de los nauplios (SOD, CAT, GPx y GST), empleadas como biomarcadores de oxidación, en comparación a lo observado en nauplios sin enriquecer.

4. El enriquecimiento de nauplios de *Artemia* con la emulsión M70 suplementada con un antioxidante hidrosoluble externo natural basado en el extracto de oliva

y especialmente rico en hidroxitirosol, provoca una mejora en la actividad de los biomarcadores de oxidación y disminuye la peroxidación lipídica.

5. El efecto protector del antioxidante externo natural basado en extracto de oliva y muy rico en hidroxitirosol, se refleja en el perfil lipídico de los nauplios de *Artemia*, produciendo niveles más elevados de DHA y una relación DHA/EPA más alta tras la adición del mismo.

6. En pruebas semi-industriales, la alimentación de paralarvas de *Octopus vulgaris* con nauplios de *Artemia* enriquecidos con M70, produjo rendimientos similares en supervivencia y crecimiento a los obtenidos siguiendo los protocolos de alimentación estandarizados que, hasta la fecha, producen los mejores resultados; a saber: nauplios de *Artemia* enriquecidos con las algas *Isochrisis galbana* y *Nannochloropsis* sp.

7. Las evidencias científicas apuntan a que el DHA es un nutriente esencial para la cría larvaria de *O. vulgaris*. La alimentación de las paralarvas con nauplios de *Artemia* enriquecidos con la emulsión M70, se presenta como una opción particularmente eficaz a la hora de aumentar el contenido en DHA de dichas paralarvas, siendo esta condición extrapolable al cultivo de otros organismos marinos.

RESUMEN

Los nauplios de *Artemia* se utilizan como presa viva en criaderos de organimos marinos de todo el mundo debido a su disponibilidad y digestibilidad, pero su valor nutricional no se ajusta a las demandas o necesidades de las larvas de peces, moluscos o crustáceos marinos, ya que carecen de los ácidos grasos esenciales (EFA, *Essential Fatty Acids*) para estos organismos. Por tanto, es indispensable llevar a cabo un enriquecimiento de los nauplios de *Artemia*. El enriquecimiento consiste en incubaciones en agua de mar en la que se dispersan productos ricos en EFA y que serán incorporados de forma pasiva por los nauplios debido a su naturaleza de filtradores. A pesar de los muchos avances que se han hecho en este campo, el enriquecimiento en EFA de nauplios de *Artemia* continúa siendo un proceso ineficaz. Mientras que el enriquecimiento en ácido araquidónico (20:4n-6, ARA) y ácido eicosapentaenoico (20:5n-3, EPA) se ha logrado fácilmente, el enriquecimiento en ácido docosahexaenoico (22:6n-3, DHA) sigue siendo dificultoso debido a factores específicos que limitan su incorporación a los nauplios de *Artemia*, como los mecanismos de autoxidación de los ácidos grasos altamente insaturados (HUFA) de la emulsión, que pueden ser especialmente severos con el DHA en comparación con otros HUFA debido a su larga cadena y alto grado de insaturación, y a la existencia de procesos de retroconversión.

Para superar los efectos adversos del enriquecimiento es necesario desarrollar nuevas estrategias que permitan aumentar el contenido en DHA de los nauplios de *Artemia*. Con el fin de hacer frente a estos problemas durante el enriquecimiento, se investigó la eficacia de una nueva emulsión lipídica (M70), con un 70 % de DHA en su composición.

Se probaron diferentes condiciones de enriquecimiento con el fin de establecer un uso óptimo de la emulsión M70. Las condiciones de enriquecimiento ensayadas fueron diferentes regímenes de oxigenación, temperaturas de incubación, concentraciones de producto enriquecedor y modos de dosificación, y de densidad naupliar. Todos estos parámetros se han considerado como relevantes durante el proceso de enriquecimiento de presas vivas. Los resultados indican que la utilización eficiente de la emulsión M70 para nauplios de *Artemia* se logra con una temperatura de incubación de 28ºC, ya que a esta temperatura se consigue un aumento en la incorporación de HUFA en los nauplios de *Artemia* en comparación con 24 ºC. Una aireación baja/moderada (1 L min^{-1}) produce mejores resultados que flujos mayores (3 L min^{-1}) ya que inducen mayores turbulencias en el medio, lo que puede afectar al comportamiento natatorio de los nauplios e incluso dañar las estructuras filtradoras, dificultando así la incorporación de las partículas del producto de enriquecimiento en los nauplios. La densidad naupliar óptima se estableció en

300 individuos por mL, ya que la eficacia del enriquecimiento no varió entre 150 y 300 individuos por mL. Por otra parte, la emulsión M70 se puede administrar a nauplios recién eclosionados mediante una única dosis de hasta 0,8 g L^{-1} al comienzo del enriquecimiento sin que la mortalidad naupliar sea evidente.

Utilizando la emulsión M70 en los enriquecimientos de nauplios de *Artemia* se consiguieron unas relaciones de DHA/EPA siempre superiores a 1,1, siendo las más bajas las correspondientes a condiciones experimentales subóptimas (excesiva aireación o bajas temperaturas). Así, la relación DHA/EPA fue particularmente alta (1,8-1,9) cuando se utilizaron las condiciones experimentales optimizadas, lo que sugiere que éstas se habían establecido adecuadamente.

Los resultados en incorporación de DHA con la emulsión M70, aunque prometedores y más elevados que los alcanzados con enriquecedores comerciales, sugieren la existencia de algún factor que impide un enriquecimiento eficiente. A pesar de que M70 contiene en su formulación un antioxidante liposoluble no se puede descartar la oxidación del DHA como causa de la limitada incorporación de este ácido graso en los nauplios de *Artemia*, ya que estudios anteriores, han establecido que la autoxidación se asocia a un descenso en la concentración de HUFA en los lípidos de nauplios de *Artemia* enriquecidos, en particular en el EPA y en el DHA. La inclusión de un

antioxidante soluble en agua, puede ser una opción a la hora de evitar o prevenir la oxidación del DHA durante el enriquecimiento de *Artemia*. El hidroxitirosol (3,4-dihidroxifeniletanol) es una molécula polar que se encuentra en la fracción acuosa resultantes tras el proceso de molienda de las olivas, con un alto poder antioxidante mayor que el de las vitaminas antioxidantes y el de algunos antioxidantes sintéticos. Teniendo en cuenta que no hay datos disponibles sobre su papel como agente protector de las emulsiones lipídicas empleadas para el enriquecimiento de presas vivas, se propuso investigar el efecto protector de la adición de dicho antioxidante utilizando el compuesto natural Hytolive Protect (Genosa I + D SI, Málaga, España) durante el enriquecimiento de nauplios de *Artemia* con M70. Para llevar a cabo este propósito, se determinaron las enzimas clave (superóxido dismutas, catalasa, glutatión peroxidasa y glutatión-S-transferasa) como biomarcadores de la actividad antioxidante, y la peroxidación lipídica (LPO) de los nauplios de *Artemia* enriquecidos. La utilización de Hytolive mejoró la actividad enzimática y la peroxidación lipídica de los nauplios de *Artemia* enriquecidos con M70 haciéndola más similar a la de los grupos control sin enriquecer. Asimismo, los resultados muestran que los niveles de DHA más elevados se obtuvieron en los nauplios enriquecidos con M70 suplementado con Hytolive. El análisis quimiométrico de los datos sugiere claramente, que la acción protectora del hidroxitirosol está teniendo lugar.

El estudio sobre la utilización de la emulsión M70 se completó con la utilización de *Artemia* enriquecida con dicha emulsión para la alimentación de paralarvas de pulpo común (*Octopus vulgaris*) en condiciones semi-industriales. En los últimos años, el pulpo ha sido un candidato prometedor para la acuicultura, pero, hasta la fecha, la cría de paralarvas está severamente limitada por la falta de éxito en la fase planctónica, con mortalidades generalizadas que se producen antes del asentamiento de los juveniles, habiéndose apuntado la carencia de ácidos grasos esenciales del alimento como una de las posibles causas.

Se comparó el uso de metanauplios enriquecidos con M70 frente a otros enriquecidos con *Isochrysis galbana* y *Nannochloropsis* sp. (tratamiento Nanno), analizando el efecto de dichas dietas sobre la composición de ácidos grasos de los lípidos totales, polares y neutros de las paralarvas.

Los resultados muestran que el peso seco promedio de las paralarvas de 28 días de edad de ambos grupos dietéticos no fue significativamente diferente y aunque la supervivencia a esta edad se estimó, aproximadamente, en el 3% para M70 y 22,5% para Nanno, el cultivo de las paralarvas de este último grupo experimental hasta los 35 días, condujo asimismo a supervivencias del 3%. Los resultados muestran sin embargo, que las paralarvas alimentadas con *Artemia* enriquecida con

M70 tenían niveles de DHA más elevados, tanto en los lípidos totales como en los polares.

A fecha de hoy, la relación entre la supervivencia y la composición cualitativa de los lípidos de la dieta de las paralarvas de pulpo está aún por esclarecer. Este experimento constató que la elevada cantidad de DHA de la emulsión lipídica M70, se trasladó a las paralarvas de pulpo alimentadas con este tratamiento, evidenciándose como un medio eficaz de incrementar el contenido en DHA en la dieta y en la composición corporal final de larvas de *O. vulgaris*, y por extrapolación, de otras especies marinas.

En conclusión, la utilización de la emulsión M70 para el enriquecimiento de nauplios de *Artemia* es una herramienta útil que produce unos excelentes resultados en cuanto a niveles de DHA alcanzados, y que incluso revierte la relación DHA/EPA de los nauplios, haciéndola más similar a la de las presas naturales de las larvas de organismos marinos. Asimismo es un medio eficaz de transferir DHA a organismos larvarios en cultivo.

ANEXO

Artículos

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Enriching *Artemia* nauplii with a high DHA-containing lipid emulsion: search for an optimal protocol

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Abstract

This study aimed to investigate practical strategies to optimize the use of a high-docosahexaenoic acid (DHA) lipid emulsion (M70), a product with great potential in live prey enrichments for marine larviculture. Considering its particularly high content in DHA (22:6n-3), the adequate utilization of the emulsion for *Artemia* enrichments was evaluated in a series of six experiments. More specifically, the bioencapsulation efficiency of M70 into *Artemia* nauplii was tested under different experimental conditions of oxygen source, aeration flow, incubation temperature, concentration and dosage, as well as nauplial densities. Our results showed that an optimal utilization of M70 is achieved with incubation temperatures of 28 °C, moderate aeration flows and nauplial densities of 300 ind per mL. In addition, the emulsion can be dispensed in the enrichment medium in one single dose of 0.8 g L^{-1}, with no apparent detrimental effects on its oxidative stability and *Artemia* nauplii survival during enrichment.

Keywords: *Artemia*, docosahexaenoic acid, essential fatty acid, lipid emulsion, live prey enrichment

Introduction

Marine fish, and particularly their larval stages, have high requirements for highly unsaturated fatty acids (HUFA), including the physiologically active arachidonic (20:4n-6, ARA), eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic acids (22:6n-3, DHA). However, the essential fatty acids (EFA) requirements of freshwater and salmonid species can be met by including the C18 polyunsaturated fatty acids (PUFA) α-linolenic acid (18:3n-3, LNA) and linoleic acid (18:2n-6, LA) (Buzzy, Henderson & Sargent 1996; Bell & Dick 2004), it is generally accepted that marine species have limited capability for the biosynthesis of HUFA from the C18 PUFA, although, exceptionally, some species have been proved to express genes encoding fatty acyl desaturases and elongases involved in these biochemical pathways (Yamamoto, Kabeya, Takeuchi, Alimuddin Haga, Satoh, Takeuchi & Yoshizaki 2010; Monroig, Webb, Ibarra-Castro, Holt & Tocher 2011; Morais, Mourente, Ortega, Tocher & Tocher 2011). In consequence, adequate levels of pre-formed ARA, EPA and DHA need to be supplied through the diet to ensure normal growth and development (Bell, Batty, Dick, Fretwell, Navarro & Sargent 1995; Estévez, McEvoy, Bell & Sargent 1998, 1999; Sargent, Tocher & Bell 2002; Bell, McEvoy, Estévez, Shields & Sargent 2003; Tocher 2003; Benitez-Santana, Masuda, Juarez, Ganuza, Valencia, Hernández-Cruz & Izquierdo 2007).

Artemia nauplii are widely used as live prey in marine fish hatcheries worldwide, due to their availability and digestibility (Léger, Bengtson, Simpson & Sorgeloos 1986). The nutritional value of *Artemia* nauplii, however, is not adequate for marine fish larvae since they are largely deficient in essential HUFA, particularly DHA (Koven, Tandler, Kissil, Sklan, Friezlander & Harel 1990).

In order to overcome the problem, *Artemia* nauplii are enriched through incubations in seawater containing HUFA-rich products that are passively incorporate by the nauplii (Léger *et al.* 1986). This procedure is called bioencapsulation (Navarro, Henderson, McEvoy, Bell & Amat 1999). Many different enrichment diets have been used to enhance *Artemia* EFA contents: microalgae (Watanabe, Ohta, Kitajima & Fujita 1982; Aragão, Conceição, Dinis & Hans-Jørgen 2004), microcapsules (Southgate & Lou 1995), liposomes (Hontoria, Crowe, Crowe & Amat 1994; McEvoy, Navarro, Hontoria, Amat & Sargent 1996; Monroig, Navarro, Amat, González, Amat & Hontoria 2003; Monroig, Navarro, Amat, González, Bermejo & Hontoria 2006a; Monroig, Navarro, Amat, Gonzalez & Hontoria 2006b,c) and lipid emulsions (Léger *et al.* 1986; Evjemo, Coutteau, Olsen & Sorgeloos 1997; Han, Geurden & Sorgeloos 2000, 2001; Han, Geurden, Van der Meeren, Bai & Sorgeloos 2005).

Lipid emulsions are arguably the most extended enrichment diets and a big variety of products are now commercially available. Composition of commercial lipid emulsions basically consists of fish oils, with other minor components including emulsifying agents, vitamins and fatty acid (FA) derivatives such as fatty acid ethyl esters (FAEE) added to compensate suboptimal natural profiles of fish oil (Monroig, Navarro, Amat & Hontoria 2007). Although the efficiency of enrichment protocols with emulsified diets is normally higher than that of other enrichment products (Coutteau & Sorgeloos 1997; Sorgeloos, Dhert & Candreva 2001), their use has been related with detrimental side effects. Among them, the autoxidation of HUFA (McEvoy, Navarro, Bell & Sargent 1995; Sargent, McEvoy & Bell 1997) and the consequent bioaccumulation of potentially toxic lipid peroxides into larvae fed emulsion-enriched *Artemia* (Monroig 2006) have been described. Furthermore, autooxidation of HUFA-rich products might deplete dissolved oxygen (DO) levels in the enrichment medium and thus can further compromise the overall performance of the enrichment process as well as favour *Artemia* mortalities (Léger, Bengtson, Sorgeloos, Simpson & Beck 1987; Figueiredo, van Woesik, Lin & Narciso 2009). Importantly, the most hindering and yet unresolved drawback from the use of emulsion-based enrichers involves specific difficulties in increasing the DHA content of nauplii.

The low efficiency of DHA enrichment in *Artemia* nauplii has been acknowledged as a major obstacle for their use as live prey for first-feeding larvae of marine fish (Bell *et al.* 2003; Haché & Plante 2011). While the bioencapsulation of other HUFA including EPA and ARA has been readily achieved (Villalta, Estévez & Bransden 2005a; Villalta, Estévez, Bransden & Bell 2005b), specific factors determining the limited DHA incorporation into *Artemia* appear to exist. On one hand, the autooxidation mechanisms mentioned above might be particularly severe for DHA compared with other HUFA, due to its longer chain length and higher unsaturation degree (Cosgrove, Church & Pryor 1987). On the other hand, biological factors related to *Artemia* physiology also need to be considered. Naturally occurring trace levels of DHA found in *Artemia* lipids (Evjemo *et al.* 1997) suggest that *Artemia* DHA requirements are low, and excessive DHA input occurring during enrichment might be compensated through DHA retroconversion to EPA (Watanabe, Kitajima & Fujita 1983; Barclay & Zeller 1996; Furuita, Takeuchi, Toyota & Watanabe 1996; Evjemo *et al.* 1997; Navarro *et al.* 1999). In order to overcome such adverse effects, novel enrichment strategies to enhance the DHA contents of *Artemia* nauplii need to be developed.

In this study, we investigated the efficiency of a DHA-rich oil emulsion (M70) as enrichment diet for *Artemia* nauplii. Considering the particular aspects derived from its DHA-rich nature, a series of experiments were carried out to establish an optimal use of M70 under different oxygenation regimes, incubation temperatures, enrichment product concentrations and dosage modes, as well as different nauplial densities. All the investigated parameters were previously shown as relevant aspects during live prey enrichment procedures (Van Stappen 1996; Han *et al.* 2000, 2001; Monroig *et al.* 2006a,b; Sui, Wille, Cheng & Sorgeloos 2007; Hamre & Harboe 2008; Figueiredo *et al.* 2009).

Materials and methods

Emulsion formulation

The experimental lipid emulsion M70 consisted of a 1:1 (v/v) oil/water suspension, of which the synthetic oil DHA Algatrium (Brudy, Barcelona, Spain) contained ~70% DHA of total FA in the form of ethyl esters. Xanthan gum (0.36%) and Tween 80 (0.10%) were used as stabilizer and

emulsifier respectively (Giner 2005). M70 formulation was developed by Archivel Technologies S.L. (Barcelona, Spain) and the The Physical and Sensory Properties Laboratory at the Instituto de Agroquímica y Tecnología de los Alimentos (IATA-CSIC, Valencia, Spain). The FA profiles of the M70 emulsion are reported in Table 1.

Artemia nauplii enrichments: general conditions

Low HUFA containing *Artemia* nauplii were obtained from the hatching of EG grade cysts (Inve, Ghent, Belgium). After an incubation period of 23 h at 28°C, nauplii were collected and rinsed with tap water to remove the hatching metabolites and debris. Newly hatched nauplii were thereafter placed in 1 L cylinder-conical glass vessels containing seawater for further enrichment with the experimental emulsion M70. Unless otherwise stated, *Artemia* enrichments were carried out at 28 °C,

Table 1 Fatty acid composition (per cent of total fatty acids) of the enrichment products M70 and DC Super Selco (DCSS, Inve) utilized in this study

Fatty acid	M70	DCSS
14:0	0.1	1.3
16:0	0.9	5.2
16:1n-7	0.4	1.9
18:0	0.4	2.7
18:1n-9	3.7	9.2
18:1n-7	0.5	1.6
18:2n-6	0.7	4.8
18:3n-3	0.6	1.0
18:4n-3	0.4	1.8
20:0	n.d.	0.4
20:1n-9	0.3	2.3
20:3n-6	0.1	0.1
20:4n-6	2.4	1.6
20:3n-3	0.1	0.2
20:4n-3	0.5	1.1
20:5n-3	7.7	31.6
22:0	0.1	0.4
22:1n-11	0.1	0.2
22:5n-3	2.7	2.2
22:6n-z3	70.3	20.6
Saturated	1.8	10.3
Monounsaturated	5.2	18.5
Polyunsaturated	91.1	66.2
HUFA n-3	81.3	55.6
HUFA n-6	8.0	2.6
DHA/EPA ratio	9.1	0.6

n.d.: not detected; HUFA n-3: ≥20:3n-3; HUFA n-6: ≥20:2n-6; DHA/EPA: docosahexaenoic and eicosapentaenoic fatty acid ratio.

aeration of 1 L min^{-1}, diffusion system consisting of a 25-cm long and 0.5-cm-diameter section glass tube applied from the bottom of the vessel, nauplial density of 300 nauplii per mL, and product concentrations of 0.8 g L^{-1} for M70 dispensed in a single dose at the beginning of the enrichment process. All the enrichment treatments were run in triplicates ($n = 3$). After an enrichment period of 21 h, samples of *Artemia* nauplii were collected by filtering the enrichment medium through a 100 μm mesh carefully washed with tap water in order to eliminate remains of emulsion adhered to the nauplial shells, and subsequently rinsed with distilled water. *Artemia* samples were frozen at −20°C and freeze-dried previous to FA analysis.

Artemia nauplii enrichment with M70: protocol optimization

Optimal conditions for the use of M70 in *Artemia* enrichments were assessed by analyzing the FA composition of the nauplii obtained from a series of experiments (1–6) varying the type and source of aeration, temperature, dosage and concentration of enrichment product and nauplial density. Conditions for each subsequent experiment were established based on the results from preceding experiments (Table 2).

Experiment 1: effects of oxygen source

The effects of air quality on the enrichment efficiency of the emulsion M70 were tested. Three different treatments, all providing a fairly constant DO content above the recommended 4 mg L^{-1} (Van Stappen 1996), were tested: 'oxygen', with pure oxygen; 'air', with compressed air; and 'mixture', with both pure oxygen and aeration.

Experiment 2: effects of aeration

In order to assess the effects of the aeration level in the *Artemia* enrichment with M70, three different air flows were tested: '1 lpm', '2 lpm' and '3 lpm' with air flows of 1.0, 2.0 and 3.0 L min^{-1}, respectively, being used. Air flows were individually controlled in each enrichment vessel by means of a rotameter (Key Instruments, Trevose, PA, USA).

Experiment 3: effects of temperature

The effects of temperature in the enrichment process were assessed by incubating the nauplii in the presence of M70 at 24°C (treatment '24C') or 28°C (treatment '28C'). The temperature was kept

Table 2 Experimental conditions set up in the experiments carried out in this study. DC Super Selco (DCSS, Inve) was used as control treatment in Experiment 6 as indicated in Materials and methods

Treatment	Oxygen source	Air flow (L min^{-1})	Temperature (°C)	Product concentration (g L^{-1})	Doses	Nauplial density (nauplii per mL)
Exp. 1						
Oxygen	Oxygen	1	28	0.8	1	300
Air	Air	1	28	0.8	1	300
Mixture	Oxygen+Air	1	28	0.8	1	300
Exp. 2						
1 lpm	Air	1	28	0.8	1	300
2 lpm	Air	2	28	0.8	1	300
3 lpm	Air	3	28	0.8	1	300
Exp. 3						
24C	Air	1	24	0.8	1	300
28C	Air	1	28	0.8	1	300
Exp. 4						
0.6	Air	1	28	0.6	1	300
0.8	Air	1	28	0.8	1	300
Exp. 5						
2 doses	Air	1	28	0.8	2	300
1 dose	Air	1	28	0.8	1	300
Exp. 6						
Low dens	Air	1	28	0.8	1	150
High dens	Air	1	28	0.8	1	300
DCSS	Air	1	28	0.6	1	300

(±1°C) constant by placing the enrichment vessels in a thermostatic bath.

Experiment 4: effects of enrichment product concentration

The effects of the M70 concentration on the enrichment efficiency were evaluated by enriching *Artemia* at two different concentrations: 0.8 g M70 L^{-1} (treatment '0.8'), this concentration being equivalent to 0.6 g L^{-1} recommended for commercial emulsions after correction for the water content difference; and 0.6 g M70 L^{-1} (treatment '0.6').

Experiment 5: effects of product dosage

In order to assess the effects of the enrichment product dosage on *Artemia* bioencapsulation, two experimental treatments were established: '2 doses', with M70 emulsion being splitted into two doses supplying 0.4 g L^{-1} at the beginning of the enrichment process and 0.4 g L^{-1} after 7 h; '1 dose', with the enrichment diet M70 being supplied in a single dose of 0.8 g L^{-1} at the beginning of the enrichment process.

Experiment 6: effects of nauplial density

The effect of nauplial density in the *Artemia* enrichment with M70 was evaluated by incubating the nauplii at two different densities: 150 (treatment 'low dens') and 300 nauplii per mL (treatment 'high dens'). As the last of our experiments aiming at optimization of M70 as enrichment diet, Experiment 6 was carried out under those experimental conditions (oxygen source, aeration flow, temperature, product concentration and product dosage) that had produced the optimal enrichment results in the preceding Experiments 1–5. Moreover, we compared the enrichment performance of M70 with that of DC Super Selco ('DCSS', Inve), a commercial product basically consisting of a fish oil emulsion. DC Super Selco enrichment was carried out under the same conditions of M70, except for the product concentration, 0.8 g L^{-1} for M70, and 0.6 g L^{-}1for DCSS, to compensate for the different water content of both products (50% and 30% for M70 and DCSS, respectively), as mentioned before.

Total lipids and FA analyses

Total lipids were extracted (Folch, Lees & Sloane Stanley 1957) from nauplii freeze-dried samples, measured gravimetrically (XS105 Mettler Toledo, Switzerland), and stored in chloroform: methanol (2:1; v/v) containing 0.01% butylated hydroxytoluene (BHT) at −20°C until further use. Total lipids

were subjected to acid catalyzed transmethylation for 16 h at 50°C using 1 mL toluene and 2 mL of 1% (v/v) sulphuric acid in methanol (Christie 2003). Fatty acid methyl esters (FAME) were extracted with hexane:diethyl ether (1:1; v/v) containing 0.01% BHT and purified by thin-layer chromatography (Silica gel G60, 20 × 20 cm glass plates, Merck, Darmstadt, Germany) using hexane:diethyl ether:acetic acid (85:15:1.5; v/v/v) as a solvent system. Fatty acid methyl esters were then analysed with a Fisons Instruments GC 8000 Series (Rodano, Italy) gas chromatograph. Peaks were recorded using the Azur software package (version 4.0.2.0. Datalys, France). Individual FAME were identified by comparison with known standards. The relative amount of each FA was expressed as a percentage of the total amount of FA.

Statistical analysis

Analytical data were expressed as means ± SD ($n = 3$). Differences between treatments were analysed by one-way ANOVA, followed by either Bonferroni's multiple comparison test or a Student's *t*-test when only two groups were compared (Sokal & Rohlf 1981). If heterogeneity of variances existed, Welch test was used to detect differences, followed by Games–Howell test to assess the differences between groups. When significance was $P \leq 0.05$, means were considered statistically different. The FA analytical data from Experiment 6, for which a comparison with the commercial product DCSS was carried out, were further analysed by multivariate Principal Components Analysis (PCA), in order to highlight the effects of factors enrichment diet and nauplial density. Statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA).

Results

Experiment 1: effect of oxygen source

Although the DHA bioencapsulation into *Artemia* nauplii did not show statistical differences among the treatments in Experiment 1, the levels of EPA and ARA in nauplii from 'air' treatment were significantly higher than those of treatments, 'oxygen' and 'mixture' (Table 3). Thus, the DHA/EPA ratio also showed statistically significant differences and *Artemia* from 'oxygen' treatment had higher DHA/EPA values (1.8 ± 0.0) in comparison with nauplii from treatments 'air' and 'mixture' (1.6 ± 0.1). As a preliminary conclusion from Experiment 1, DHA levels were similar in nauplii from all treatments and thus use of pure oxygen did not improve the enrichment efficiency of a more readily available source like compressed air.

Experiment 2: effect of aeration

The FA profiles of the *Artemia* nauplii enriched under different air flows ('1 lpm', '2 lpm' and '3 lpm') are shown in Table 3. Our results suggested that M70 enrichments are more efficient under relatively low air flows. Thus, the DHA contents of *Artemia* from treatment '1 lpm' were significantly higher than those of *Artemia* from treatment '3 lpm'. This was also reflected in the DHA/EPA ratio of both treatments, being 1.4 and 1.3 for '1 lpm' and '3 lpm' treatments respectively. Experiment 2 results allowed us to conclude that enrichment procedures at 1 L min^{-1} improved M70 bioencapsulation efficiency.

Experiment 3: effect of temperature

Table 3 shows the FA profiles of *Artemia* nauplii enriched with M70 at 24 and 28°C. Our results indicated that the contents of ARA, EPA and DHA, as well the ratio DHA/EPA, were significantly higher in nauplii incubated at 28°C than at 24°C. Overall, 28°C was confirmed to be a more adequate incubation temperature for M70 enrichments compared to 24°C.

Experiment 4: effect of product concentration

Experiment 4 compared the HUFA bioencapsulation performance of M70 dispensed to *Artemia* nauplii at product concentrations of 0.6 or 0.8 g L^{-1}. Generally, no differences among any of each individual FA analysed were detected between both treatments ($P > 0.05$), possibly due to a remarkably high variability in the FA contents of '0.6' nauplii. Despite no significantly different, average DHA contents in nauplii from treatment '0.8' (8.3 ± 0.2) were still higher than those of '0.6' nauplii (6.6 ± 2.0) (Table 3). Moreover, statistically significant increases of the DHA/EPA ratio were observed for M70 concentrations of 0.8 g L^{-1}. We could therefore conclude that an

Table 3 Fatty acids (percentage of total fatty acids) from enriched *Artemia* nauplii collected from Experiment 1–6 (see Materials and methods for treatment details). Data represent means ± SD ($n = 3$). Treatments in each Experiment with different letter are significantly different ($P \leq 0.05$). If no superscript appears, values are not different

% fatty acid	Experiment 1			Experiment 2			Experiment 3		Experiment 4		Experiment 5		Experiment 6	
	Oxygen	Air	Mixture	1 lpm	2 lpm	3 lpm	24C	28C	0.6	0.8	2 doses	1 dose	Low dens	High dens	DCSS
14:0	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.3	0.6 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	1.0 ± 0.7	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0^{b}	0.6 ± 0.0^{b}	0.8 ± 0.0^{b}
15:0	0.5 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.9 ± 0.0	0.9 ± 0.1	0.9 ± 0.0	0.7 ± 0.1	0.7 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.4 ± 0.3	0.6 ± 0.0	0.3 ± 0.3
16:0	9.5 ± 0.1	9.3 ± 0.0	9.6 ± 0.7	10.1 ± 0.1	10.5 ± 0.4	10.4 ± 0.1	10.0 ± 0.0	9.7 ± 0.2	11.1 ± 1.1	10.4 ± 0.1	10.0 ± 0.1	9.8 ± 0.1	9.9 ± 0.2	10.0 ± 0.1	10.0 ± 0.4
16:1n-9	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.0^{a}	0.6 ± 0.0^{ab}	0.7 ± 0.0^{b}	0.6 ± 0.0	0.6 ± 0.0	0.5 ± 0.1	0.6 ± 0.0	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.0^{a}	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}
16:1n-7	1.9 ± 0.0	1.9 ± 0.0	2.1 ± 0.3	4.7 ± 0.0^{a}	4.7 ± 0.2^{ab}	4.5 ± 0.0^{b}	2.9 ± 0.1	2.9 ± 0.1	2.7 ± 0.9	2.1 ± 0.0	4.4 ± 0.2	4.4 ± 0.1	2.1 ± 0.1^{a}	2.1 ± 0.0^{a}	2.3 ± 0.0^{a}
16:2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	0.7 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}	0.4 ± 0.0^{a}
16:3	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.7 ± 0.2	0.9 ± 0.1	0.8 ± 0.1	0.6 ± 0.0	0.6 ± 0.0	0.2 ± 0.3	0.0 ± 0.0	0.9 ± 0.1	0.7 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
18:0	4.7 ± 0.2	4.6 ± 0.1	4.5 ± 0.1	4.1 ± 0.0	4.3 ± 0.2	4.3 ± 0.1	4.2 ± 0.0	4.3 ± 0.1	4.4 ± 0.2	4.7 ± 0.0	4.1 ± 0.0	4.20 ± 0.1	4.5 ± 0.4^{a}	4.4 ± 0.0^{a}	4.1 ± 0.2^{a}
18:1n-9	16.1 ± 0.2	16.0 ± 0.2	16.0 ± 0.1	22.4 ± 0.2	23.2 ± 1.1	23.5 ± 0.1	19.2 ± 0.1^{a}	18.7 ± 0.1^{b}	16.8 ± 0.6	16.8 ± 0.1	22.2 ± 0.3	21.7 ± 0.2	16.4 ± 0.2^{a}	16.4 ± 0.1^{a}	17.0 ± 0.2^{a}
18:1n7	6.0 ± 0.1	6.1 ± 0.1	6.1 ± 0.1	8.2 ± 0.1	8.6 ± 0.5	8.8 ± 0.1	7.5 ± 0.2	7.4 ± 0.1	6.3 ± 0.4	6.3 ± 0.0	8.3 ± 0.1	8.4 ± 0.3	6.3 ± 0.0^{a}	6.3 ± 0.0^{a}	5.5 ± 0.1^{a}
18:2n-6	4.9 ± 0.1	4.7 ± 0.0	4.7 ± 0.1	4.3 ± 0.0	4.4 ± 0.2	4.4 ± 0.0	4.8 ± 0.0^{ab}	4.6 ± 0.0^{b}	4.9 ± 0.5	5.0 ± 0.0	4.3 ± 0.0	4.3 ± 0.0	4.8 ± 0.1^{a}	4.9 ± 0.0^{a}	6.3 ± 0.0^{b}
18:3n-3	24.2 ± 0.9	24.6 ± 0.3	24.4 ± 0.9	19.1 ± 0.3	19.9 ± 1.0	19.8 ± 0.2	25.6 ± 0.1^{a}	23.9 ± 0.4^{b}	25.2 ± 3.2	25.4 ± 0.1	19.1 ± 0.2	19.3 ± 0.1	24.8 ± 0.6^{a}	24.6 ± 0.1^{a}	19.8 ± 0.0^{b}
18:4n-3	3.0 ± 0.1	2.9 ± 0.1	3.0 ± 0.1	1.8 ± 0.0	1.9 ± 0.1	1.7 ± 0.0	3.0 ± 0.0	2.6 ± 0.1	3.1 ± 0.2	3.2 ± 0.1	1.8 ± 0.0	1.9 ± 0.0	3.1 ± 0.1^{a}	3.1 ± 0.0^{a}	2.8 ± 0.1^{a}
20:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:1n-9	0.6 ± 0.1	0.8 ± 0.0	0.6 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0^{b}	0.5 ± 0.0^{b}	0.9 ± 0.0^{a}
20:2n-6	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:3n-6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4n-6	1.3 ± 0.0^{ab}	1.5 ± 0.0^{a}	1.4 ± 0.0^{ab}	1.5 ± 0.0	1.4 ± 0.1	1.5 ± 0.0	1.2 ± 0.0^{b}	1.5 ± 0.0^{a}	1.0 ± 0.1	1.1 ± 0.0	1.6 ± 0.0	1.7 ± 0.0	1.2 ± 0.1^{a}	1.2 ± 0.1^{a}	1.0 ± 0.0^{b}
20:3n-3	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.1	0.6 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.6 ± 0.0^{a}	0.6 ± 0.0^{a}	0.5 ± 0.0^{b}
20:4n-3	0.6 ± 0.0	0.8 ± 0.0	0.6 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.2 ± 0.0	0.1 ± 0.1	0.6 ± 0.0^{a}	0.6 ± 0.0^{a}	0.8 ± 0.0^{a}
20:5n-3	5.7 ± 0.2^{b}	6.2 ± 0.1^{a}	6.4 ± 0.2^{a}	6.2 ± 0.1	5.6 ± 0.6	5.7 ± 0.0	5.2 ± 0.0^{b}	6.2 ± 0.2^{a}	5.0 ± 1.3	4.6 ± 0.0	6.2 ± 0.1	6.5 ± 0.0	5.2 ± 0.2^{b}	5.1 ± 0.1^{b}	13.8 ± 0.3^{a}
22:0	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.2	0.0 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.2 ± 0.0^{b}	0.2 ± 0.0^{b}	0.3 ± 0.0
22:5n-3	0.7 ± 0.0	0.8 ± 0.0	0.6 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.2	0.1 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.1 ± 0.1	0.1 ± 0.2	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.0^{a}
22:6n-3	10.4 ± 0.4	10.2 ± 0.1	10.3 ± 0.5	8.2 ± 0.2^{a}	6.2 ± 2.8^{b}	7.3 ± 0.1^{b}	6.9 ± 0.1^{b}	9.1 ± 0.3^{a}	6.6 ± 2.0	8.3 ± 0.2	9.0 ± 0.6	9.4 ± 0.1	9.2 ± 1.0^{a}	9.6 ± 0.3^{a}	4.9 ± 0.2^{b}
Saturated	15.7 ± 0.4	15.5 ± 0.1	15.8 ± 1.1	15.8 ± 0.2	16.6 ± 0.7	16.2 ± 0.0	15.6 ± 0.3	15.3 ± 0.3	17.5 ± 1.7	16.7 ± 0.1	15.7 ± 0.2	15.4 ± 0.2	15.8 ± 0.5	16.0 ± 0.1	15.2 ± 0.6
Monounsat.	25.8 ± 0.2	25.7 ± 0.2	25.8 ± 0.5	36.3 ± 0.3	37.7 ± 1.8	37.8 ± 0.2	30.6 ± 0.2^{a}	30.0 ± 0.2^{b}	27.0 ± 0.6	26.5 ± 0.2	35.9 ± 0.4	35.5 ± 0.6	26.0 ± 0.2^{b}	26.0 ± 0.1^{b}	27.1 ± 0.3^{b}
Polyunsat.	53.3 ± 0.5	53.7 ± 0.1	53.6 ± 1.3	43.4 ± 0.4	41.7 ± 2.4	42.4 ± 0.2	49.2 ± 0.2	50.1 ± 0.6	49.3 ± 1.8	50.6 ± 0.4	44.1 ± 0.6	44.9 ± 0.4	51.0 ± 0.7	51.7 ± 0.4	51.8 ± 0.9
HUFA n-3	18.1 ± 0.6	18.3 ± 0.2	18.5 ± 0.4	15.2 ± 0.3	12.4 ± 3.6	13.5 ± 0.1	13.2 ± 0.1^{b}	16.3 ± 0.5^{a}	13.2 ± 3.0	14.6 ± 0.2	15.7 ± 0.7	16.3 ± 0.4	16.2 ± 1.2^{a}	16.5 ± 0.4^{a}	20.6 ± 0.7^{a}
HUFA n-6	2.3 ± 0.1	2.3 ± 0.1	2.2 ± 0.1	1.7 ± 0.0	1.5 ± 0.2	1.5 ± 0.0	1.4 ± 1.1	1.6 ± 0.1	1.7 ± 0.2	1.9 ± 0.0	1.7 ± 0.1	1.8 ± 0.1	2.0 ± 0.1^{a}	2.1 ± 0.0^{a}	1.6 ± 0.0^{b}
DHA/EPA	1.8 ± 0.0^{a}	1.8 ± 0.1^{b}	1.6 ± 0.1^{b}	1.4 ± 0.0^{a}	1.1 ± 0.4	1.3 ± 0.0	1.3 ± 0.0^{b}	1.5 ± 0.0^{a}	1.4 ± 0.4^{b}	1.8 ± 0.0^{a}	1.4 ± 0.1	1.4 ± 0.0	1.8 ± 0.1^{a}	1.9 ± 0.0^{a}	0.3 ± 0.0^{b}
% lipids (DW)	31.4 ± 0.8^{a}	27.8 ± 0.5^{b}	27.5 ± 1.0^{b}	25.7 ± 1.6	24.0 ± 2.0	26.4 ± 0.6	22.7 ± 0.2	22.9 ± 0.4	25.2 ± 0.4	26.6 ± 1.8	31.6 ± 0.7	31.3 ± 0.8	25.7 ± 1.0^{b}	22.4 ± 1.2^{c}	28.8 ± 0.6^{a}

HUFA n-3: ≥20:3n-3; HUFA n-6: ≥20:2n-6; DHA/EPA: docosahexaenoic and eicosapentaenoic acid ratio; FAME: Fatty acid methyl ester.

M70 concentration of 0.8 g L^{-1} produced better bioencapsulation results than 0.6 g L^{-1}.

Experiment 5: effect of product dosage

The results from Experiment 5 indicated that dispensation of the enrichment product M70 in one or two doses did not produce differences in the FA profiles from *Artemia* lipids (Table 3). It was concluded that dispensing the product M70 in one single dose at the beginning of the enrichment process was a more practical and simpler strategy.

Effects of nauplial density

No effect of the nauplial density (150 and 300 nauplii per mL) on the enrichment performance with M70 was observed. Thus, no significant differences among any of the FA analysed could be established. Interestingly, a notable impact on *Artemia* FA profiles was observed when comparing the enrichment products M70 and the commercial emulsion DCSS, the control treatment in Experiment 6. The enrichment comparisons with DCSS showed that differences were mainly attributable to the effect of enrichment product (Table 3). This was further corroborated by the PCA, with the first component (PC1) explaining 92.5% and the second 6.5% (Fig. 1a), and subsequent score plot (Fig. 1b) that revealed two groups separated in the first component on the basis of the enrichment product used, whereas no separation based on the nauplial density was achieved. The association of the two groups to their respective variables allowed to identify 18:1n-9, LA and EPA with DCSS and 16:0, 18:0, 18:1n-7, LNA, ARA and DHA with M70 in the first component.

Discussion

It has been often reported in the literature that bioencapsulation of HUFA into *Artemia* nauplii is particularly difficult to achieve (Navarro *et al.* 1999; Bell *et al.* 2003). Among the factors believed to account for such limited efficiency for HUFA delivery, some have a particular relevance when DHA-rich enrichment products are used. Here, we investigated diverse practical strategies to optimize the use of the emulsion M70, a product with great potential for its use in *Artemia* enrichments.

Dissolved oxygen availability in the culture medium is critical for *Artemia* survival during enrichment, and DO concentrations above 4 mg L^{-1} have been proposed (Van Stappen 1996). While standard enrichment procedures involve the use of compressed air to provide adequate levels DO, pure oxygen supply is particularly recommended when DO levels can be potentially compromised. That might be the case of live prey enrichments with high-DHA contents, as oxygen depleting processes such as lipid peroxidation are especially favoured (McEvoy *et al.* 1995; Ries 2009). We therefore compared the performance of pure oxygen vs. compressed air when using M70 as enrichment diet, and conclude that no difference in either the HUFA bioencapsulation into *Artemia* or nauplial mortality was observed. Altogether, Experiment 1 allowed us to conclude that oxygen supplied with compressed air is an effective diffusion system to meet the DO demands in enrichment procedures with M70, and that other more expensive alternatives such as pure oxygen are not required.

Other factors like air flow and temperature during the enrichment procedure also need to be considered when a compromise between lipid peroxidation, oxygen supply and enrichment efficiency is pursued. In addition to oxygen solubility and its potential consequences in lipid peroxidation, the air flow during enrichment also determines the overall turbulence in the medium. It has been suggested that highly turbulent conditions during enrichment might impede the normal swimming behaviour of nauplii and damage the nauplial filtratory structures, thus ultimately hindering the incorporation of the enrichment product particles (Navarro *et al.* 1999; Monroig *et al.* 2006b). In agreement with those findings, the emulsion M70 exhibited a higher HUFA bioencapsulation efficiency when utilized at moderate air flows ('1 lpm') than that at highly turbulent conditions ('3 lpm'). In contrast with the results reported by Monroig *et al.* (2006b), who investigated the enrichment efficiency of HUFA-rich liposomes under the same air flow conditions set for Experiment 2, the enrichment with M70 did not produce an excessive foam in the medium and thus the *Artemia* mortality caused by such an effect did not occur for any of the air flow studied.

Temperature of the enrichment medium is a key factor for efficient bioencapsulation of HUFA into *Artemia*. Beyond controlling the oxygen solubility and lipid peroxidation mechanisms, temperature also determines the metabolic capability of *Artemia*

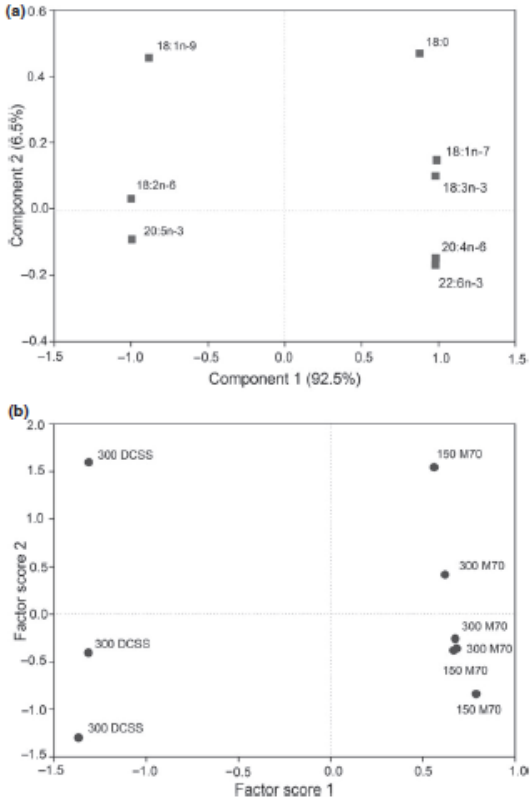

Figure 1 Component plot (a) and factor score plot (b) of the multivariate Principal Components Analysis of selected FA from total lipids of enriched *Artemia* nauplii from Experiment 6.

nauplii and therefore important development landmarks such as the mouth opening and vitelum absorption, as well as the onset of filtering/natatory appendixes (Hochanchka & Somero 1984; Anger 2001). Our results indicated that enhanced HUFA enrichment was achieved at 28°C compared with 24°C, this being consistent with the vast majority of studies in which 28°C has been established as preferred enrichment temperature (Harel, Ozkizilcik, Lund, Behrens & Place 1999; Han *et al.* 2001; Sui *et al.* 2007; Boglino, Darias, Ortiz-Delgado, Özcan, Estévez, Andree, Hontoria, Sarasquete & Gisbert 2012). In contrast, relatively low temperatures (21–22°C) were established in other studies (Garcia, Parrish & Brown 2008; Figueiredo *et al.* 2009), but unfortunately no specific reasons for the choice of these low temperatures, and the causes of their effects were given. Since the enrichment efficiency of M70 did not appear to be compromised by potentially more pro-oxidant temperatures ('28C' vs. '24C'), we continued our investigations evaluating other experimental parameters potentially affecting the oxidative stability of M70 and consequently its HUFA bioencapsulation efficacy.

Another strategy to preserve DHA-rich enrichment diets from oxidation involves an adequate dosage along the enrichment protocol. While one single dose at the beginning of the enrichment

process appears to be the most extended dosage mode (Estévez *et al.* 1999; Villalta *et al.* 2005a,b), two doses, one at the beginning of the enrichment process and another one at mid-period (Evjemo *et al.* 1997; Estévez *et al.* 1998; Han *et al.* 2000, 2001; Evjemo, Danielsen & Olsen 2001; Sui *et al.* 2007; Hamre & Harboe 2008) might contribute to reduce lipid peroxidation as the time of exposure to pro-oxidant conditions (continuous light, high temperature and limited DO) is minimized. The results from this study confirmed that no differences in HUFA bioencapsulation existed when M70 was dispensed in one or two doses, and thus the oxidative stability of M70 enables this enrichment diet to be dispensed in a unique dose without any evident detrimental effect in terms of HUFA incorporation over 21 h of incubation.

Our experiments investigating the effects of the M70 concentration and the nauplial density on *Artemia* bioencapsulation efficiency evidenced that M70 behaved similar to other enrichment products, and no special specification derived from its particularly high-DHA content needs to be considered. Thus, a dose of 0.8 g L^{-1} of M70, equivalent in dry weight basis to the recommended 0.6 g L^{-1} for commercial products (McEvoy *et al.* 1995, 1996; McEvoy, Navarro, Amat & Sargent 1997; Smith, Ritar, Phleger, Nelson, Mooney, Nichols & Hart 2002; Villalta *et al.* 2005a,b; Sui *et al.* 2007), resulted in increased DHA bioencapsulation into *Artemia* compared to lower concentration enrichments. At first glance, no significant differences in DHA contents between *Artemia* enriched at M70 concentrations of 0.6 and 0.8 g L^{-1} would have indicated that a concentration of 0.6 g M70 L^{-1} was sufficient to produce as much DHA enrichment as 0.8 g L^{-1}. However, the high variability observed in the FA profiles from *Artemia* enriched at concentration of 0.6 g L^{-1} could have certainly masked a lowered bioencapsulation efficiency in comparison with that obtained at 0.8 g L^{-1}. Indeed we did observe significant higher DHA/EPA ratio, as well as higher average contents of individual DHA for *Artemia* enriched at 0.8 g M70 L^{-1}. These results enabled us to choose 0.8 g L^{-1} of the M70 emulsion as a more adequate enricher concentration than 0.6 g L^{-1}. In addition, our experiment assessing the effect of nauplial density on HUFA enrichment revealed that M70 can be used at nauplial densities of 300 nauplii per mL, with the potential limitation of DO not causing any apparent effect on nauplial motility (Southgate & Lou 1995). Qualitative (FA profile) enrichment efficiency of M70 did not vary between 150 and 300 nauplii per mL. However, from a quantitative point of view (lipid %), a higher uptake of enrichment product was achieved at 150 nauplii per mL. Thus, any positive cost-effectiveness aspects related, among others, to a more restricted utilization of enrichment product, pointing at 300 nauplii per mL as the recommended nauplial density for M70 enrichments, has to be counterbalanced by the higher uptake achieved at lower nauplial density.

The DHA/EPA ratio is a common biochemical parameter used to evaluate the nutritional suitability of diets for marine finfish larviculture (Reitan, Rainuzzo & Olsen 1994; Evjemo *et al.* 1997). For instance, the yolk of marine fish eggs and the polar lipids of copepodites, natural preys of marine fish larvae in the wild, have DHA/EPA ratios around 2.0 (Fraser, Sargent & Gamble 1989; McEvoy *et al.* 1997; Sargent *et al.* 1997; Sorgeloos *et al.* 2001; Evjemo, Reitan & Olsen 2003; Van der Meeren, Olsen, Hamre & Fyhn 2008). Generally, the results from this study showed that the emulsion M70 allowed us to obtain in most cases *Artemia* nauplii with DHA/EPA ratios above 1.3 and only suboptimal experimental conditions (i.e. excessive aeration or relatively low temperature) resulted in lower values. It is worth mentioning that the DHA/EPA ratios of *Artemia* nauplii were particularly high (1.8–1.9) for Experiment 6, supporting that the final (optimized) experimental conditions developed for a more efficient use of M70 as enrichment diet had been adequately established. Moreover,
M70-enriched nauplii had higher DHA individual contents and DHA/EPA ratios than nauplii enriched with commercial enrichment products like the one utilized in this study (DCSS) and previous studies (Léger *et al.* 1986; Woods 2003; Lund, Steenfeldt & Hanses 2007; Naz 2008). Interestingly, Haché and Plante (2011) recently reported DHA/EPA ratios of 3.6 in *Artemia* nauplii enriched with Algamac 3050, a commercial product based on spray-dried cells of the marine protist *Schizochytrium* sp. (Barclay & Zeller 1996). Since DHA concentration of Algamac 3050 (~40% of total FA) is lower than that of M70 (~70%), factors possibly related to the physical and biochemical nature of Algamac 3050 might account for such unexpectedly high HUFA bioencapsulation. Never-

theless, the results obtained from this study clearly show that M70 emulsion was able to consistently produce DHA/EPA ratios above 1 in *Artemia* lipids, with particularly high values when optimized conditions are used.

In summary, the results from this study indicated that an efficient utilization of the emulsion M70 as enrichment diet for *Artemia* nauplii is achieved with incubation temperatures of 28°C, low/moderate aeration (1 L min^{-1}) and nauplial densities of 150 ind per mL. Moreover, the emulsion M70 can be administered to newly hatched nauplii through a single dose of 0.8 g L^{-1}, with no detrimental effects such as DHA autooxidation and nauplial mortalities becoming apparent.

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References

Anger K. (2001) The biology of decapod crustacean larvae. In: *Crustacean Issues*, Vol. **14** (ed. by R. Vonk), p. 419. A. A. Balkema Publishers, Lisse.

Aragão C., Conceição L.E.C., Dinis M.T. & Hans-Jørgen F. (2004) Amino acid pools of rotifers and *Artemia* under different conditions: nutritional implications for fish larvae. *Aquaculture* **234**, 429–445.

Barclay W. & Zeller S. (1996) Nutritional enhancement of n-3 and n-6 fatty acids in rotifers and *Artemia* nauplii by feeding spray-dried *Schizochytrium* sp. *Journal of the World Aquaculture Society* **27**, 214–322.

Bell M.V. & Dick J.R. (2004) Changes in capacity to synthesise 22:6n-3 during early development in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **235**, 393–409.

Bell M.V., Batty R.S., Dick J.R., Fretwell K., Navarro J.C. & Sargent J.R. (1995) Dietary deficiency of docosahexaenoic acid impairs vision at low light intensities in juvenile herring (*Clupea harengus* L.). *Lipids* **30**, 2443–449.

Bell J.G., McEvoy L.A., Estévez A., Shields R.J. & Sargent J.R. (2003) Optimising lipid nutrition in first-feeding flatfish larvae. *Aquaculture* **227**, 211–220.

Benitez-Santana T., Masuda R., Juarez E., Ganuza E., Valencia A., Hernández-Cruz C.M. & Izquierdo M.S. (2007) Dietary n-3 HUFA deficiency induces a reduced visual response in gilthead seabream Sparus aurata larvae. *Aquaculture* **264**, 408–417.

Boglino A., Darias M.J., Ortiz-Delgado J.B., Özcan F., Estévez A., Andree K.B., Hontoria F., Sarasquete C. & Gisbert E. (2012) Commercial products for *Artemia* enrichment affect growth performance, digestive system maturation, ossification and incidence of skeletal deformities in Senegalese sole (*Solea senegalensis*) larvae. *Aquaculture* **324**, 290–302.

Buzzy M., Henderson R.J. & Sargent J.R. (1996) The desaturation and elongation of linolenic acid and eicosapentaenoic acid by hepatocytes and liver microsomes from rainbow trout (*Oncorhynchus mykiss*) fed diets containing fish oil or olive oil. *Biochimica et Biophysica Acta* **1299**, 235–244.

Christie W.W. (2003) *Lipid analysis*(3rd edn). Oily Press, Bridgwater.

Cosgrove J.P., Church D.F. & Pryor W.A. (1987) The kinetics of the autoxidation of polyunsaturated fatty acids. *Lipids* **22**, 299–304.

Coutteau P. & Sorgeloos P. (1997) Manipulation of dietary lipids, fatty acids and vitamins in zooplankton cultures. *Freshwater Biology* **38**, 501–512.

Estévez A., McEvoy L.A., Bell J.G. & Sargent J.R. (1998) Effects of temperature and starvation time on the pattern and rate of loss of essential fatty acids in *Artemia* nauplii previously enriched using arachidonic acid and eicosapentaenoic acid-rich emulsions. *Aquaculture* **165**, 295–311.

Estévez A., McEvoy L.A., Bell J.G. & Sargent J.R. (1999) Growth, survival, lipid composition and pigmentation of turbot (*Scophthalmus maximus*) larvae fed live-prey enriched in Arachidonic and Eicosapentaenoic acids. *Aquaculture* **180**, 321–343.

Evjemo J.O., Coutteau P., Olsen Y. & Sorgeloos P. (1997) The stability of docosahexaenoic acid in two *Artemia* species following enrichment and subsequent starvation. *Aquaculture* **155**, 135–148.

Evjemo J.O., Danielsen T.L. & Olsen Y. (2001) Losses of lipid, protein and n-3 fatty acids in enriched *Artemia franciscana* starved at different temperatures. *Aquaculture* **193**, 65–80.

Evjemo J.O., Reitan K.I. & Olsen Y. (2003) Copepods as live food organisms in the larval rearing of halibut larvae (*Hippoglossus hippoglossus* L.) with special emphasis on the nutritional value. *Aquaculture* **227**, 191–210.

Figueiredo J., van Woesik R., Lin J. & Narciso L. (2009) *Artemia franciscana* enrichment model – How to keep

them small, rich and alive? *Aquaculture* **294**, 212–220.

Folch J., Lees M. & Sloane Stanley H. (1957) A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry* **226**, 497–509.

Fraser A.J., Sargent J.R. & Gamble J.C. (1989) Lipid class and fatty acid composition of *Calanus finmarchicus* (Gunnerus), *Pseudocalanus* sp. and *Temora longicornis* Muller from a nutrient-enriched seawater enclosure. *Journal of Experimental Marine Biology and Ecology* **130**, 81–92.

Furuita H., Takeuchi T., Toyota M. & Watanabe T. (1996) EPA and DHA requirements in early juvenile sea bream using HUFA enriched *Artemia* nauplii. *Fisheries Science* **62**, 246–251.

Garcia A.S., Parrish C.C. & Brown J.A. (2008) Growth and lipid composition of Atlantic cod (*Gadus morhua*) larvae in response to differently enriched *Artemia franciscana*. *Fish Physiology and Biochemistry* **34**, 77–94.

Giner E. (2005) *Desarrollo de emulsiones de alto contenido en ácidos grasos poliinsaturados para complementar el valor nutritivo de presas vivas en acuicultura*, p. 75. Universidad Politécnica de Valencia, Valencia.

Haché R. & Plante S. (2011) The relationship between enrichment, fatty acid profiles and bacterial load in cultured rotifers (*Brachionus plicatilis* L-strain) and *Artemia* (*Artemia salina* strain *franciscana*). *Aquaculture* **311**, 201–208.

Hamre K. & Harboe T. (2008) *Artemia* enriched high n-3 HUFA may give a large improvement in performance of Atlantic halibut (*Hippoglossus* L.) larvae. *Aquaculture* **277**, 239–243.

Han K., Geurden I. & Sorgeloos P. (2000) Enrichment strategies for *Artemia* using emulsions providing differents levels of n-3 highly unsaturated fatty acids. *Aquaculture* **183**, 335–347.

Han K., Geurden I. & Sorgeloos P. (2001) Fatty acid changes in enriched and subsequently starved *Artemia franciscana* nauplii enriched with different essential fatty acids. *Aquaculture* **199**, 93–105.

Han K., Geurden I., Van der Meeren P., Bai S.C. & Sorgeloos P. (2005) Particle size distribution in two lipid emulsion used for the enrichment of *Artemia* nauplii as a function of their preparation method and storage time. *Journal of the World Aquaculture Society* **36**, 196–202.

Harel M., Ozkizilcik S., Lund E., Behrens P. & Place A.R. (1999) Enhanced absorption of docosahexaenoic acid (DHA, 22:6n3) in *Artemia* nauplii using a dietary combination of DHA-rich phospholipids and DHA-sodium salts. *Comparative Biochemistry and Physiology B* **124**, 169–176.

Hochachka P. W. & Somero G. N. (ed.) (1984) *Temperature adaptation. In: Biochemical Adaptations*, pp. 355–449. Princeton University Press, Princeton, NJ.

Hontoria F., Crowe J.H., Crowe L.M. & Amat F. (1994) Potential use of liposomes in larviculture as a delivery system through Artemia nauplii. *Aquaculture* **127**, 255–264.

Koven W.M., Tandler A., Kissil G.W., Sklan D., Friezlander O. & Harel M. (1990) The effect of dietary (*n*-3) polyunsaturated fatty acids on growth, survival and swim bladder development in *Sparus aurata* larvae. *Aquaculture* **91**, 131–141.

Léger P., Bengtson D.A., Simpson K.L. & Sorgeloos P. (1986) The use and nutritional value of *Artemia* as a food source. *Oceanography and Marine Biology: An Annual Review* **24**, 521–623.

Léger P., Bengtson D.A., Sorgeloos P., Simpson K.L. & Beck A.D. (1987) The nutritional value of Artemia: a review: p. 357–372. In: *Artemia Research and its Applications*, Vol. **3** (ed. by P. Sorgeloos, D.A. Bengtson, W. Declier & E. Jaspers), p. 556. Universa Press, Wetteren, Belgium.

Lund I., Steenfeldt S.J. & Hanses B.W. (2007) Effect of dietary arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid on survival, growth and pigmentation in larvae of common sole (*Solea solea* L.). *Aquaculture* **273**, 532–544.

McEvoy L.A., Navarro J.C., Bell J.G. & Sargent J.R. (1995) Autoxidation of oil emulsions during the *Artemia* enrichment process. *Aquaculture* **134**, 101–112.

McEvoy L.A., Navarro J.C., Hontoria F., Amat F. & Sargent J.R. (1996) Two novel *Artemia* enrichment diets containing polar lipid. *Aquaculture* **144**, 339–352.

McEvoy L.A., Navarro J.C., Amat F. & Sargent J.R. (1997) Application of soya phosphatidylcholine in tuna orbital oil enrichment emulsions for *Artemia*. *Aquaculture International* **5**, 517–526.

Monroig O. (2006) Diseño y optimización de liposomas para su uso como sistema de suministro de nutrientes a larvas de peces marinos. PhD thesis, University of Valencia, Spain 239 p.

Monroig O., Navarro J.C., Amat L., González P., Amat F. & Hontoria F. (2003) Enrichment of *Artemia* nauplii in PUFA, phospholipids, and water-soluble nutrients using liposomes. *Aquaculture International* **11**, 151–161.

Monroig O., Navarro J.C., Amat F., González P., Bermejo A. & Hontoria F. (2006a) Enrichment of *Artemia* nauplii in essential fatty acids with different types of liposomes and their use in the rearing of gilthead sea bream (*Sparus aurata*) larvae. *Aquaculture* **251**, 491–508.

Monroig O., Navarro J.C., Amat F., Gonzalez P. & Hontoria F. (2006b) Effect of aeration on the efficiency of *Artemia* enrichment with EFA-rich emulsion and liposomes. *Aquaculture* **257**, 382–392.

Monroig O., Navarro J.C., Amat F., Gonzalez P. & Hontoria F. (2006c) Effects of nauplial density, product concentration and product dosage on the survival of the

nauplii and EFA incorporation during *Artemia* enrichment with liposomes. *Aquaculture* **261**, 659–669.

Monroig O., Navarro J.C., Amat F. & Hontoria F. (2007) Enrichment of *Artemia* nauplii in vitamin A, vitamin C and methionine using liposomes. *Aquaculture* **269**, 504–513.

Monroig O., Webb K., Ibarra-Castro L., Holt G.J. & Tocher D.R. (2011) Biosynthesis of long-chain polyinsaturated fatty acids in marine fish: characterization of an Elovl4-like elongase from cobia *Rachycentron canadum* and activation of the pathway during early life stages. *Aquaculture* **312**, 145–153.

Morais S., Mourente G., Ortega A., Tocher J.A. & Tocher D.R. (2011) Expression of fatty acyl desaturase and elongase genes, and evolution of DHA:EPA ratio during development of unfed larvae of Atlantic bluefin tuna (*Thunnus Thynnus* L.). *Aquaculture* **313**, 129–139.

Navarro J.C., Henderson R.J., McEvoy L.A., Bell M.V. & Amat F. (1999) Lipid conversions during enrichment of *Artemia*. *Aquaculture* **174**, 155–166.

Naz M. (2008) The changes in the biochemical composition and enzymatic activities of rotifer (*Brachionus plicatillis*, Müller) and *Artemia* during the enrichment and starvation periods. *Fish Physiology and Biochemistry* **34**, 391–404.

Reitan K.I., Rainuzzo J.R. & Olsen Y. (1994) Influence of lipid composition of live feed on growth, survival and pigmentation of turbot larvae. *Aquaculture International* **2**, 22–48.

Ries D. (2009) Studies on the antioxidant activity of milk proteins in model oil-in-water emulsions. PhD thesis, Riddet Institute (Massey University), Palmerston North, New Zealand 256pp.

Sargent J.R., McEvoy L.A. & Bell J.G. (1997) Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. *Aquaculture* **155**, 117–127.

Sargent J.R., Tocher D.R. & Bell J.G. (2002) The lipids. In: *Fish Nutrition* (ed. by J.E. Halver & R.W. Hardly), pp. 181–257. Academic Press, San Diego, CA, USA.

Smith G.G., Ritar A.J., Phleger C.F., Nelson M.M., Mooney B., Nichols P.D. & Hart P.R. (2002) Changes in gut content and composition of juvenile *Artemia* after oil enrichment and during starvation. *Aquaculture* **208**, 137–158.

Sokal R.R. & Rohlf F.J. (1981) *Biometry*. Freeman, New York, 859 pp.

Sorgeloos P., Dhert P. & Candreva P. (2001) Use of the brine shrimp, *Artemia* spp., in marine fish larviculture. *Aquaculture* **200**, 147–159.

Southgate P.C. & Lou D.C. (1995) Improving the *n* - 3 HUFA composition of *Artemia* using microcapsules containing marine oils. *Aquaculture* **134**, 91–99.

Sui L., Wille M., Cheng Y. & Sorgeloos P. (2007) The effect of dietary n-3 HUFA levels and DHA/EPA ratios on growth, survival and osmotic stress tolerance of Chinese mitten crab *Eriocheir sinensis* larvae. *Aquaculture* **273**, 139–150.

Tocher D.R. (2003) Metabolism and functions of lipids and fatty acids in teleost fish. *Reviews in Fisheries Science* **11**, 107–184.

Van der Meeren T., Olsen R.E., Hamre K. & Fyhn H. (2008) Biochemical composition of copepods for evaluation of feed quality in production of juvenile marine fish. *Aquaculture* **274**, 375–397.

Van Stappen G. (1996) Introduction, biology and ecology of *Artemia*. In: *Manual on the Production and Use of live Food for Aquaculture*, (ed. by P. Lavens & P. Sorgeloos), FAO Fisheries Technical Paper (361), Roma.

Villalta M., Estévez A. & Bransden M.P. (2005a) Arachidonic acid enriched live prey induces albinism in Senegal sole (*Solea senegalensis*) larvae. *Aquaculture* **245**, 193–209.

Villalta M., Estévez A., Bransden M.P. & Bell J.G. (2005b) The effect of graded concentrations of dietary DHA on growth, survival and tissue fatty acid profile os Senegal Sole (*Solea senegalensis*) larvae during the *Artemia* feeding period. *Aquaculture* **249**, 353–365.

Watanabe T., Ohta M., Kitajima C. & Fujita S. (1982) Improvement of dietary value of brine shrimp *Artemia salina* for fish larvae by feeding them on w3 highly unsaturated fatty acids. *Bulletin of the Japanese Society of Scientific Fisheries* **48**, 1775–1782.

Watanabe T., Kitajima C. & Fujita S. (1983) Nutritional values of live organisms used in Japan for mass propagation of fish: a review. *Aquaculture* **34**, 115–143.

Woods C.M.C. (2003) Effects of varying *Artemia* enrichment on growth and survival of juvenile seahorses, *Hippocampus abdominalis*. *Aquaculture* **220**, 537–548.

Yamamoto Y., Kabeya N., Takeuchi Y., Alimuddin Haga Y., Satoh S., Takeuchi T. & Yoshizaki G. (2010) Cloning and nutritional regulation of polyunsaturated fatty acid desaturase and elongase of a marine teleost, the nibe croaker *Nibea mitsukurii*. *Fisheries Science* **76**, 463–472.

Aquaculture Research

Title

Antioxidant activity and lipid peroxidation in *Artemia* nauplii enriched with a DHA-rich oil emulsion and the effect of adding an external antioxidant based on hydroxytyrosol

Running head

Effect of hydroxytyrosol during *Artemia* enrichment

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Keywords: Antioxidant enzymes, *Artemia*, docosahexaenoic acid, enrichment, hydroxytyrosol, oxidation.

Abstract

Artemia nauplii catabolise polyunsaturated fatty acids (PUFA); in particular, they retroconvert docosahexaenoic acid (DHA, 22:6n-3), so enrichment is a continuous quest towards increasing PUFA through the use of PUFA-rich enrichment products. However, optimal conditions during enrichment (aeration, illumination and temperatures around 28 ºC) tend to accelerate autoxidation of PUFA, and the formation of potentially toxic oxidation products. Water-soluble antioxidants like the polyphenolic compound hydroxytyrosol (3,4-dihydroxyphenylethanol), a polar molecule found in the water fraction resulting after the milling process of olives, arise as promising compounds to prevent oxidation during *Artemia* enrichments. We investigated the antioxidant activity and lipid peroxidation in *Artemia* nauplii during enrichment, and the effect of adding an external antioxidant based on hydroxytyrosol

during the enrichment with a PUFA-rich emulsion (M70). For this purpose, the activity of antioxidant enzymes (catalase, superoxide dismutase, glutathione-S-transferase, glutathione peroxidase), as well as lipid peroxidation, were determined in enriched and unenriched *Artemia* nauplii. To validate antioxidant activity and lipid peroxidation, in a first experiment, nauplii were enriched with microalgae (*Tetraselmis suecica*), yeast (*Saccharomyces cerevisiae*) and M70 emulsion. In a second experiment, enrichment with a commercial emulsion (DC Super Selco), M70, and a combination of M70 and hydroxytyrosol (Hytolive, HYT) added as an external antioxidant was performed. The combination of M70 with HYT produced the best results, in terms of activity of antioxidant enzymes. The analysis of the fatty acids from total lipids showed that the addition of hydroxytyrosol preserved the DHA percentage of enriched nauplii.

1. Introduction

Adequate provision of polyunsaturated fatty acids (PUFA) is important in the diet of marine organisms, in particular during early life stages, where these compounds accumulate in rapidly developing tissues such as brain and eye (Bell et al., 1995, 2003; Benitez-Santana et al., 2007; Tocher, 2010). Thus, enrichment of live preys like *Artemia*, which are naturally deficient of essential PUFA such as docosahexaenoic acid (22:6n-3, DHA), is critical for the survival and normal development of larval

stages of marine organisms, and thus the production of good quality fingerlings (Hamre & Harboe, 2008; Tocher, 2010).

McEvoy et al. (1995) investigated the stability of PUFA in lipid emulsions throughout the enrichment process since optimal enrichment conditions such as vigorous aeration, illumination and temperature of 28 ºC, tend to accelerate autoxidation of PUFA and the formation of potentially toxic oxidation products. It was found that, at later stages of the enrichment process, all the emulsions tested showed autoxidation associated to notable decreases in the concentration of PUFA in the lipids of *Artemia* nauplii, particularly for eicosapentaenoic acid (EPA, 20:5n-3) and DHA. Interestingly, it was reported that autoxidation of enrichment diets occurred even in commercial emulsions that are formulated with antioxidants. Beyond the decrease in the enrichment efficiency described above, the study of autoxidation in enrichment diets is also important because it implies that *Artemia* nauplii can accumulate potentially toxic oxidation products that can compromise the health and survival of fish and crustacean larvae. In fact, McEvoy et al. (1995) recommended that *Artemia* enrichments, particularly those with DHA-rich emulsions, should be shortened so that the alluded to accumulation of potentially toxic compounds in nauplii could be reduced.

We have recently investigated the efficiency of a DHA-rich oil emulsion (M70) as enrichment diet for *Artemia* nauplii

(Viciano et al., 2015). M70 consists of a 1:1 (v/v) oil/water suspension, based on the synthetic oil DHA Algatrium (Brudy Technology, Barcelona, Spain) that contains 70% of total fatty acids as DHA in the form of ethyl esters (Viciano et al., 2015). Additionally, the emulsion M70 is also formulated with Tocobiol Plus (BTSA Biotecnologías Aplicadas, Madrid, Spain), a liposoluble antioxidant. Considering the particular aspects derived from its DHA-rich nature, a series of experiments were carried out to optimise the use of M70 under different enrichment conditions including oxygenation regime, incubation temperature, nauplial density and enrichment product concentration and dosage mode, which have been regarded as important factors determining the enrichment efficiency (Van Stappen, 1996; Han et al., 2000, 2001; Monroig et al., 2006a, b; Sui et al., 2007; Hamre & Harboe, 2008; Figueiredo et al., 2009). Importantly, our results suggested that, despite the likely preservative action of the liposoluble antioxidant Tocobiol Plus, oxidation of DHA contained in M70 could not be ruled out as suggested by relatively low DHA incorporation in *Artemia* nauplii (Viciano et al., 2015).

Pro-oxidative conditions during enrichment could partly explain massive mortalities events reported in the literature during enrichments (Monroig et al., 2006b), since they can alter the capacity of live preys for catalyzing oxidative reactions, leading to the production of reactive oxygen species (ROS) as a

general pathway of toxicity causing oxidative stress (Livingstone, 2001; Barata et al., 2005b). ROS include superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) (Correia et al., 2003), compounds that are involved in cellular damage and with the capacity to promote morphological and physiological failures and peroxidation of PUFA, all this leading to pathologies and compromising normal development (Tovar-Ramirez et al., 2010). To minimize oxidative damage to cellular components, organisms have developed key antioxidant enzyme defences that can be used as biomarkers (Barata et al., 2005a, b).

Water-soluble antioxidants appear to have potential for preventing DHA oxidation during *Artemia* enrichments. In a number of studies, the polyphenolic compound hydroxytyrosol (3,4-dihydroxyphenylethanol) (Hao et al., 2010), has been shown to be more efficient than antioxidant vitamins and synthetic antioxidants (Gordon et al., 2001; González, 2005; Deiana et al., 2008; Fernández-Bolaños et al., 2008). Hydroxytyrosol is a polar molecule found in the water fraction resulting after the milling process of olives (Schaffer et al., 2007). Several studies have investigated the potent activity of hydroxytyrosol to neutralize free radicals and slow oxidative stress (Chimi et al., 1991; Visioli et al., 1998, 2000). To the best of our knowledge, there are no data available concerning its

potential role as protective agent of oil emulsions used for live prey enrichments.

The global aim of the present study was to investigate the protective effects of the addition of an external water-soluble natural olive fruit extract, rich in the polyphenol hydroxytyrosol (HYT, Hytolive Protect, Genosa I+D S.I., Malaga, Spain), to the enrichment of *Artemia* nauplii with a DHA-rich oil emulsion (M70). For this purpose, key enzymes (superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase) as biomarkers of antioxidant activity, and the lipid peroxidation of enriched *Artemia* nauplii were determined. To the best of our knowledge, such an approach has never been used before to study the effects of oxidation during enrichment. Two experiments were performed. Experiment 1 aimed at establishing the baseline of lipid oxidation in the enrichment of *Artemia* nauplii. We analyzed the antioxidant activity and lipid peroxidation of *Artemia* nauplii enriched with M70, and compared them with those of nauplii enriched with yeast *Saccharomyces cerevisiae* or the green microalgae *Tetraselmis suecica*, as well as those of non-enriched nauplii. Yeast and green microalgae are known to have antioxidant properties (Greetham & Grant, 2009; Lee et al., 2009). Experiment 2, aimed at studying the effect of the external antioxidant HYT during enrichment with high PUFA emulsions. For this purpose we determined the antioxidant activity, lipid peroxidation and

fatty acid composition of: a) non-enriched nauplii, used as a control; b) nauplii enriched with a commercial emulsion (DC Super Selco, DCSS, Inve, Dendermonde, Belgium), as reference of conventional enrichment; c) nauplii enriched with M70; and d) nauplii enriched with M70 plus the water-soluble antioxidant HYT.

2. Materials and methods

2.1 Experimental design

Artemia nauplii were obtained from the hatching of EG grade cysts (Inve, Dendermonde, Belgium). After an incubation period of 23 h at 28 ºC, nauplii were collected and rinsed with tap water to remove the hatching debris and metabolites. Newly hatched nauplii were thereafter placed in 1 L cylinder-conical vessels containing seawater.

The *Artemia* enrichments were performed for 21 h with incubation temperatures of 28 ± 1 ºC, low/moderate aeration (1 L min^{-1}), the air diffusion system consisting of a 25 cm long and 0.5 cm-diameter section glass tube applied from the bottom of the vessel. Nauplial densities were 300 nauplii mL^{-1} (Monroig et al., 2006a, b), and illumination was 1500 lux (Viciano et al., 2015). The temperature was kept (±1 ºC) constant by placing the hatching and enrichment vessels in a thermostatic bath. The enrichment product was dispensed in a single dose at the beginning of the incubation.

All the enrichment treatments were run in triplicates (n=3). After an enrichment period, samples of *Artemia* nauplii were collected by filtering the enrichment medium through a 100 µm mesh carefully washed with tap water and subsequently rinsed with distilled water.

Experiment 1: Baseline of M70 oxidation

Antioxidant activity and lipid peroxidation levels were determined in *Artemia* nauplii enriched with: a) 0.6 g L^{-1} *Saccharomyces cerevisiae* (fresh yeast (Levital, Lesaffre Ibérica S.A., Valladolid, Spain) dispersed in sea water using a mixer for 2 minutes), b) *Tetraselmis suecica* (algal culture stock from IATS facilities, at 300000 ± 20000 cells mL^{-1}), c) 0.8 g L^{-1} M70 emulsion (dispersed in sea water using a mixer for 2 minutes in a single dose at the beginning of the enrichment period), and d) a control group consisting of unenriched *Artemia* nauplii kept in the same conditions.

Experiment 2: External antioxidant

Antioxidant activity, fatty acid profile and lipid peroxidation of *Artemia* nauplii enriched with 0.6 g L^{-1} commercial emulsion DCSS, 0.8 g L^{-1} M70 emulsion, 0.8 g L^{-1} M70 in combination with 0.04 g L^{-1} of exogenously added water-soluble antioxidant HYT (hereafter named M70+HYT treatment) and a control group of unenriched *Artemia* nauplii were compared. All enrichment media were dispersed in sea water using a domestic blender for 2 min.

2.2 Sample preparation for antioxidant enzymes

After enrichment, nauplii were filtered through a 100 µm mesh, washed with distilled water and kept at -80 ºC until analysis. Samples were processed as soon as possible and always within 3 h of the end of the enrichment period to avoid possible oxidation during the storage. *Artemia* samples were homogenized in a 1:4 wet weight: buffer volume ratio in 100 mM phosphate buffer, pH 7.4 at 4 ºC, containing 100 mM KCl and 1 mM ethylenediaminetetrasacetic acid (EDTA). Homogenates were further centrifuged at 10000 g for 10 min, and supernatants were immediately used as enzyme sources. Supernatant proteins were measured by the Bradford method (Bradford, 1976) using bovine serum albumin as standard.

2.3 Enzymatic activities

The following enzymatic activities were measured in *Artemia* homogenates: Superoxide dismutase, SOD (EC 1.15.1.1, converts O_2^- to H_2O_2); catalase, CAT (EC 1.11.1.6, reduces H_2O_2 to water); glutathione peroxidise, GPx (EC 1.11.1.9, detoxifies H_2O_2 or organic hydroperoxides produced e.g. by lipid peroxidation); and Glutathione-S-transferase, GST (EC 2.5.1.18, plays a role preventing oxidative damage by conjugating breakdown products of lipid peroxides to glutathione (GSH)) (Barata et al., 2005a, b; Tovar-Ramirez et al., 2010).

All the enzymatic assays were measured as described by Barata et al. (2005a, b). The final results for enzymatic activities

were normalized by protein content. Assays were run at least in duplicate.

2.4 Lipid peroxides

Another factor affecting the potential oxidative damage is the level of target molecules like PUFA, which are easily oxidized by ROS in lipid peroxides (Di Giulio et al., 1995). Lipid peroxidation was evaluated analysing the lipid peroxidation levels (LPO). Malondialdehyde (MDA) is a common substance produced in lipid peroxidation. LPO method is based in the reaction of MDA with N-methyl-2-phenylindole (NMPI) to form a coloured complex with known absorbance (586 nm) (Esterbauer et al., 1991). By this method the interference from other lipid peroxidation products (such as 4-hydroxyalkenals) is minimized.

2.5 Fatty acid analysis

Fatty acid analyses were performed to check any protective effect of the external antioxidant (mainly on PUFA). Thus, in Experiment 2, two samples of each replicate of every treatment (*Artemia* enriched with the different diets) were obtained, one was assigned to the analysis of enzymatic activity (processed as specified above), and the other was freeze-dried to proceed with the analysis of total lipids and fatty acids. Total lipids were extracted following the method of Folch et al. (1957), and analyses of fatty acids were carried out following the methods described in Viciano et al. (2015).

2.6 Statistical analysis

Analytical data were expressed as means ± standard deviation (SD) (n=3). When more than two means were compared, differences with control group were analysed by Dunnett test (Dunnett, 1964). Dunnett test is a multiple comparison procedure and is performed by computing a Student t-statistic for each experimental or treatment group, where the statistic compares the treatment group to a single control group. When significance was $P \leq 0.05$, means were considered statistically different. For pair-wise comparisons a Student t test was used.

Enzyme activities analytical data, and highly unsaturated fatty acids (HUFA), fatty acids of 20 or more atoms of carbon and two or more double bonds, which are considered to have the highest oxidative potential (Barata et al., 2005c), were included as variables in multivariate Principal Component Analyses (PCA), to highlight the effects of the different treatments on the patterns of enzyme activities and on the fatty acid profiles composition. With such a parsimonious approach, the dataset of variables is reduced into a smaller set of factors or components. Parsimony is achieved by explaining the maximum amount of common variance in a correlation matrix using the smallest number of explanatory concepts. Factors are statistical entities that can be visualized as classification axes along which measurement variables can be plotted, providing an idea of their correlation with the corresponding factor (loading). Score plots are a graphical representation of individual (treatment groups)

scores in the new subset of measurement variables (factors). They illustrate the relationship among individual cases (treatment groups), and the variables, and help in the analysis of data by showing graphical display. Statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA).

3. Results

Experiment 1: Baseline of M70 oxidation

Figure 1 illustrates the levels of enzymatic activities and LPO measured in the control group and in the *Artemia* nauplii enriched with different treatments. The *Tetraselmis* group was not significantly different from control group in any of the enzymatic activities or LPO measured. Yeast treatment presented lower activities of SOD, GPx-Tot and GPx-Se, but a high GST activity. M70 group showed a decreased activity of SOD and GPx-Tot and a significant ($P<0.05$) increase, near two-fold relatively to control animals, in LPO.

The component plot (Figure 2a) obtained from PCA shows variables that were responsible for separation along the two principal components. The first two components of the PCA explained more than 68% of total variance (43.9% and 24.2% for the first and second component, PC1 and PC2, respectively). The factor score plot (Figure 2b) showed groups separated in the second component on the basis of lipid peroxidation. M70 treatment appeared to be related with high loads of LPO and

clearly separated from the other treatments. Control and *Tetraselmis* treatments appeared very close to each other and on the opposite side of M70 treatment, indicating less lipid peroxidation, and higher activities of GPx-Tot, GPx-Se and SOD than M70. The Yeast treatment was related to high GST activity. This graph allowed us to establish the baseline of antioxidant activity for the M70 emulsion, and to investigate the effect of including an external antioxidant into the enrichment media.

Experiment 2: External antioxidant

Figure 3 shows the enzyme activities and levels of LPO of the control and enriched *Artemia* nauplii with and without the antioxidant HYT. Observed patterns of antioxidant enzyme activities and LPO levels for M70 versus controls were quite similar to those reported in Figure 1, except for GST. *Artemia* nauplii incubated with the two tested enriched media (DCSS and M70) showed similar enzymatic activity and LPO patterns. The addition of the antioxidant HYT decreased the activities of CAT and GST, and increased those of GPx-Tot of *Artemia* nauplii enriched with M70.

LPO were significantly ($P<0.05$) higher in nauplii enriched with DCSS and M70 treatments. Adding an external antioxidant (HYT) dramatically reduced LPO.

Table 1 lists the main fatty acids (% of total fatty acids) from total lipids of *Artemia* nauplii enriched with different treatments. EPA values were significantly ($P<0.05$) higher in DCSS treatment (12.2 ± 2.6) compared to all other treatments. The n-3 HUFA increased in all treatments when compared with the control group, but n-6 HUFA were only significantly ($P<0.05$) higher in M70+HYT treatment. DHA levels were significantly ($P<0.05$) higher in M70+HYT treatment compared to other treatments, and this result is reflected in the highest DHA/EPA ratio among all treatments.

Figure 4a illustrates the component plot for the enzymatic activity and HUFA data. The two first components of PCA accounted for the 66.9 % of variation, PC1 explaining 45.0% and PC2 explaining 21.9% of the variation of the data set. Figure 4b shows the factor score plot based on enrichment and antioxidant scores from the constituent variables. All treatments were clearly separated from each other, with control treatment to the left, DCSS and M70+HYT in the middle, and M70 to the right. The PCA grouping indicates that M70 treatment has higher LPO than the other treatments. Figure 4 also shows that the scores of M70+HYT treatment were associate to both n-3 and n-6 HUFA variables pointing at the protective role of HYT during enrichment.

Analysing the PCA results of both experiments, the M70 group is always linked to lipid peroxidation as evidenced by the

association of the scores to the location of the LPO variable in the components graph. The association is lost by adding HYT to the enrichment. The separation of the two groups (M70 and M70+HYT) was achieved, with the LPO values of M70+HYT treatment approaching those of the commercial emulsion DCSS and control group.

4. Discussion

High levels of DHA are important in the development of nervous and sensory system during early development of vertebrates, including fish (Hamre & Harboe, 2008; Tocher, 2010). For that reason, enrichment of live preys including *Artemia* that naturally lack DHA is critical for the successful rearing of fish larvae, particularly those from marine species (Tocher, 2010). DHA levels achieved in *Artemia* nauplii with the M70 and M70+HYT treatments were different. The DHA/EPA ratio is a common biochemical parameter used to evaluate the nutritional suitability of diets for marine finfish larviculture (Evjemo et al., 1997), and high DHA/EPA ratios were also reported as important to promote growth, stress resistance and pigmentation (Mourente et al., 1993; Reitan et al., 1994; Sorgeloos et al., 2001). Rodríguez et al. (1997) observed significantly higher growth rates in sea bream larvae fed rotifers with a DHA/EPA ratio of 1.5 compared to those fed DHA/EPA ratio < 0.6. In natural preys such as copepods, this ratio is around 2.0 (Fraser et al., 1989; Sorgeloos et al., 2001; Van der

Meeren et al., 2011). Currently satisfactory results in an enrichment process with *Artemia* nauplii is achieved with DHA/EPA ratios of 2 and higher (Dhert et al., 1993; Sorgeloos et al., 2001) but, on the other hand, this has been acknowledged as a major challenge due to an apparent inability of *Artemia* nauplii to retain DHA (Evjemo et al., 1997). In fact, *Artemia* nauplii actively retroconvert DHA to EPA during and after enrichment (Navarro et al., 1999). Thus, enrichment of *Artemia* nauplii is a continuous quest towards increasing PUFA and DHA, and despite the use of high DHA emulsions like M70 which consistently produces *Artemia* DHA/EPA ratios higher than 1 (Viciano et al., 2015), the fact that it only produces moderate levels of DHA in the enriched nauplii (5-8 % of total fatty acids) illustrates the problems of DHA retroconversion in *Artemia*.

Artemia nauplii possess antioxidant enzymes required to metabolize O_2^- (SOD), H_2O_2 (CAT and GPx) and organic hydroperoxides activity (GPx-Se) (Nunes et al., 2006). These enzymes allow the organism to prevent, intercept and repair the damage caused by the free radicals (Olsen et al., 2013). However, during live feed enrichments, it is possible that these endogenous protective mechanisms are not sufficient to prevent oxidative damage as HUFA contained in the enrichment diet are very prone to oxidation, particularly under physical-chemical conditions set up during the enrichment procedures (McEvoy et al., 1995; Monroig et al., 2007), thus contributing to the low

efficiency in HUFA enrichment in general and DHA in particular.

SOD is the first defence mechanism in the detoxification process and, along with CAT, is regarded as playing an important antioxidant role in aquatic invertebrates (Livingstone, 1991; Barata et al., 2005b). In Experiment 1, SOD activity was inhibited in the nauplii enriched with yeast and M70, in comparison with those enriched with *Tetraselmis*, and the control group (unenriched nauplii). These differences can be explained by SOD *modus operandi*, with SOD activity increasing in early stages of oxidative stress when ROS abundance increases. If the oxidative stress persists, SOD becomes depleted and unable to cope with free radicals (Lukaszewicz-Hussan & Moniuszko-Jakoniuk, 2004). This is consistent with increased LPO levels achieved in *Artemia* nauplii enriched with M70 and yeast in Experiment 1. In Experiment 2 differences in SOD activity were not observed, although lipid peroxidation in M70 treatment was very high. Inter experiment difference in SOD supports the previous argument that antioxidant enzyme activities are transient (Livingstone, 1991; Barata et al., 2005b).

The response to oxidative stress has been related to increased CAT activities (Tovar-Ramirez et al., 2010) and therefore, the activity of this enzyme is expected to increase with the presence of substrates that enhance or produce ROS (Barata et al., 2005a; Tovar-Ramirez et al., 2010). In this study,

CAT activity in *Artemia* nauplii from the control group remained at low levels, increasing in those nauplii enriched with yeast, *Tetraselmis*, DCSS or M70 emulsion. Nauplii enriched with a combination of the DHA-rich emulsion M70 and the antioxidant Hytolive (HYT) treatment showed significantly lower CAT activities than those enriched with the same emulsion (M70) without antioxidant, and a commercial emulsion (DCSS). Clearly, these results strongly suggest the protective effect of Hytolive added to the enrichment diet. CAT and GPx have complementary roles in the detoxification of hydrogen peroxides, but have different subcellular localization (peroxisomal and cytosolic, respectively) and affinity for H_2O_2 levels. Thus, high levels of H_2O_2 produce an increased affinity for CAT, whereas low levels of H_2O_2 produce an increased affinity for GPx (Orbea et al., 2000; Barata et al., 2005b). Interestingly, GPx activity (GPx-Tot and GPx-Se) reached the highest values in unenriched nauplii (control). On the contrary, *Artemia* nauplii enriched with M70 showed low activity values for both GPx enzymes, suggesting high levels of H_2O_2 and oxidative stress. Addition of the antioxidant HYT increased the GPx activity in nauplii enriched with M70 and, together with the decreased CAT activity mentioned above, made the nauplii enriched with M70+HYT exhibit the best antioxidant status among all enrichment diets tested and most closely resembled the control *Artemia*. These results clearly indicate that the Hytolive exerts an effective antioxidant effect during *Artemia*

enrichment and, in addition to lipophilic antioxidants that are commonly used in the formulation of enrichment diets, inclusion of water-soluble antioxidants may also help live preys to cope with exposure to toxic oxidized compounds.

GSTs are a family of Phase II detoxification enzymes that catalyze the conjugation of glutathione (GSH) to a wide variety of endogenous and exogenous electrophilic compounds (Townsend & Tew, 2003). GST activity detoxifies endogenous compounds such as peroxidized lipids and enables the breakdown of xenobiotics (Sherrat & Hayes, 2001). GST activity was higher than the experimental control in nauplii enriched with yeast, DCSS, M70 (Expt. 2) and M70+HYT treatment. This is consistent with previous reports showing that GST activity increases in response to exogenous chemical sources including those promoting ROS (Barata et al., 2005b). Increased GST activity indicates indirect conjugation to its endogenous substrate during Phase II detoxification, which would help eliminate toxic metabolites (Townsend & Tew, 2003). Phenolic compounds from olive oil can restore redox balance by free radical quenching (El-Azem, 2013). By neutralizing the free radicals formed, the amount of antioxidant enzymes such as GSH is preserved in the body (Masella et al., 2004; El-Azem, 2013). The previous effect may explain the slight but significant reduction of GST in *Artemia* nauplii co-exposed to M70 and HYT.

Because ROS have extremely short half-lives, they are difficult to measure directly and therefore oxidative stress can be inferred through measurement of several products of the damage produced (Pryor, 1991). Lipid peroxidation (LPO) can be considered an index of oxidative damage to lipids produced by toxic compounds (Fernández et al., 2012). Results from this study showed that *Artemia* nauplii enriched with M70 and DCSS emulsions presented significantly higher LPO levels than those supplemented with HYT and the control group.

It is reasonable to assume that lipid peroxides and other potentially toxic oxidation products are filtered by *Artemia* nauplii during the enrichment procedure (McEvoy et al., 1995). *Artemia* enrichment is largely regarded as a "bioencapsulation" process whereby the *Artemia* ingest enrichment diet particles until the gut is full (Figueiredo et al., 2009). The supplementation of M70 enrichments with HYT was hypothesised to decrease the lipid peroxidation and therefore the presence of oxidized lipid available in the enrichment media. Consequently, the PCA results strongly suggest that such a protective action from HYT took place, as the HYT-supplemented treatment clearly separated from the M70 group, the latter containing higher levels of the oxidation biomarker LPO. There are no previous studies using hydroxytyrosol on live prey enrichment. In humans, hydroxytyrosol is rapidly absorbed in a dose-dependent manner in the small intestine and colon by bidirectional passive diffusion (El-Azem, 2013), and then is

mostly conjugated as glucuronoconjugates, the most abundant form found in plasma. From this conjugated form, it displays its antioxidant potential. Hydroxytyrosol is rapidly metabolized and, after five minutes of administration, it is possible to find derivatives in plasma (Miro-Casas et al., 2003). McEvoy et al. (1995) demonstrated autoxidation in three enrichment emulsions when *Artemia* were present, however the oil emulsions in the control media without *Artemia* did not show autoxidation within the experimental period. It is expected that once introduced into the enrichment media, with the brine shrimp present, the protective effect of hydroxytyrosol begins due to its water soluble nature, protecting against oxidation of the fatty acids. Thereby oxidation may be reduced before the fatty acids entrance in the digestive tract of *Artemia*, ensuring that the enzymatic system is not oversaturated and remains active. This is shown in the improvement of the enzymatic activity when hydroxytyrosol was added in the enrichment medium.

In conclusion, the addition of a natural water-soluble olive fruit extract with a high amount of hydroxytyrosol to the lipid emulsion enrichment improves the antioxidant defence of *Artemia* nauplii and decreases the levels of the oxidative biomarker LPO. Moreover, the addition of this olive fruit extract makes the activity of all antioxidant enzymes in *Artemia* enriched with a DHA-rich oil emulsion resemble those of homeostasis as defined by unenriched nauplii. Such an antioxidant protective role of hydroxytyrosol is reflected in the

lipid profile of *Artemia*, since higher levels of DHA and a higher DHA/EPA ratio were found in nauplii enriched in the presence of hydroxytyrosol. As a first approach, here, only a concentration of 0.04 g L^{-1} was tested and, although the benefits prove significant, probably higher concentrations would produce even better results, since there is evidence of an inhibitory effect of HYT in LPO at increasing concentrations (Gargouri et al., 2011). Thus further dose dependent studies are advisable in order to establish if the antioxidant potential can be further enhanced and to verify how this improvement may be applicable in large-scale enrichments.

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6. Bibliography

Barata, C., Lekumberri, I., Vila-Escalé, M., Prat, N., Porte, C., 2005a. Trace metal concentration, antioxidant enzyme activities and susceptibility to oxidative stress in the tricoptera larvae *Hydropsyche exocellata* from the Llobregat river basis (NE Spain). Aquatic Toxicology 74, 3-19.

Barata, C., Varo, I., Navarro, J.C., Arun, S., Porte, C., 2005b. Antioxidant enzyme activities and lipid peroxidation in the freshwater cladoceran *Daphnia magna* exposed to redox cycling compounds. Comparative Biochemistry and physiology Part C: Toxicology & pharmacology 140, 175-186.

Barata, C., Navarro, J.C., Varo, I., Riva, M.C., Arun, S., Porte, C., 2005c. Changes in antioxidant enzyme activities, fatty acid composition and lipid peroxidation in *Daphnia magna* during the aging process. Comparative Biochemistry and Physiology Part B 140, 81-90.

Bell, M.V., Batty, R.S., Dick, J.R., Fretwell, K., Navarro, J.C., Sargent, J.R., 1995. Dietary deficiency of docosahexaenoic acid impairs vision at low light intensities in juvenile herring (*Clupea harengus* L.). Lipids 30, 443-449.

Bell, J.G., McEvoy, L.A., Estévez, A., Shields, R.J., Sargent, J.R., 2003. Optimising lipid nutrition in first-feeding flatfish larvae. Aquaculture 227, 211-220.

Benítez-Santana, T., Masuda, R., Juárez Carrillo, E., Ganuza, E., Valencia, A., Hernández-Cruz, C.M, Izquierdo, M.S., 2007. Dietary n-3 HUFA deficiency induces a reduced visual response in gilthead seabream *Sparus aurata* larvae. Aquaculture 264, 408-417.

Bradford, M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry 72, 248-254.

Chimi, H., Cillard, J., Cillard, P., Rahmani, M., 1991. Peroxyl and hydroxyl radical scavenging activity of some natural phenolic antioxidants. JAOCS 68, 307-312.

Correia, A.D., Costa, M.H., Luis, O.J., Livingstone, D.R., 2003. Age-related changes in antioxidant enzyme activities, fatty acid composition and lipid peroxidation in whole body *Gammarus locusta* (Crustacea: Amphipoda). J. Exp. Mar. Biol. and Ecol. 289, 83-101.

Deiana, M., Incani, A., Rosa, A.,Corona, G., Atzeri, A., Loru, D., Melis, M.P., Dessi, A., 2008. Protective effect of hydroxytyrosol and its metabolite homovanillic alcohol on H_2O_2 induced lipid peroxidation in renal tubular epitelial cells. Food and Chem. Toxixology 46, 2984-2990.

Dhert, P., Sorgeloos, P., Devresse, B., 1993. Contributions towards a specific DHA enrichment in the live food *Brachionus plicatilis* and *Artemia* sp. In: Reinertsen, H., Dahle, L.A., Jorgensen, L., Tvinnereim, K. (Eds.), Fish Farming Technology. Balkema, Rotterdam, Netherlands, pp. 109-115.

Di Giulio, R.T., Benson, W.H., Sanders, B.M., Van Veld, P.A., 1995. Biochemical mechanisms: metabolism, adaptation and toxicity. In: Rand, G. (Ed.), Fundamentals of aquatic toxicology, effects, environmental fate and risk assessment, Taylor & Francis, London, pp. 523-561.

Dunnett, C.W., 1964. New tables for multiple comparisons with a control. Biometrics 20, 482-491.

El-azem, N., 2013. Efecto del hidroxitirosol sobre la cardiotoxicidad producida por doxorubicina. PhD. Universidad de Granada. Pp. 202.

Esterbauer, H., Schaur, R.J., Zollner, H., 1991. Chemistry and biochemistry of 4-hydroxynonenal malonaldehyde and related aldehydes. Free radic. Biol. Med. 11, 81-128.

Evjemo, J.O., Coutteau, P., Olsen, Y., Sorgeloos, P., 1997. The stability of docosahexaenoic acid in two *Artemia* species following enrichment and subsequent starvation. Aquaculture 155, 135-148.

Fernández, B., Campillo, J.A., Martínez-Gómez, C., Benedicto, J., 2012. Assessment of the mechanisms of detoxification of chemical compounds and antioxidant enzymes in the digestive gland of mussels, *Mytilus galloprovincialis*, from Mediterranean coastal sites. Chemosphere 87, 1235-1245.

Fernández-Bolaños, J.G., López, Ó., Fernández-Bolaños, J., Rodríguez-Guitiérrez, G., 2008. Hydroxytyrosol and derivates: Isolation, synthesis, and biological properties. Curr. Org. Chem. 12, 442-463

Figueiredo, J., Van Woesik, R., Lin, J., Narciso, L., 2009. *Artemia franciscana* enrichment model – How to keep them small, rich and alive? Aquaculture 294, 212-220.

Folch, J., Lees, M., Sloane Stanley, H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509.

Fraser, A.J., Sargent, J.R., Gamble, J.C., 1989. Lipid class and fatty acid composition of *Calanus finmarchicus* (Gunnerus), *Pseudicalanus* sp. and *Temora longicornis* Muller from a nutrient-enriched seawater enclosure. J. Exp. Mar. Biol. Ecol. 130, 81-92.

Gargouri, B., ben Mansour, R., Jmal, M.M., Boujelben, R.N., Allouch, N., Lassoued, S., Hamden, K. 2011. Hydroxytyrosol supplementation inhibits oxidative DNA damage, suppresses protein-lipid oxidation and modulates antioxidant enzymes in human peripheral blood lymphocytes. J. Medicinal Plants Research 5, 4863-4869.

González, M.P., 2005. Estudio de los efectos cardiovasculares y la absorción oral del hidroxitirosol en modelos animales y humanos. Ph D. University of Granada, Spain. 201 pp.

Gordon, M.H., Paiva-Martins, F., Almeida, M., 2001. Antioxidant activity of hydroxytyrosol acetate compared with that of other olive oil polyphenols. J. Agric. Food Chem. 49, 2480-2485.

Greetham, D. & Grant, C.M., 2009. Antioxidant activity of the yeast mitochondrial one-cys peroxidation is depedent on thioredoxin reductase and glutathione in vivo. Mol. Cell. Boil. 29: 3229-40

Hamre, K. & Harboe, T., 2008. *Artemia* enriched with high n-3 HUFA may give a large improvement in performance of Atlantic halibut (*Hippoglossus hippoblossus* L.) larvae. Aquaculture 277, 239-243

Han, K., Geurden, I., Sorgeloos, P., 2000. Enrichment strategies for *Artemia* using emulsions providing different levels of n-3 highly unsaturated fatty acids. Aquaculture 183, 335-347.

Han, K., Geurden, I., Sorgeloos, P., 2001. Fatty acid changes in enriched and subsequently starved *Artemia franciscana* nauplii enriched with different essential fatty acids. Aquaculture 199, 93-105.

Hao, J., Shen, W., Yu, G., Jia, H.,Li, X., Feng, Z., Wang, Y., Weber, P., Wertz, Z., Sharman, E, Liu, J., 2010. Hydroxytyrosol promotes mitochondrial biogenesis and mitochondrial function in 3T3-L1 adypocites. J. Nutr. Biochem. 21: 634-44.

Lee, S., Chang, D., Lee, B., Jeon, Y., 2009. Antioxidant activity of solubilized *Tetraselmis suecica* and *Chlorella ellipsoidea* by enzymatic digest. J. Food Sci. and Nutrition 14, 21-28.

Livingstone, D.R., 1991. Organic xenobiotics metabolism in marine invertebrates. Adv. Comp. Environ. Physiol. 7, 45-185.

Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Marine pollution bulletin 42, 656-666.

Lukaszewicz-Hussain, A. & Moniuszko-Jakoniuk, J., 2004. Liver catalase, glutathione peroxidase and reductase activity, reduced glutathione and hydrogen peroxide levels in acute intoxication with chlorfenviphos, an organophosphate insecticide. Pol. J. Environ. Studies 13, 303-309.

Masella, R., Varí, R., D'Archivio, M., Di Benedetto, R., Matarrese, P., Malorni, W., Scazzocchio, B., Giovannini, C., 2004. Extra virgin olive oil biophenols inhibit cell-mediated oxidation of LDL by increasing the mRNA transcription of glutathione-related enzymes. J. Nutr. 134, 785-791.

McEvoy, L.A., Navarro, J.C., Bell, J.G., Sargent, J.R., 1995. Autoxidation of oil emulsions during the *Artemia* enrichment process. Aquaculture 134, 101-112.

Miro-Casas, E., Covas, M.I., Farre, M., Fito, M., Ortuño, J., Weinbrenner, T., Roset, P., de la Torre, R., 2003. Hydroxytyrosol disposition in humans. Clin. Chem. 49, 945-952.

Monroig, Ó., Navarro, J.C., Amat, F., González, P., Bermejo, A., Hontoria, F., 2006a. Enrichment of *Artemia* nauplii in essential fatty acids with different types of liposomes and their use in the rearing of gilthead sea bream (*Sparus aurata*) larvae. Aquaculture 251, 491-508.

Monroig, Ó., Navarro, J.C., Amat, F., González, P., Hontoria, F., 2006b. Effect of aeration on the efficiency of *Artemia* enrichment with EFA-rich emulsion and liposomes. Aquaculture 257, 382-392.

Monroig, Ó, Navarro, J.C., Amat, F., González, P., Hontoria, F., 2007. Oxidative stability and changes in the particle size of liposomes used in the *Artemia* enrichment. Aquaculture 266, 200-210.

Mourente, G., Rodríguez, A., Sargent, J.R., 1993. Effects of dietary docosahexaenoic acid (DHA; 22:6*n*-3) on lipid and fatty acid composition and growth in gilthead sea bream (*Sparus aurata* L.) larvae during first feeding. Aquaculture 112, 79-98.

Navarro, J.C., Henderson, R.J., McEvoy, L.A., Bell, M.V., Amat, F., 1999. Lipid conversion during enrichment of *Artemia*. Aquaculture 174, 155-166.

Nunes, B., Carvalho, F., Guilhermino, L., 2006. Effects of widely used pharmaceuticals and a detergent on oxidative stress biomarkers of the crustacean *Artemia parthenogenetica*. Chemosphere 62, 581-594.

Olsen, E.K., Hansen, E., Isaksson, J., Andersen, J.H., 2013. Cellular antioxidant effect of four bromophenols from the red algae, *Vertebrata lanosa*. Mar. Drugs 11, 2769-2784.

Orbea, A., Fahimi, H.D., Cajaraville, M.P., 2000. Immunolocalization of four antioxidant enzymes in digestive glands of mollusks and crustaceans and fish liver. Histochem. Cell. Biol. 114, 393-404.

Pryor, W., 1991. The antioxidant nutrients and disease prevention – What do we know and what do we need to find out?. Am. J. Clin. Nutr 53, 391S-393S.

Reitan, K.I., Rainuzzo, J.R., Olsen, Y., 1994. Influence of lipid composition of live feed on growth, survival and pigmentation of turbot larvae. Aquacult. Int. 2, 33-48.

Rodríguez, C., Perez, J.A., Diaz, M., Izquierdo, M.S, Fernández-Palacios, H., Lorenzo, A., 1997. Influence of EPA/DHA ratio in rotifers on gilthead seabream (*Sparus aurata*) larval development. Aquaculture 150, 77-89.

Schaffer, S., Podstawa, M., Visioli, F., Bogani, P., Müller, W.E., Eckert, G.P., 2007. Hydroxytyrosol-rich olive mill wastewater extract protects brain cells in vitro and ex vivo. J. Agric. Food Chem. 55, 5043-5049.

Sherrat, P.J & Hayes, J.D., 2001. Glutathione-S-transferases. In: Ioannides, C. (Ed.), Enzyme system that metabolise drugs and other xenobiotics. John Wiley & Sons Ltd. pp. 319-352.

Sorgeloos, P., Dhert, P., Candreva, P., 2001. Use of the brine shrimp *Artemia* spp., in marine fish larviculture. Aquaculture 200, 147-159.

Sui, L., Wille, M., Cheng, Y., Sorgeloos, P., 2007. The effect of dietary n-3 HUFA levels and DHA/EPA ratios on growth, survival and osmotic stress tolerance of Chinese mitten crab *Eriocheir sinensis* larvae. Aquaculture 273, 139-150.

Tocher, D., 2010. Fatty acid requirements in ontogeny of marine and freshwater fish. Aquacult. Res. 41, 717-732.

Tovar-Ramirez, D., Mazurais, D., Gatesoupe, J.F., Quazuguel, P., Cahu, C.L., Zambonino-Infante, J.L., 2010. Dietary probiotic live yeast modulates antioxidant enzyme activities and gene expression of sea bass (*Dicentrarchus labrax*) larvae. Aquaculture 300, 142-147.

Townsend, D.M. & Tew, K.D., 2003. The role of glutathione-S-transferase in anti-cancer drug resistance. Oncogene 22, 7369-7375.

Van der Meeren, T., Olsen, R.E., Hamre, K., Fyhn, H., 2011. Biochemical composition of copepods for evaluation of feed quality in production of juvenile marine fish. Aquaculture 274, 375-397.

Van Stappen, G., 1996. Introduction, biology and ecology of *Artemia*. In: Manual on the production and use of live food for Aquaculture, (ed. by P. Lavens & P. Sorgeloos), FAO Fisheries Technical Paper (361), Roma.

Viciano, E., Monroig, Ó., Salvador, A., Amat, J., Fiszman, S, Navarro; J.C., 2015. Enriching *Artemia* nauplii with a high DHA-containing lipid emulsion: search for an optimal protocol. Aquaculture Research 46, 1066-1077.

Visioli, F., Bellomo, G., Galli, C., 1998. Free radical-scavenging properties of olive oil polyphenols. Biochem. Biophys. Res. Commun. 247, 60-64.

Visioli, F., Caruso, D., Galli, C, Viappiani, S, Galli, G., Sala, A., 2000. Olive oils rich in natural catecholic phenols decrease isoprostane excretion in humans. Biochem. Biophys. Res. Commun. 278, 797-799.

Tables

Table 1. Selected fatty acids (percentage of total fatty acids) from enriched *Artemia* nauplii from Experiment 2. Data represent means ± SD (n=3). Treatments with asterisk are significantly different ($P \leq 0.05$) from control treatment (Dunnett test). If no superscript appears, values are not different. T-Student test was performed between M70 and M70+HYT treatments, and statistical differences were marked with a capital letter superscript (A, B)

% Fatty acids	Control	DCSS	M70	M70+HYT
14:0	1.1±0.1	1.0±0.1	1.2±0.2	1.1±0.1
16:0	12.2±0.7	10.0±0.5*	10.8±0.3*	10.8±0.4*
16:1n-9	0.6±0.1	0.5±0.1	0.7±0.1	0.7±0.2
16:1n-7	2.4±0.1	2.3±0.2	2.4±0.1	2.3±0.1
18:0	6.7±0.0	5.0±0.1*	5.0±0.1*	5.0±0.1*
18:1n-9	17.5±0.9	16.4±0.8	15.7±0.7*	15.5±0.3*
18:1n-7	8.6±0.4	6.7±0.5*	7.1±0.5*	6.8±0.2*
18:2n-6	5.4±0.1	5.9±0.4	5.1±0.3	4.9±0.1
18:3n-3	25.1±0.6	22.0±0.8*	25.5±0.7A	23.8±0.4B
18:4n-3	3.2±0.1	2.8±0.2	3.1±0.3	3.0±0.0
20:0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
20:2n-6	0.2±0.0	0.3±0.0	0.2±0.0	0.2±0.0
20:3n-6	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
20:4n-6	0.8±0.1	1.0±0.2	1.1±0.1*	1.2±0.0*
20:3n-3	0.7±0.0	0.6±0.0	0.7±0.1	0.6±0.0
20:4n-3	0.6±0.0	0.8±0.0*	0.6±0.0	0.6±0.0
20:5n-3	2.2±0.4	12.0±2.6*	4.3±0.7	4.6±0.2
22:0	0.4±0.0	0.3±0.0*	0.3±0.0*	0.3±0.0*
22:5n-3	0.0±0.0	0.4±0.1*	0.4±0.1*	0.5±0.0*
22:6n-3	0.1±0.0	2.4±0.3*	5.1±0.8*A	8.4±0.5*B
Saturated	22.8±0.8	18.1±0.8*	19.5±0.4*	19.0±0.6*
Monounsaturated	31.0±0.9	28.6±1.0*	27.4±1.2*	26.7±0.5*
Polyunsaturated	40.0±1.4	48.0±2.5*	47.9±0.9*	49.1±1.0*
HUFA n-3	3.6±0.5	15.5±5.2*	12.0±3.0*	14.3±1.3*
HUFA n-6	1.4±0.3	1.6±0.2	1.9±0.2	2.2±0.2*
DHA/EPA ratio	0.0±0.0	0.2±0.1*	1.2±0.1*A	1.8±0.0*B
Total FAME (mg g^{-1})	43.7±7.9	138.3±12.1*	105.7±17.1*	121.3±9.2*
% Lipid (DW)	20.9±0.4	27.8±1.3	27.4±0.5	26.2±0.4

HUFA n-3: ≥20:3n-3; HUFA n-6: ≥20:2n-6; DHA/EPA: docosahexaenoic/eicosapentaenoic fatty acid ratio

Figure captions

Figure 1. Activity of Catalase (CAT), superoxide dismutase (SOD), total glutathione peroxidase (GPx Total), Selenium dependent glutathione peroxidase (GPx Se-dep), glutathione-s-transferase (GST) and lipid peroxidation (LPO) in whole body of *Artemia* nauplii enriched with M70 emulsion, *Tetraselmis suecica* algae, yeast (*Saccharomyces cerevisiae*) and non-enriched *Artemia*. Asterisk shows statistical differences between the different treatments and the control group (Dunnett test).

Figure 2. Component plot (a) and factor score plot (b) of the multivariate Principal Components Analysis of lipid peroxidation (LPOprot) and antioxidant enzymes (Catalase (CAT), glutathione-s-transferase (GST), superoxide dismutase (SOD), total glutathione peroxidase (GPxTot), Selenium dependent glutathione peroxidase (GPxSe)) of enriched *Artemia* nauplii with M70 emulsion, *Tetraselmis suecica* algae (Tetra), yeast (*Saccharomyces cerevisiae*) and non-enriched *Artemia* from Experiment 1.

Figure 3. Activity of Catalase (CAT), superoxide dismutase (SOD), total glutathione peroxidase (GPx Total), Selenium dependent glutathione peroxidase (GPx Se-dep), glutathione-s-transferase (GST) and lipid peroxidation (LPO) in whole body of non-enriched *Artemia* nauplii and *Artemia* nauplii enriched with commercial emulsion DCSS, M70 emulsion and M70 emulsion in combination with an external antioxidant HYT, M70+HYT treatment. Asterisk shows statistical differences between the treatment and the control group (Dunnett test) and lowercase letter superscript shows statistical differences between M70 and M70+HYT treatment (Student t-test).

Figure 4. Component plot (a) and factor score plot (b) of the multivariate Principal Components Analysis of lipid

peroxidation (LPOprot), antioxidant enzymes (Catalase (CAT), glutathione-s-transferase (GST), superoxide dismutase (SOD), total glutathione peroxidase (GPxTot), Selenium dependent glutathione peroxidase (GPxSe)) and total HUFA (n-3 and n-6 series) of enriched *Artemia* nauplii with commercial emulsion DCSS, M70 emulsion, M70 emulsion in combination with an external antioxidant, HYT (M70+HYT treatment) and non-enriched *Artemia* from Experiment 2.

Figure 1

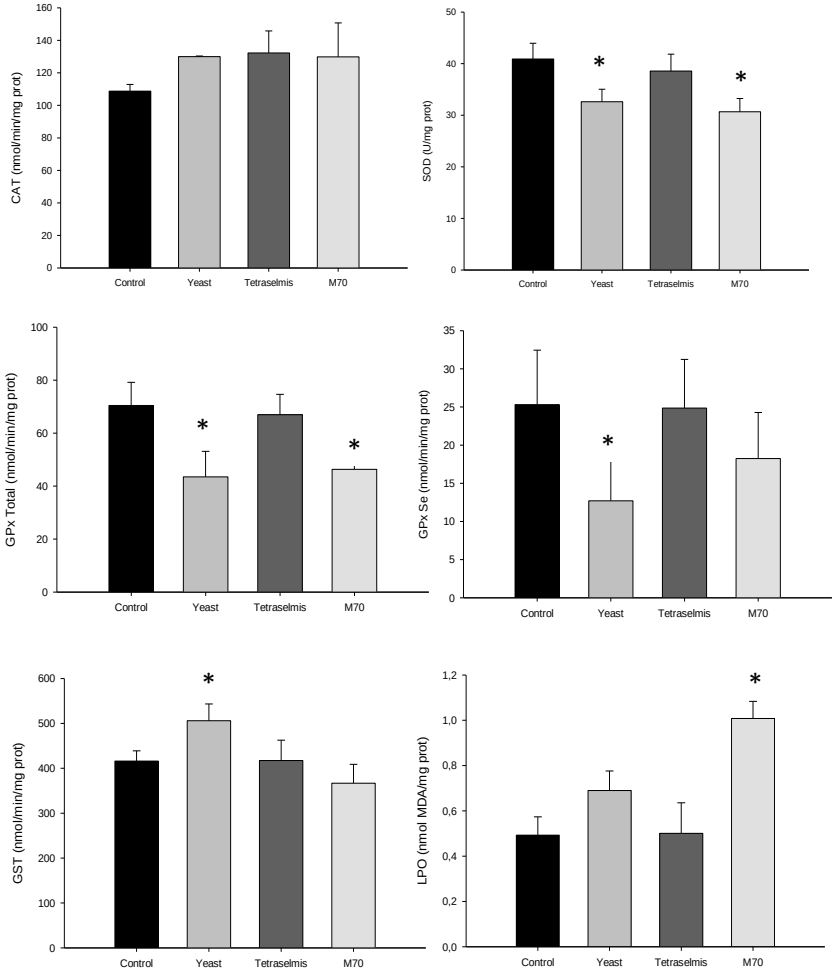

CAT (nmol/min/mg prot)
SOD (U/mg prot)
GPx Total (nmol/min/mg prot)
GPx Se (nmol/min/mg prot)
GST (nmol/min/mg prot)
LPO (nmol MDA/mg prot)
Control
Yeast
Tetraselmis
M70
*

Figure 2

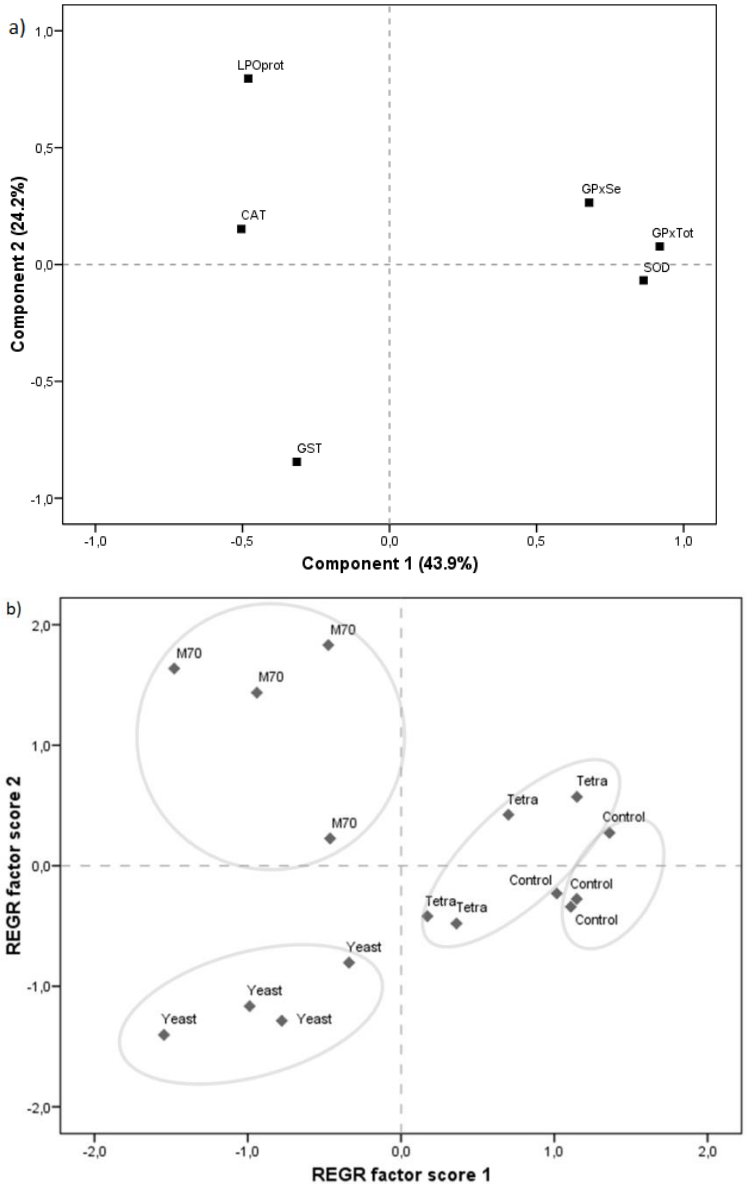

a)
LPOprot
CAT
GST
GPxSe
GPxTot
SOD
Component 2 (24.2%)
Component 1 (43.9%)
b)
M70
M70
M70
M70
Tetra
Tetra
Tetra
Tetra
Control
Control
Control
Control
Control
Yeast
Yeast
Yeast
Yeast
REGR factor score 2
REGR factor score 1

Figure 3

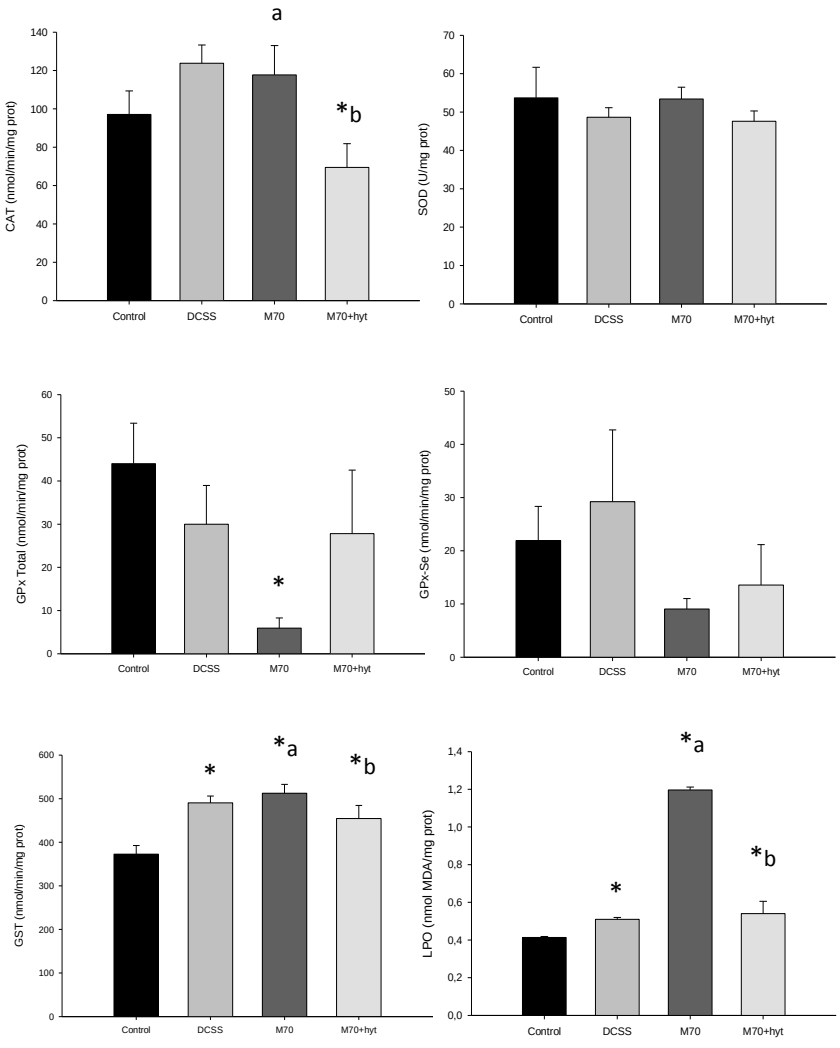
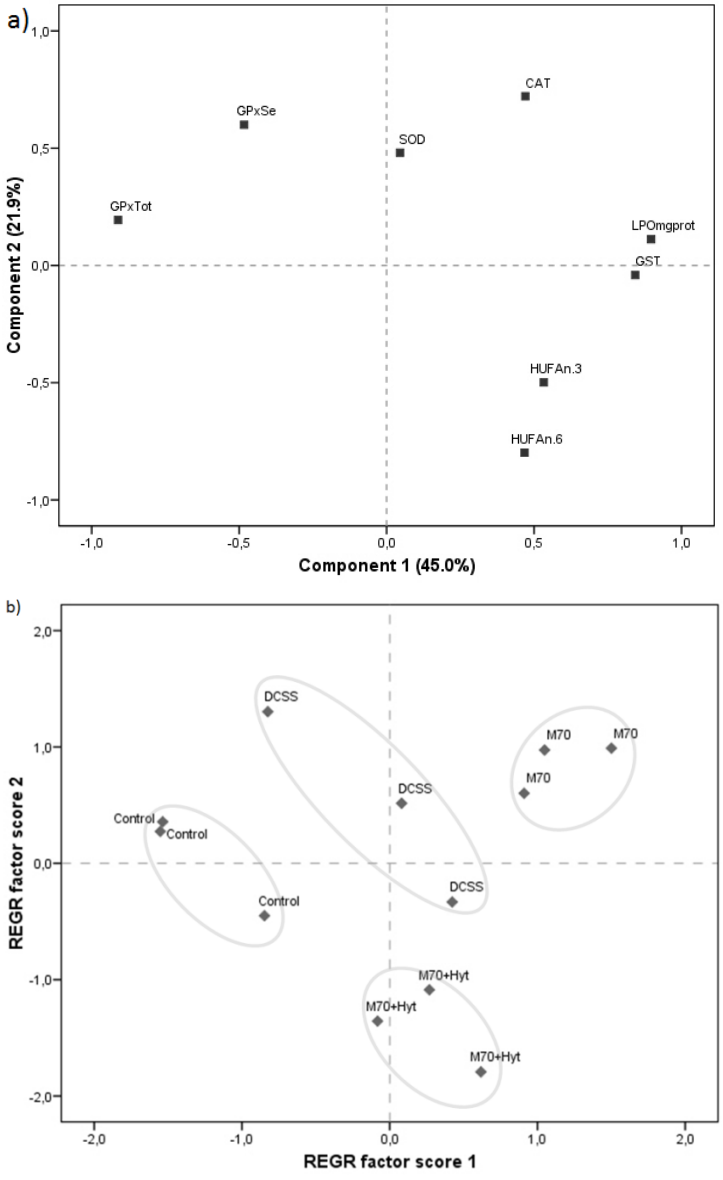

a
*b
CAT (nmol/min/mg prot)
Control
DCSS
M70
M70+hyt
SOD (U/mg prot)
GPx Total (nmol/min/mg prot)
*
GPx-Se (nmol/min/mg prot)
*a
*b
GST (nmol/min/mg prot)
LPO (nmol MDA/mg prot)

Figure 4


a)
CAT
GPxSe
SOD
GPxTot
LPOmgprot
GST
HUFAn.3
HUFAn.6
Component 2 (21.9%)
Component 1 (45.0%)
b)
DCSS
M70
M70
M70
DCSS
Control
Control
Control
DCSS
M70+Hyt
M70+Hyt
M70+Hyt
REGR factor score 2
REGR factor score 1

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Fatty acid composition of polar and neutral lipid fractions of *Octopus vulgaris* Cuvier, 1797 paralarvae reared with enriched on-grown *Artemia*

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Abstract

Rearing of common octopus *Octopus vulgaris* is limited by the lack of success during the paralarval stage, with generalized mortalities occurring before the settlement of the juveniles. The use of on-grown *Artemia* cultured with the microalga *Isochrysis galbana* and further enriched with *Nannochloropsis* sp. has led to a certain degree of success. The present work aims at studying the effects of this rearing protocol (Nanno) on the fatty acid composition of paralarvae, by comparison with a diet based on on-grown *Artemia* further enriched with a high polyunsaturated fatty acid oil emulsion (M70). After 28 days, survival was estimated at 3% for M70 and 22.5% for Nanno, whereas the average dry weight was not significantly different (Nanno: 1.76 ± 0.28 mg; M70: 1.88 ± 0.22 mg). Although apparently no clear association between the fatty acid composition of the enriched preys and that of the total lipids of paralarvae could be established, further fractionation and fatty acid analysis of the total lipids into polar and neutral classes, followed by principal components analysis, revealed that irrespective of the diet, both lipid fractions showed distinct fatty acid patterns. Besides, the fatty acid composition of the polar lipids was more conservative, whereas that of the neutral lipids was more influenced by the diet, showing more variation among dietary treatments.

Keywords: *Octopus vulgaris*, paralarvae, lipids, fatty acids, *Artemia*

Introduction

In recent years, common octopus *Octopus vulgaris* has been targeted as a promising candidate for aquaculture but, to date, paralarvae rearing is severely limited by the lack of success during the planktonic stage, with generalized mortalities occurring before the settlement of the juveniles.

The life cycle of *O. vulgaris* under conditions of captivity was completed for the first time in 2001 (Iglesias, Otero, Moxica, Fuentes & Sánchez 2004), using *Artemia* and *Maja* zoeae as prey. Although some successful cultures of a reduced number of paralarvae up to juveniles and even sub-adults have been achieved (Moxica, Linares, Otero, Iglesias & Sánchez 2002; Iglesias *et al.* 2004; Carrasco, Arronte & Rodríguez 2006), these experiences have been mainly occasional, anecdotic and, needless to say, economically profitless.

According to Navarro and Villanueva (2000, 2003), among others, a lack of balance in the lipid and fatty acid composition of the food could be responsible for the high mortalities encountered in the rearing of the paralarval stages of *O. vulgaris*. These authors studied the lipid requirements of early stages of cephalopods to conclude that a nutritional imbalance in the lipid and fatty acid profile of the artificial feeding protocol, based on *Artemia*, may be responsible for the high mortalities encountered. *Octopus vulgaris* require feeding on low-lipid preys rich in polar lipids, long-chain polyunsaturated fatty acids (PUFA) and possibly cholesterol (Navarro & Villanueva 2000, 2003;

Okumura, Kurihara, Iwamoto & Takeuchi 2005; Kurihara, Okumura, Iwamoto & Takeuchi 2006; Seixas 2009; Seixas, Rey-Méndez, Valente & Otero 2010). This closely resembles the composition of a 'natural' diet (totally unpractical from an aquaculture approach) based on crustacean larvae (e.g. *M.* zoeae) and other marine planktonic forms like copepods, but is considerably different from the typical composition of enriched *Artemia* in any of its forms. In fact, early stages of cephalopods are particularly rich in polar lipids and cholesterol, and artificial feeding under culture conditions increases their triacylglyceride content drastically (Navarro & Villanueva 2000, 2003).

When using only *Artemia* as food, the only experiment that has attained *Octopus* adult stages was reported by Moxica, Fuentes, Hernández, Iglesias, Lago, Otero and Sánchez (2006), who obtained 67% survival and 1.89 mg of dry weight for 1-month-old paralarvae using on-grown *Artemia* (> 1.5 mm) cultured with the microalga *Isochrysis galbana* and further enriched with *Nannochloropsis* sp. Imamura (1990) and Hamazaki, Fukunaga, Yoshida and Maruyama (1991) reported a limited production of presettlement individuals on adding *Nannochloropsis* sp. to the culture tanks. Hamasaki and Takeuchi (2000) and Hamasaki and Morioka (2002) also reported success (although also limited), on adding *Nannochloropsis* sp. to the culture tanks and as food for *Artemia* respectively. The reasons for the improvements obtained with the rearing protocols involving the use of *Nannochloropsis* sp. remain obscure, but a striking feature of this microalga is its high eicosapentaenoic acid (EPA, 20:5n-3) content (Sukenik, Zmora & Carmeli 1993), an essential fatty acid for marine animals (Sargent, McEvoy, Estévez, Bell, Bell, Henderson & Tocher 1999). Other hypotheses about the beneficial effects of *Nannochloropsis* sp. relate to their potentiality to inhibit the microflora growth and improve the culture conditions (Iglesias personal observation).

The present work aims at studying the effects of a rearing protocol based on *Artemia* on-grown with *Isochrysis galbana* and enriched with *Nannochloropsis* sp. on the fatty acid composition of paralarvae. It will be carried out by comparison with the effects of a diet based on the same on-grown *Artemia* further enriched with a high PUFA oil emulsion especially rich in docosahexaenoic acid (DHA, 22:6n-3). Thus, the work will ultimately focus on the dietary effects of DHA-rich and EPA-rich diets on the total, polar and neutral lipid fatty acid composition of the paralarvae.

Materials and methods

Octopus vulgaris paralarvae were obtained from broodstock kept in captivity using the technology described by Moxica *et al.* (2002). They were fed for 28 days with on-grown *Artemia* (1.5–2 mm) cultured with *I. galbana* and further enriched for 24 h with either *Nannochloropsis* sp. (Nanno treatment) or a PUFA-rich oil emulsion based on a speciality oil containing approximately 70% of DHA, 50/50 oil/water, xanthan gum as a stabilizer and Tween 80 as an emulsifier (M70 treatment).

Cultures were carried out in 1000-L black, circular tanks (diameter 130 cm), at a larval density of 5 individuals L^{-1} (5000 paralarvae per tank). The mean water temperature was 21–22 °C and salinity was 34–35 g L^{-1}; a continuous illumination of 800–1000 lx provided by two 36 W day-light fluorescent tubes was supplied to the tanks. During the first week, the rearing tanks were maintained under standing conditions, with gentle central aeration, and a concentration of 1 million cells mL^{-1} of *Nannochloropsis* sp. From day 8, a water flow of 200 L h^{-1} was partially opened (4 h d^{-1}) every 2 days.

Enriched *Artemia* was added (three to four times per day) at a density of 0.5 prey mL^{-1} until day 11 and at 0.2 prey mL^{-1} onwards. Artemia was on-grown with 300 000 cells mL^{-1} of *I. galbana* for 4 days, and further enriched for 24 h with 10 million cells mL^{-1} of *Nannochloropsis* sp.

Dissolved oxygen, nitrites and ammonium were measured daily. The dry weights of ten paralarvae after washing with distilled water and drying at 90 °C 24 h were recorded at the end of the experiment. Survival was recorded at day 28.

At the end of the trial, the lipids of diets and paralarvae (triplicate pools) were extracted from freeze-dried samples using the method of Folch, Lees and Sloane-Stanley (1957). The total lipids were determined gravimetrically (0.0001 g, Mettler Toledo, Barcelona, Spain) and stored in chloroform/methanol (2:1, v/v) with 0.01% BHT as an antioxidant. An aliquot of total lipids was transmethylated after the addition of 19:0 as an internal standard. Fatty acid methyl esters (FAME) were purified by thin-layer chromatography (Silica gel G 60, Merck, Darmstadt, Germany) and injected on-column in a Fisons 8000 gas chromatograph equipped with a fused silica 30 m × 0.25 mm open tubular column (Tracer, TR-WAX, film thickness: 0.25 µm, Teknokroma, Barcelona, Spain). Helium was used as a carrier gas, and the analyses were run in a 50–220 °C thermal gradient. Peaks

were recorded and integrated in a personal computer using AZUR software (Azur, Datalys, France), and identified by comparison with well-characterized standards. A further aliquot of the total lipids was fractionated into polar and neutral lipids by thin-layer chromatography, and the fatty acids were transmethylated and analysed as described for the total lipids.

The fatty acid profiles thus obtained were subsequently analysed chemometrically by principal component analysis (PCA). The score plot obtained after the generation of the two principal components was used to identify patterns of similarity among the cases.

For each lipid class, the mean values of the fatty acid composition of the paralarvae from the two dietary treatments were compared using Student's *t*-tests. Statistics were carried out using SPSS 17.0 software (SPSS).

Results and discussion

The average dry weight of 28-day paralarvae from both dietary groups was not significantly different (Nanno: 1.76 ± 0.28 mg; M70: 1.88 ± 0.22 mg) and is similar to the values reported by Moxica *et al.* (2006) using the same microalgal enrichment. They are clearly lower than those reported for paralarval of the same age (30 days post hatch) using crustacean zoeae as complementary food by Moxica *et al.* (2002): 2.42 mg; Iglesias *et al.* (2004): 3.33 mg; Carrasco *et al.* (2006): 2.83 mg; and higher than those obtained by other authors using different feeding regimes: Villanueva, Koueta, Riba and Boucaud-Camou (2002) reported weights from 0.79 to 1.49 mg using enriched Artemia co-fed with millicapsules; Seixas (2009) obtained 0.83 mg paralarvae at day 25 using on-grown Artemia enriched with a mixture of *I. galbana* y *Rhodomonas lens*; Fuentes, Sánchez, Otero, Lago and Iglesias (2009) reported values between 0.96 and 1.12 mg with Artemia and frozen wild zooplankton as food; and Estévez, Gairin and Berger (2009) produced 0.33 mg paralarvae using live zooplankton as food.

These differences can be explained by the different culture conditions and foods. It is evident that crustacean zoeae seem to be a superior food, and the data reported here are better than those obtained with other experimental feeding protocols.

Survival was roughly estimated at 3% for M70 and 22.5% for Nanno. Culture of this last group was followed until 35 days, reaching an average paralarval weight of 1.83 ± 0.28 mg ($n = 10$) and a 3% survival. These data indicate that the biometrical outcome of both dietary treatments was very similar and that any hypothetical advantage of the Nanno group was lost 1 week later.

The lipid content of diets [% dry weight (DW)] was significantly different ($P < 0.05$): 21.01 ± 0.84 for Nanno and 18.23 ± 0.70 for M70. Analysis of the main fatty acids (Table 1) showed that the main differences were due to the Nanno treatment being higher in 16:1n-7 (which resulted in a higher monoene content) and 20:5n-3, whereas M70 showed higher 18:2 n-6 (thus increasing the total n-6 content) and 22:6n-3 contents, which was not detected in the other diet (Table 1).

Differences in the lipid content of diets did not translate ($P > 0.05$) into the lipid content of the paralarvae [Total lipid (% DW): M70 = 16.31 ± 1.04; Nanno = 17.5 ± 0.23; total FAME (mg g^{-1} DW): M70 = 45.71 ± 1.82; Nanno = 47.29 ± 1.51]. Similarly, a first glance at the fatty acid profiles of the total lipids of both dietary groups did not reveal streaking differences between them (Table 2). Especially notable is the case of 16:1n-7, very different in the diets. Besides, the paralarvae seem to be able to cope with a

Table 1 Selected fatty acids (% of total lipid fatty acids) and total FAME of enriched on-grown *Artemia* ($n = 3$)

	M70		Nanno	
Fatty acid	**Mean**	**SD**	**Mean**	**SD**
14:0	1.43	0.15	1.13	0.01
16:0	10.54	0.47	15.71	0.04
16:1n-7	5.19	0.13	23.69	0.62
18:0	7.26	0.38	5.40	0.12
18:1n-9	16.22	0.79	12.31	0.37
18:1n-7	9.27	0.70	7.89	0.37
18:2n-6	12.15	1.06	2.34	0.04
18:3n-3	5.05	0.13	0.38	0.01
20:4n-6	2.75	0.09	2.88	0.04
20:3n-3	0.07	0.07	ND	
20:4n-3	0.25	0.04	ND	
20:5n-3	11.99	0.43	22.55	0.52
22:5n-3	0.52	0.26	0.02	0.03
22:6n-3	8.10	1.10	ND	
Sat	20.38	0.70	23.33	0.30
Mono	31.58	0.80	44.19	0.18
Poly	44.42	1.76	28.96	0.50
n-3	27.92	1.15	23.04	0.54
n-6	16.67	1.03	5.62	0.13
HUFAn-3	20.93	0.99	22.57	0.55
HUFAn-6	3.83	0.09	3.16	0.02
FAME	60.49	4.35	116.97	12.89

Sat, saturates; mono, monoenes; poly, polyunsaturated; HUFA, highly unsaturated fatty acids (> 20 °C); SD, standard deviation; FAME, total fatty acid methyl esters (mg g^{-1} DW); ND, not detected.

Table 2 Selected fatty acids (% of total fatty acids) of the total, polar and neutral lipid of 28 days *Octopus vulgaris* paralarvae fed two enriched on-grown *Artemia* diets ($n = 3$)

	Total lipid				Polar lipid				Neutral lipid			
	M70		Nanno		M70		Nanno		M70		Nanno	
Fatty acid	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
14:0	0.74	0.06	1.62*	0.05	0.58	0.13	1.09*	0.17	2.56	0.93	5.19*	0.23
16:0	21.09	1.12	21.23	0.07	18.53	0.67	18.25	0.65	20.21	3.18	18.22	1.50
16:1n-7	5.02	0.64	4.31	0.45	2.07	0.42	2.47	0.29	16.22	4.27	13.01	1.08
18:0	12.61	0.28	12.51	0.29	14.21	0.28	13.56	0.42	10.50	3.35	8.19	1.25
18:1n-9	6.93	1.17	8.32	0.22	3.94	0.35	5.33*	0.34	12.36	1.78	17.25*	1.08
18:1n-7	5.58	0.11	5.28*	0.06	4.08	0.21	3.88	0.18	6.27	2.57	6.10	0.14
18:2n-6	1.50	0.32	2.95*	0.06	1.11	0.02	2.08*	0.21	2.52	0.63	7.06*	0.48
18:3n-3	0.94	1.20	0.77	0.04	0.22	0.11	0.56*	0.03	0.24	0.21	1.81*	0.31
20:4n-6	6.35	0.33	5.50*	0.08	7.69	0.34	6.31*	0.22	1.19	0.53	0.49	0.12
20:3n-3	1.25	0.04	1.37*	0.02	1.51	0.13	1.58	0.05	ND		ND	
20:4n-3	0.03	0.06	ND		0.01	0.02	0.05	0.05	ND		ND	
20:5n-3	21.89	1.54	20.33	0.36	25.73	0.43	23.31*	0.64	5.33	1.48	2.31*	0.14
22:5n-3	1.20	0.08	1.09	0.05	1.48	0.06	1.43	0.05	ND		ND	
22:6n-3	5.55	0.63	5.21	0.07	6.44	0.74	5.63	0.35	ND		ND	
Sat	34.57	1.28	35.54	0.15	33.73	0.59	33.35	0.59	34.62	6.19	31.94	1.96
Mono	20.20	1.26	20.45	0.66	12.81	1.02	14.55	0.82	36.41	7.43	37.19	1.76
Poly	41.57	0.54	40.48	0.89	46.06	1.66	43.42	1.11	11.96	3.83	13.99	1.20
n-3	30.98	0.62	29.09*	0.57	35.45	0.83	32.75*	0.94	5.57	1.67	4.56	0.78
n-6	9.73	0.08	10.72*	0.12	10.75	0.28	10.56	0.36	4.16	1.51	9.25*	1.29
HUFAn-3	29.92	0.87	28.00*	0.46	35.16	0.75	32.01*	0.94	5.33	1.48	2.31*	0.14
HUFAn-6	7.02	0.29	6.35*	0.14	8.38	0.25	7.12*	0.39	1.64	1.06	1.60	1.13

For abbreviations see Table 1.
*Significant difference between means within lipid class (*t*-test $P < 0.05$).

22:6n-3-deficient diet, although the poor performance of the two dietary groups could also point towards a dietary deficiency of both treatments.

A closer look at the polar and neutral lipid fatty acid composition showed that the polar lipids were much richer in 20:5n-3 and 22:6n-3 (absent in the neutrals), which was reflected in a higher polyunsaturated, n-3 and highly unsaturated fatty acid (HUFA) n-3 content. Neutrals in turn were richer in monounsaturated fatty acids. Within the polar lipids, M70 treatment generally increased the polyunsaturated, n-3 and HUFA n-3 contents with respect to Nanno. Within the neutral lipids, Nanno treatment could be associated with a higher n-6 content, whereas M70 seemed to increase HUFA n-3.

These trends were confirmed by the results of the PCA. The first component explained 76% of the variance and was associated with variables 14:0, 18:1n-9, 18:2n-6 and 16:1n-7 on the positive side and variables 20:5n-3, 20:4n-6, 22:6n-3 and 18:0 on the negative side. The second component explained only 11% of the variance. The results of the score plot (Fig. 1) showed two main clouds of scores corresponding to polar (associated with variables on the negative side) and total lipids on one side, clearly distinct from neutral lipids (associated with variables on the positive side). Within the polar and neutral lipids, the scores corresponding to the different dietary treatments were distinguishable, whereas those corresponding to the total lipids did not separate the two dietary groups. The scores of the polar lipids were more grouped than those of the neutral lipids, indicating a higher similarity in the fatty acid patterns, which indicates the structural role of this lipid class (Gurr & Harwood 1991). Recently, Quintana (2009) have reported on the effects of the broodstock diets on the fatty acid composition of 3-day-old paralarvae, fed and starved, to conclude that the neutral lipids of the paralarvae reflect better than the polars any difference in the fatty acid profile of the breeders' diets. This is a further indication of the importance of the polar lipids as a structural and conservative lipid class for this species. In fact, the mantle being essentially protein and membranes, any changes in the neutral lipid fraction may be indicative of the contribution of this lipid class to the lipid composition of the digestive gland and ultimately of the lipid metabolism and turnover linked to the dietary fatty acid composition.

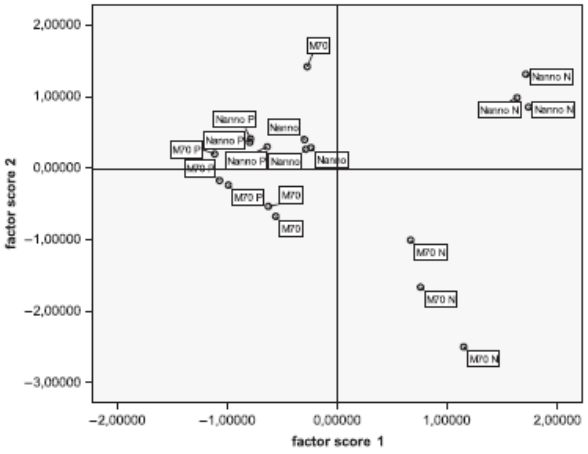

Figure 1 Score plot generated after principal component analysis of the fatty acid patterns of 28-day *Octopus vulgaris* paralarvae fed two enriched on-grown *Artemia* diets. Nanno, M70, total lipid; Nanno N, M70 N, neutral lipid; Nanno P, M70 P, polar lipid.

To a certain point, the above result seems to contradict the results of Navarro and Villanueva (2003), who found that the main dietary changes were already very evident in the total lipids of *O. vulgaris* paralarvae, although they were also clear in the polar lipids (unfortunately, the neutral lipid composition was not analysed). Thus, the dietary changes induced and their influence on the structural lipids may be dependent on the composition of the diets and on the coverage of the essential requirements. From this point of view, both Nanno and M70 diets fulfil the essential requirements of the species, and the presence of DHA in Nanno fed paralarvae may be indicative of a certain degree of synthetic capacity. Alternatively, the levels of DHA may be a remnant from the original content at hatching, a result of a conservative strategy for the retention of an essential lipid.

Paralarval DHA levels not correlating with the amount in food have been reported previously (Navarro & Villanueva 2000; Seixas 2009; Seixas *et al.* 2010). In fact, Seixas *et al.* (2010) have recently pointed out the importance of an adequate ratio protein/lipid before the essential lipid requirements of the species may be considered. Given the generalized low performances of the paralarval cultures, it is difficult to focus on a single source of variation, i.e. lipids, in a cause–effect design. Obviously, factors other than lipids may be affecting the final outcome, and even the fine-tuning of the lipid requirements may have a drastic influence. It should suffice to recall that for example, in larval sea bream (*Sparus aurata*), Rodríguez, Pérez, Díaz, Izquierdo, Fernández-Palacios and Lorenzo (1997) reported that the essential PUFA requirements were considerably dependent on the supply of an adequate DHA/EPA ratio. In this sense, it is interesting to note that EPA requirements of *O. vulgaris* may be particularly important, because this fatty acid has been found in very high amounts (and in 1:1 ratios with DHA) in the phosphatidylcholine fraction of the early stages (Quintana 2009).

Conclusions

Although apparently no clear association between the fatty acid composition of the enriched preys and that of the total lipids of paralarvae could be established, further fractionation and fatty acid analysis of the total lipids of the paralarvae into polar and neutral classes, followed by PCA, revealed that irrespective of the diet, both lipid fractions showed distinct fatty acid patterns. Besides, the fatty acid composition of the polar lipids was more conserva-

tive, whereas that of the neutral lipids was more influenced by the diet and showed more variation among dietary treatments. Further research is needed to determine the lipid requirements of this species.

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References

Carrasco J.F., Arronte J.C. & Rodríguez C. (2006) Paralarval rearing of the common octopus, *Octopus vulgaris* (Cuvier). *Aquaculture Research* **37**, 1601–1605.

Estévez A., Gairín J.I. & Berger E. (2009) Wild zooplankton for *Octopus vulgaris* larval rearing. In: *Larvi '09 – Fish and Shellfish Larviculture Symposium*. European Aquaculture Society Special Publication No. 38 (ed. by C.I. Hendry, G. Van Stappen, M. Wille & P. Sorgeloos), pp. 228–229. European Aquaculture Society, Oostende, Belgium.

Folch J., Lees N. & Sloane-Stanley G.H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry* **226**, 497–509.

Fuentes L., Sánchez F.J., Otero J.J., Lago M.J. & Iglesias J. (2009) Utilización de zooplancton y artemia en el cultivo larvario de pulpo *Octopus vulgaris*. In: *Actas del XII Congreso Nacional de Acuicultura* (ed. by D. Beaz, M. Villarroel & S. Cárdenas), pp. 122–123. CICEGRAF, Madrid, Spain.

Gurr M.I. & Harwood J.L. (1991) *Lipid Biochemistry. An introduction*. Chapman and Hall, London, UK.

Hamasaki K. & Morioka T. (2002) Effects of temperature on egg incubation period, paralarval survival and growth of common octopus, *Octopus vulgaris* reared in the laboratory. *Suisanzoshoku* **50**, 407–413.

Hamasaki K. & Takeuchi T. (2000) Effects of the addition of *Nannochloropsis* to the rearing water on survival and growth of planktonik larvae of *Octopus vulgaris*. *Saibai-Giken* **28**, 65–68.

Hamazaki K., Fukunaga K., Yoshida Y. & Maruyama K. (1991) Effects of marine microalgae *Nannochloropsis* sp., on survival and growth of rearing pelagic paralarvae of *Octopus vulgaris*, and results of mass culture in the tank of 20 metric tons. *Saibai–giken* **19**, 75–84.

Iglesias J., Otero J.J., Moxica C., Fuentes L. & Sánchez F.J. (2004) The completed life cycle of the octopus (*Octopus vulgaris*, Cuvier) under culture conditions: paralarval rearing using *Artemia* and Zoeae, and first data on juvenile growth up to 8 months of age. *Aquaculture International* **12**, 481–487.

Imamura S. (1990) Larval rearing of octopus (*Octopus vulgaris* Cuvier). The progress of technological development and some problems remained. *Collecting and Breading* **52**, 339–343.

Kurihara A., Okumura S., Iwamoto A. & Takeuchi T. (2006) Feeding pacific sandeel enhances DHA level in common octopus paralarvae. *Aquaculture Science* **54**, 413–420.

Moxica C., Linares F., Otero J.J., Iglesias J. & Sánchez F.J. (2002) Cultivo intensivo de paralarvas de pulpo, *Octopus vulgaris* Cuvier, 1797, en tanques de 9 m3. *Boletín del Instituto Español de Oceanografía* **18**, 31–36.

Moxica C., Fuentes L., Hernández J., Iglesias J., Lago M.J., Otero J.J. & Sánchez F.J. (2006) Efecto de *Nannochloropsis* sp. en la supervivencia y crecimiento de paralarvas de pulpo *Octopus vulgaris*. In: *Libro de Resúmenes del IX Foro dos Recursos Mariños e da Acuicultura das Rías Galegas* (ed. by M. Rey-Méndez, J. Fernández & M. Izquierdo), pp. 255–260. IX Foro Marino, O Grove, Spain.

Navarro J.C. & Villanueva R. (2000) Lipid and fatty acid composition of early stages of cephalopods: an approach to their lipid requirements. *Aquaculture* **183**, 161–177.

Navarro J.C. & Villanueva R. (2003) The fatty acid composition of *Octopus vulgaris* paralarvae reared with live and inert food: deviation from their natural fatty acid profile. *Aquaculture* **219**, 613–631.

Okumura S., Kurihara A., Iwamoto A. & Takeuchi T. (2005) Correlations among arm sucker count, wet and dry weight of reared common octopus paralarvae. *Aquaculture Science* **53**, 329–330.

Quintana D. (2009) *Valoración de los requerimientos nutricionales de reproductores de pulpo común (Octopus vulgaris)*. Tesis doctoral, Universidad de La Laguna.

Rodríguez C., Pérez J.A., Díaz M., Izquierdo M.S., Fernández-Palacios H. & Lorenzo A. (1997) Influence of the EPA/DHA ratio in rotifers on gilthead seabream (*Sparus aurata*) larval development. *Aquaculture* **150**, 77–89.

Sargent J.R., McEvoy L., Estévez A., Bell J.G., Bell M., Henderson J. & Tocher D.R. (1999) Lipid nutrition of marine fish during early development: current status and future directions. *Aquaculture* **179**, 217–230.

Seixas P. (2009) *Composición bioquímica y crecimiento de paralarvas de pulpo (Octopus vulgaris Cuvier, 1797), alimentadas con juveniles de Artemia enriquecidos con microalgas y otros suplementos nutricionales*. Tesis doctoral, Universidad de Santiago de Compostela, p. 255.

Seixas P., Rey-Méndez M., Valente L.M.P. & Otero A. (2010) High DHA content in Artemia is ineffective to improve *Octopus vulgaris* paralarvae rearing. *Aquaculture* **300**, 156–162.

Sukenik A., Zmora O. & Carmeli Y. (1993) Biochemical quality of marine unicellular algae with special emphasis on lipid composition. II. *Nannochloropsis* sp. *Aquaculture* **117**, 313–326.

Villanueva R., Koueta N., Riba J. & Boucaud-Camou E. (2002) Growth and proteolitic activity of *Octopus vulgaris* paralarvae with different food rations during first feeding, using *Artemia* nauplii and compound diets. *Aquaculture* **205**, 269–286.

